

Expression of Myofibrillar Proteins and Parvalbumin Isoforms in White Muscle of the Developing Turbot *Scophthalmus Maximus* (Pisces, Pleuronectiformes)

Bruno Focant, S. Collin, P. Vandewalle⁽¹⁾, and F. Huriaux

Laboratoire de Biologie cellulaire et tissulaire, Université de Liège, Liège, and (1) Laboratoire de Morphologie fonctionnelle et évolutive, Institut de Chimie-B6C, Sart-Tilman, Université de Liège, Liège, Belgique

Abstract

Expression of polymorphic myofibrillar and sarcoplasmic proteins was investigated in the fish *Scophthalmus maximus* (L.) undergoing metamorphosis. A range of electrophoretic techniques was used to monitor sequential synthesis of isoforms from hatching to the adult stage. Two isoforms (larval and adult) of myosin light chain LC2 and troponin-I were successively detected during turbot growth, in addition to variations in the peptide composition of myosin heavy chains. Two isoforms of troponin-T also appeared sequentially, but the first to make its appearance was not detected until the juvenile stage. The composition of alkali light chains, actin, tropomyosin, and troponin-C did not seem to change as the fish progressed through the different stages. Parvalbumin isoforms were isolated and their physico-chemical parameters defined. As in the other fish examined so far, there appeared a succession of larval (PA IIa and PA IIb) and adult (PA V) parvalbumin isoforms through the life of the fish. All these biochemical changes occurred gradually in the course of turbot development, and did not appear particularly related to metamorphosis but rather to physiological needs of the different growth stages.

Key words: development, metamorphosis, myofibrillar proteins, parvalbumin isoforms, *Scophthalmus maximus*, turbot.

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Various biochemical, histochemical, and immunohistochemical investigations have revealed that fish muscle development is associated with sequential synthesis of a range of myofibrillar protein isoforms [4, 5, 9, 10, 21, 22, 24, 25, 26, 30], as also shown in avian and mammalian muscles [29]. Similarly two classes of parvalbumins, or calcium-binding muscle proteins, are expressed in the course of the fish growth [9, 13, 19, 20]. The so-called "larval" isoforms (PA II) appear early with the myofibrils and predominate during the larval stage, while the isoforms of the second class (mainly PA III, PA IV, and PA V) appear later and are characteristic of the adult stage. These two classes of parvalbumins do not differ conclusively as regards their physico-chemical properties and do not seem strictly related to any particular transition in fish development. As calcium-binding proteins may promote muscle relaxation in cold-blooded vertebrates [17], their succession is probably related to the specific physiological requirements of different life stages.

Flatfish such as the turbot are characterised by major morphological, functional, and behavioural changes during growth because of a dramatic metamorphosis from larva to juvenile. This metamorphosis involves transformation from a bilaterally symmetrical body to a laterally flattened asymmetrical one, with migration of the right eye to the left side of the body [1, 14]. These morphological transformations are associated with the functional development of various structures related to the evolution of larval behaviours. The flatfish habitat changes from pelagic to benthic, and this entails a modified use of the trunk musculature. Adaptations must thus appear in muscle morphology and biochemical composition.

Investigators have studied the influence of development and rearing temperature on the distribution and structure of muscle fibre types in various flatfish including the turbot [2, 3, 16, 35], but very few studies have focused on muscle fibre composition at the molecular level. In the Japanese flounder *Paralichthys olivaceus* (Temminck and

Muscle proteins in developing turbot

Schlegel) and plaice *Pleuronectes platessa* L., larval myosin light chains LC2 are gradually replaced by adult-type LC2 after completion of metamorphosis [2, 32]. Changes in myosin heavy-chain composition appear later, at the late juvenile or adult stage. Differential synthesis of troponin-T isoforms has also been observed in the course of flounder metamorphosis [34]. Developmental changes in muscle tissue in the metamorphosing flounder are regulated and stimulated by thyroid hormone [34, 35]. Administration of thyroxine causes early onset of histological changes and of the biochemical transitions of myosin light chain LC2 and troponin-T from the larval to the adult types. As far as we know, production of parvalbumin isoforms in the course of development has not yet been studied in flatfish.

The aim of the present study was to investigate myofibrillar proteins and parvalbumins in white muscle of the developing turbot *Scophthalmus maximus* (L., Pleuronectiformes, Scophthalmidae), from hatching to the adult stage, with emphasis on the metamorphosis period. The biochemical composition of myofibrils was analysed by high-resolution electrophoresis. Sarcoplasmic parvalbumin isoforms were identified, purified, and physico-chemically characterised; their polymorphism was also monitored in the course of fish growth.

Materials and Methods

Fish samples

Larvae, juveniles, and adults of the species *Scophthalmus maximus* were reared in a pilot-scale hatchery under controlled conditions (T=15°C) (NATA, Noirmoutier Aquaculture Techniques Avancées, France). They were stored dry and frozen until used. Ages in

days, total lengths or weights, developmental stages, and numbers of specimens pooled in order to obtain a sufficient amount of muscle material for analysis are reported in Table I. Morphological stages of development were established according to [1]. Metamorphosis started on day 16 and ended around day 50. For fish up to day 20, the head, yolk sac, and viscera were removed (stages 1 to 4). From day 30 to day 50 (stage 5), the muscle was dissected free of skin, superficial muscle fibres, fins, and tail. For juveniles and adults, a piece of trunk dorsal white muscle (around 1 g) was taken.

Muscle samples were processed according to [9]. For isolation of parvalbumin isoforms, the whole white muscle (dorsal side) was dissected from a big fresh adult fish (2200 g) obtained from a local fish dealer.

Preparation of proteins

Myofibrils were prepared and incubated in several solutions for electrophoresis as described in [21]. Isolation of myosin heavy chains by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and digestion with *Staphylococcus aureus* V8 protease were carried out according to the same authors. Crude parvalbumin extracts were obtained according to [13]. Parvalbumin isoforms were prepared from 200 g fresh turbot muscle according to [19]. To isolate PA IV and PA V, we used an additional preparative PAGE in BIORAD's Model 491 Prep-Cell as described for *Chrysichthys auratus* (Geoffroy St.Hilaire) parvalbumins [13].

Analytical methods

Analytical PAGE separations of myofibrillar proteins and parvalbumin isoforms were performed under various conditions. SDS-PAGE was carried out according to [23]

Table I. Data on the fish samples.

Age (days)	Length (mm)	Weight (g)	Developmental stage	Number of fish
2	3.2		larval	30
4	3.5		larval	30
8	3.8		larval	30
12	4.8		larval	30
16	6.3		larval	30
20	9.3		larval	30
30	18.3		larval	20
40	25		larval	10
50	28		juvenile	10
75	54.4		juvenile	10
100	78.8		juvenile	5
210		25	juvenile	2
520		80	juvenile	2
880		700	adult	2

Day 16 to day 22: start of metamorphosis, morphological transformation of larvae, migration of the eye, flattening of the body, loss of the symmetry.

Day 28 to day 50: completion of metamorphosis with the change to benthic life.

Muscle proteins in developing turbot

in gels containing 20% acrylamide and 0.1% bis-acrylamide at pH 8.4, except in the case of myosin heavy chains which were separated in SDS-polyacrylamide gels containing 6% acrylamide, 0.1% bis-acrylamide, and 25 or 40% (v/v) glycerol (pH 8.4 or 8.8) [6]. IEF-PAGE of myofibrils was done at 2500Vh on gels made with 7.1% acrylamide, 0.4% bis-acrylamide, 1.6% Servalyt 3-6, 0.4% Servalyt 3-10, 9.2 M urea, and 1% (v/v) Igepal. IEF-PAGE of parvalbumin isoforms was performed under the same conditions except that we used BIORAD ampholytes (1.6% Bio-Lyte 4-6 and 0.4% Bio-Lyte 3-10) and no Igepal. NEIEF-PAGE was carried out at 400 Vh on 7.1% acrylamide, 0.4% bis-acrylamide gels containing 1% Servalyt 7-9, 1% Servalyt 9-11, 9.2 M urea, and 1% (v/v) Igepal [28]. Alkali-PAGE was done in gels containing 10% acrylamide, 0.26% bis-acrylamide, and 8 M urea, at pH 8.6 [7]. Non-denaturing-PAGE of parvalbumin isoforms was done under the same conditions but with 10% glycerol (v/v) instead of urea [8]. Two-dimensional electrophoresis was carried out using IEF-, NEIEF-, or alkali-PAGE as the first dimension (from left to right) and SDS-PAGE as the second dimension (from top to bottom) [21].

The different myofibrillar proteins were identified by comparing their mobilities in the different gel systems according to their physicochemical characteristics [21]. Determination of their apparent relative molecular masses (M_r) and isoelectric points (pI) was done according to the same authors.

For parvalbumin isoforms, the methods described in [13] were used to determine M_r values by SDS-PAGE and electrospray ionisation mass spectroscopy (ESI-MS), to measure pI values and UV spectra, and to assay sulfhydryl groups.

Results

Evolution of myofibril composition in the course of development

One-dimensional SDS-PAGE revealed myofibrillar components from day 20, at the beginning of metamorphosis (Fig. 1). Additional unidentified bands were observed in the first-stage samples, contaminated by non-muscle tissues. Throughout turbot development, each myofibrillar isoform showed the same apparent relative molecular mass (Table II).

Myosin heavy chains migrated as a single band. In SDS-PAGE carried out on high-porosity gels at different pH values and glycerol concentrations (not shown), the band appeared the same for all stages analysed. Bacterial V8 protease peptide mapping, however, revealed differences in myosin heavy-chain composition between larvae, juveniles, and adults (Fig. 2).

Two-dimensional SDS-PAGE confirmed the presence of the same alkali light-chain isoforms at the different stages of turbot growth (LC1: M_r = 27.2 kDa, pI = 4.97;

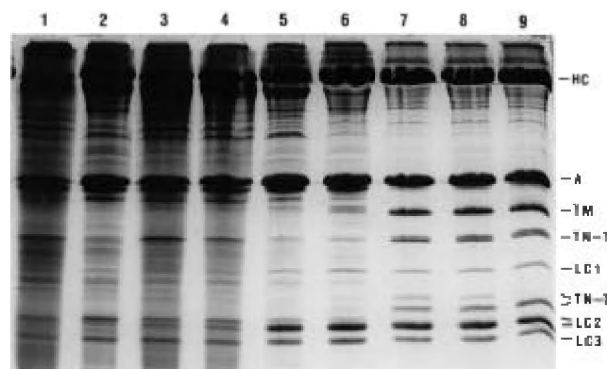


Figure 1. SDS-PAGE of myofibrillar proteins from turbot specimens aged: (1) 20 days, (2) 30 days, (3) 40 days, (4) 50 days, (5) 75 days, (6) 100 days, (7) 210 days, (8) 520 days and (9) 880 days. HC: myosin heavy chain; A: actin; TM: tropomyosin; TN-T: troponin-T; LC1: myosin light chain 1; TN-I: troponin-I; LC2: myosin light chain 2; LC3: myosin light chain 3.

LC3: M_r = 17.3 kDa, pI = 4.54) (Fig. 3), but the excess of LC3 with respect to LC1 was twice to three times higher in larvae than in juveniles and adults (Fig. 4). In contrast, there were very marked changes in the composition of the phosphorylatable light chain LC2: two isoforms appeared successively, differing by their molecular masses and isoelectric points (a 20.4-kDa LC2, pI = 4.89, and a 19.1-kDa LC2, pI = 5.05) (Figs. 3 and 4). On day 20, the 20.4-kDa LC2 represented 80% of the total LC2 light chain; by day 50 (end of metamorphosis), the proportion of this larval isoform had decreased to 50%. By day 210, it had been almost totally replaced by the 19.1-kDa LC2 (adult isoform).

Larval, juvenile, and adult muscles displayed the same, single isoform of tropomyosin (M_r = 37.7 kDa, pI = 4.97) (Fig. 3), migrating close to rabbit alpha-tropomyosin (not shown).

Table II. Apparent relative molecular masses of the main myofibrillar proteins.

Protein	M_r (kDa)
Actin	44.5
Tropomyosin	37.7
Myosin light chain 1	27.2
Myosin light chain 2	20.4
	19.1
Myosin light chain 3	17.3
Troponin-T	32.9
	32.3
Troponin-I	23.2
	21.9
Troponin-C	19.0*

*Troponin-C comigrates with adult LC2 when subjected to SDS-PAGE.

Muscle proteins in developing turbot

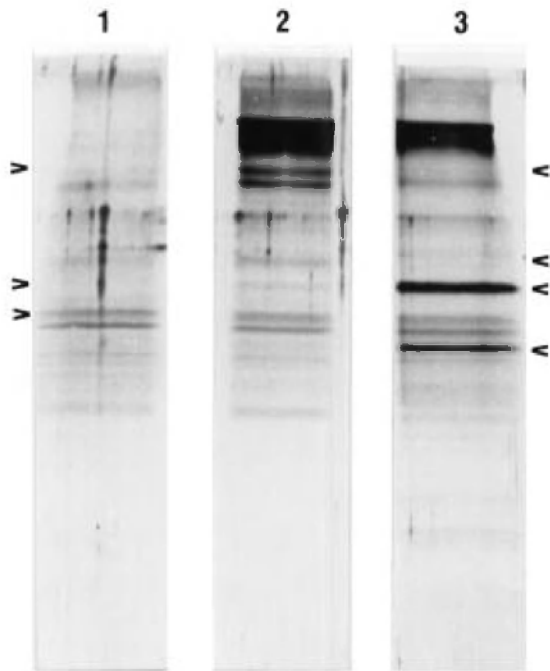


Figure 2. Peptide maps (SDS-PAGE) of myosin heavy chains from (1) a 30-day larva, (2) a 100-day juvenile and (3) an 880-day adult. Arrowheads to the left of the lanes indicate differences between larval and juvenile muscles, those to the right indicate differences between juvenile and adult muscles.

One- and two-dimensional PAGE of the basic proteins showed that different isoforms appeared in the course of turbot development, differing by their apparent Mr values and their electric charges at alkaline pH (Figs. 1 and 5). There appeared two troponin-T (TN-T) isoforms with close Mr values (32.9 kDa and 32.3 kDa) and two troponin-I (TN-I) isoforms (23.2 kDa and 21.9 kDa) (Table II). TN-I was detected from day 40 onward (larval stage), but TN-T only from day 100 (juvenile stage)



Figure 3. Two-dimensional PAGE of acidic proteins with an IEF gel as the first dimension (pH 5.8 on the left side and pH 4.0 on the right side of the window of the electrophoretogram) from turbot specimens aged: (a) 20 days, (b) 50 days, (c) 100 days, and (d) 880 days. TM: tropomyosin; LC1: myosin light chain 1; LC2_l: larval myosin light chain 2; LC2_a: adult myosin light chain 2; LC3: myosin light chain 3.

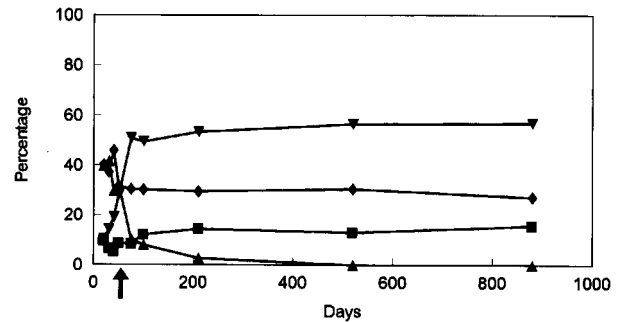


Figure 4. Evolution of the relative proportions of the myosin light chains as a function of turbot age. The data were obtained from the two-dimensional PAGE. □, LC1; □, larval LC2; □, adult LC2; □, LC3. The arrow on the abscissa indicates the end of the metamorphosis period.

onward (Fig. 6). The 23.2-kDa isoform of TN-I was the first to appear. This larval isoform was then gradually replaced by the 21.9-kDa adult isoform, amounts of the two isoforms being about equal on day 100. As for TN-T, myofibrils isolated on day 100 contained only the 32.3-kDa isoform, which diminished in proportion during ulterior growth as the proportion of 32.9-kDa TN-T increased. Both TN-T isoforms were present, in fairly equal amounts, in adult muscles.

Troponin-C (TN-C) was identified on alkali-polyacrylamide gels in the presence of EGTA (first dimension of Fig. 7). In all samples, this calcium-binding protein appeared as a minor constituent because of its higher solubility. It displayed an apparent Mr similar to that of the adult LC2 light chain and an isoelectric point

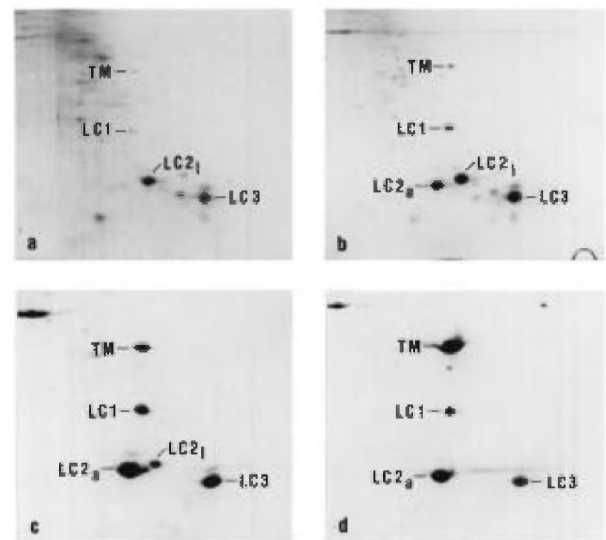


Figure 5. Two-dimensional PAGE of basic myofibrillar proteins from turbot specimens aged: (a) 100 days, (b) 210 days, (c) 880 days. NEIEF-PAGE was used as the first dimension with proteins migrating to the cathode (right). T: troponin-T; I_l: larval troponin-I; I_a: adult troponin-I.

Muscle proteins in developing turbot

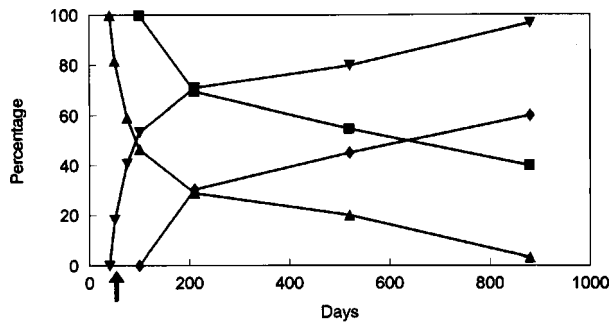


Figure 6. Evolution of the relative proportions of the two troponin-T and two troponin-I isoforms as a function of turbot age. The data were obtained from one-dimensional SDS-PAGE using the usual migration time for troponin-I but twice the usual migration time for troponin-T. □, 32.9 kDa troponin-T; ○, 32.3 kDa troponin-T; △, 23.2 kDa larval troponin-I; ◇, 21.9 kDa adult troponin-I. The arrow on the abscissa indicates the end of the metamorphosis period.

of about 4.3 (Fig. 7).

Identification of parvalbumin isoforms

Parvalbumins from larval (30 days), juvenile (100 days), and adult (880 days) turbot specimens were examined by non-denaturing PAGE (Fig. 8a). The different isoforms were numbered by comparing their migration with known parvalbumin isoforms from other fish. Larval and early juvenile muscles were found to contain only isoform PA II, while late-juvenile and adult muscles displayed four parvalbumin isoforms (PA II, PA III, PA IV, and PA V) with a marked predominance of PA II. PA IV and especially PA III appeared in low amounts. They might be aggregated or modified forms of PA V, since levels of these proteins

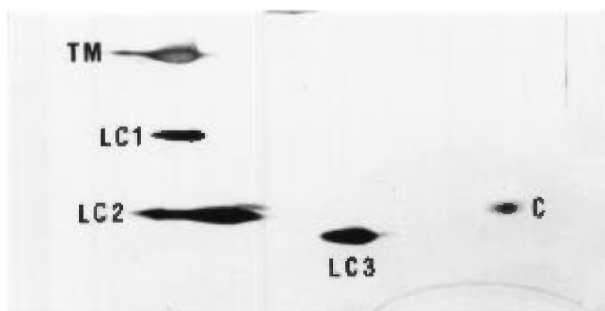


Figure 7. Two-dimensional PAGE of acidic myofibrillar proteins from a 210-day-old turbot with an alkali gel as the first dimension. In alkali-PAGE, the proteins were incubated in the presence of 0.005 M EGTA and migrated to the anode (right). The gel was silver-stained. TM: tropomyosin; LC1: myosin light chain 1; LC2: myosin light chain 2; LC3: myosin light chain 3; C: troponin-C.

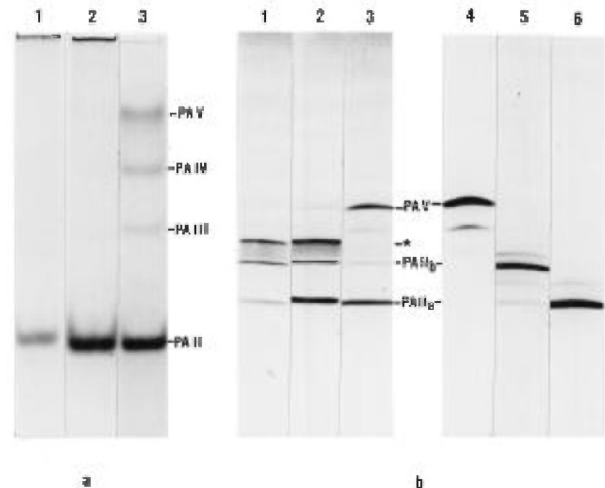


Figure 8. Non-denaturing-PAGE, pH 8.6 (a) and IEF-PAGE (b) of parvalbumin isoforms. (1) 30-day larva, (2) 100-day juvenile, (3) 880-day adult; (4) PA V; (5) PA IIb; (6) PA IIa. *, higher-Mr contaminant.

fluctuated like that of PA V.

IEF-PAGE revealed the heterogeneity of PA II: two isoforms (PA IIa and PA IIb) were present with very different isoelectric points (Fig. 8b, 1-3). PA III and PA IV were hardly visible. A band above PA IIb in larvae and juveniles corresponded to a protein contaminant of higher molecular weight, as shown by cutting out this band from the IEF-PAGE gels and subjecting it to SDS-PAGE.

Characterisation of parvalbumin isoforms

Parvalbumin isoforms were isolated from the trunk white muscle of a big adult turbot. Two peaks eluted successively from the DEAE-cellulose column at pH 5.7, when a 0-0.15 M NaCl gradient was applied. The first peak contained PA V contaminated by PA IV and traces of PA III, while the second contained both isoforms of PA II. PA IIa and PA IIb were separated by a second chromatography of this second peak under the same conditions, but with a narrower salt gradient (0.05 to 0.12 M NaCl) for elution. Pure PA IV and PA V were recovered by subjecting the first peak from the DEAE-cellulose column to preparative non-denaturing PAGE. The final yield from 200 g fresh muscle was 91 mg PA IIa, 17 mg PA IIb, 7 mg PA IV, and 76 mg PA V (Fig. 8b, 4-6).

The physico-chemical properties of the four parvalbumin isoforms are listed in Table III. For PA IIa, PA IV, and PA V, SDS-PAGE and ESI-MS yielded similar Mr values, 350-400 Da higher for PA IV and PA V than for PA IIa. For PA IIb, however, the two techniques gave surprisingly different values: SDS-PAGE yielded a value near that measured for PA IIa and ESI-MS a value closer to those measured for PA IV and PA V. The UV spectra of all four isoforms displayed the usual peaks typical of phenylalanine residues (Fig. 9). They showed the pres-

Muscle proteins in developing turbot

Table III. Physicochemical properties of the isolated parvalbumin isoforms.

PA isoforms	Molecular mass		pI	Est. nb of AA			PA type
	SDS	ESI-MS		Tyr	Trp	Cys	
PA V	11.750 \pm 50	11.796	5.12 \pm 0.09	0	0	1	A
PA IV	11.800 \pm 40	11.796	4.95 \pm 0.05	0	0	1	A
PA IIb	11.400 \pm 20	11.787	4.75 \pm 0.02	1	0	1	L
PA IIa	11.380 \pm 20	11.433	4.58 \pm 0.07	1	0	3	L

PA, Parvalbumin; SDS, sodium dodecyl sulfate; ESI-MS, electrospray ionisation mass spectrometry; pI, isoelectric point; AA, amino acid; Tyr, tyrosine; Trp, tryptophan; Cys, cysteine; A, adult parvalbumin isoform; L, larval parvalbumin isoform.

ence of one tyrosine residue in each PA II isoform and the absence of tyrosine in PA IV and PA V. All isoforms appeared to lack tryptophan. PA IIa was distinguishable by the presence of three cysteine residues.

Evolution of parvalbumin isoforms in the course of development

Levels of parvalbumin isoforms were monitored from day 2 post-hatching until the adult stage. They formed distinct bands when subjected to non-denaturing PAGE, SDS-PAGE, or IEF-PAGE. The isoforms were quantified by densitometric scanning of the stained gels (percentage) (Figs. 10a and 11a), and the results were adjusted for a same total sarcoplasmic protein concentration (relative parvalbumin content in arbitrary units) (Figs. 10b and 11b).

On non-denaturing polyacrylamide gels (Fig. 10), a single parvalbumin band corresponding to the PA II isoform was detectable from day 20 (larval stage) (cf. Fig. 8a). The relative concentration of this isoform peaked during the juvenile stage (210 days), but PA II

still constituted 60% of the total parvalbumin content in the adult. PA V, with PA III and PA IV as by-products, appeared on day 210. Levels of these three proteins rose together very slowly to total about 40% at the adult stage. This trend was confirmed by SDS-PAGE (not shown). IEF-PAGE revealed different time profiles for the two PA II isoforms (Fig. 11). At the beginning of metamorphosis, PA IIb averaged about 70% of the total parvalbumin content, then it decreased in proportion in favour of PA IIa. Both PA II isoforms displayed the same relative concentration around the end of metamorphosis. During later growth, the PA IIa level was always higher than the PA IIb

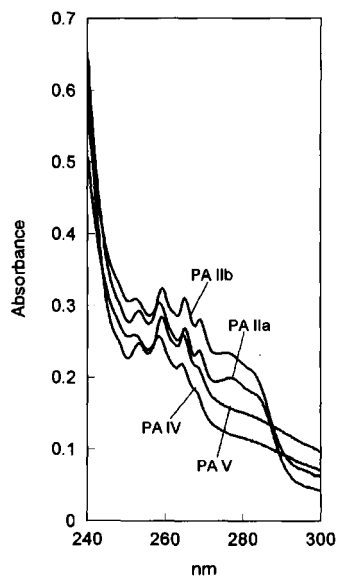


Figure 9. Ultraviolet absorption spectra (1-cm light path) of the parvalbumin isoforms (3 mg ml^{-1}) in $0.05 \text{ M NH}_4\text{HCO}_3$.

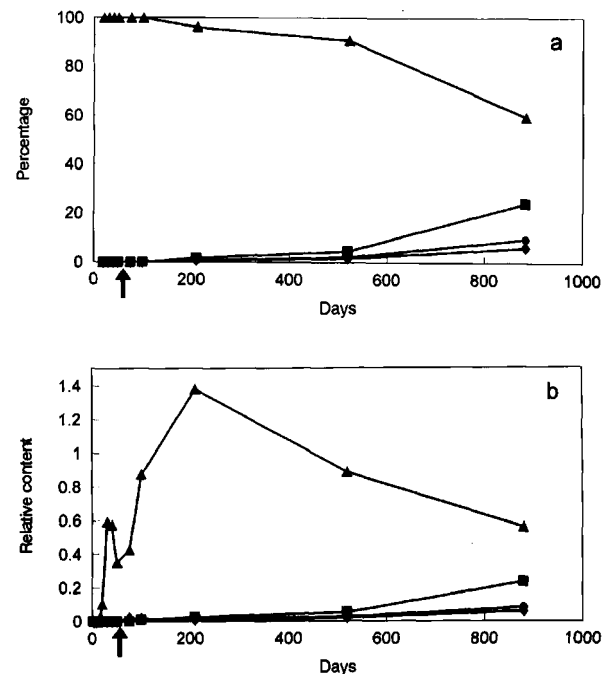


Figure 10. Evolution of the percentage (a) and relative content (b) of each parvalbumin isoform as a function of turbot age, as determined by non-denaturing PAGE. \square : PA II; \circ : PA III; \diamond : PA IV; \square : PA V. The arrow on the abscissa indicates the end of the metamorphosis period.

Muscle proteins in developing turbot

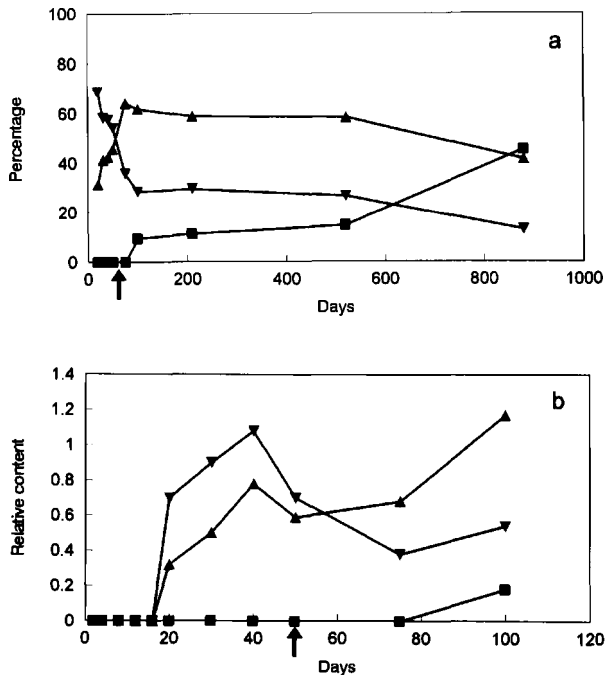


Figure 11. Evolution of the percentage (a) and relative content (b) of each parvalbumin isoform as a function of turbot age, as determined by IEF-PAGE. For clarity, the scale of the abscissa of (b) was stopped at 100 days. □: PA IIa; ▢: PA IIb; ▴: PA V (+ PA III and PA IV). The arrow on the abscissa indicates the end of the metamorphosis period.

level, being three times higher in the adult.

Discussion

The turbot is currently recognised as one of the most promising candidates for mariculture in Europe. Numerous studies have concentrated on its developmental morphology and behaviour and also on rearing systems [1, 3, 14, 15, 16, 33]. Yet biochemical knowledge of its muscle development is still very fragmentary, despite the important morphological transformations appearing at metamorphosis. Such a major adaptation is likely to be reflected in muscle morphology and fibre composition. We therefore investigated, from hatching to the adult stage, changes in the composition of the different myofibrillar subunits and parvalbumin isoforms in the trunk muscle of *Scophthalmus maximus*. All these proteins could be monitored only from day 20, corresponding to the beginning of metamorphosis. In various other fish, they have been detected earlier, in larvae with the yolk sac (see introduction). Perhaps the larval isoforms are very labile in the first life stages of the turbot, or present in very low amount or not extracted under our experimental conditions. A similar delayed appearance has been observed in the case of *Dicentrarchus labrax* (L.) myosin and parvalbumins [11, 19]. During the development of this fish, Scapolo et al. [30]

likewise detected no mATPase activity histochemically in the larval stages (up to day 65), even though the muscle fibres of specimens at all stages studied contained abundant well-organised myofibrils. The authors attributed this to an intrinsic feature of the myosins concerned.

Our peptide mapping analysis has shown that turbot white muscle contains different myosin heavy-chain isoforms according to the developmental stage: transition of myosin heavy chains can be distinguished between metamorphosing larvae (day 30) and juveniles (day 100) as well as between juvenile and adult stages. Similar changes between larvae, late juveniles and adults have been found in plaice *Pleuronectes platessa* whereas changes occurred only between juvenile and adult stages in flounder *Paralichthys olivaceus* [2, 32]. Developmental transition of myosin heavy chains appears thus not to correlate directly with metamorphosis. Moreover, this was not regulated by thyroid hormone in flounder as in higher vertebrates [35]. Myosin alkali light chains appear constant in composition in the course of turbot development, the LC3 titre being always higher than the LC1 titre as in adult-fish myosins. It is noteworthy that the particularly high ratio of LC3 to LC1 observed in the early growth stages of the turbot has already been observed in trout and two catfish (*Chrysichthys auratus* and *Heterobranchius longifilis*) species [4, 10, 21]. This might be related to an increased maximum shortening velocity of trunk muscle during this life period as suggested for rabbit fast fibres [27, 31]. In contrast, there appears a succession of two different regulatory light chain LC2 isoforms, suggesting independent regulation of the syntheses of the different myosin subunits. The larval LC2 is gradually replaced by an adult LC2 with a lower apparent relative molecular mass and higher isoelectric point. As their molecular masses are different, these two isoforms cannot be the phosphorylated and non-phosphorylated forms of the same LC2 as found in the catfish *Heterobranchius longifilis* Valenciennes [21]. Successive synthesis of larval and adult LC2 has been previously described in flounder and plaice development, the biochemical characteristics of these isoforms being similar to those of their turbot counterparts [2, 32, 35]. In the plaice, however, two larval and two post-metamorphic LC2 proteins have been distinguished, whereas the turbot seems to produce only one of each.

The thin-filament components actin, tropomyosin, and the calcium-binding protein troponin-C, appear unchanged throughout turbot growth. Tropomyosin is present as a single alpha-type isoform as in other flatfish such as the winter flounder *Pseudopleuronectes americanus* (Walbaum) and sole *Solea solea* (L.) [18].

Two isoforms each of TN-T and TN-I are synthesised during turbot development. These basic proteins could be detected with certainty only from the time of meta-

Muscle proteins in developing turbot

metamorphosis in the case of TN-I and from the juvenile stage for TN-T. The time course of the transition from the larval/juvenile to the adult isoform is very different for the two troponins, as already found in *Heterobranchus longifilis*. This supports the hypothesis that their syntheses are independently regulated. As regards the timing of the appearance of TN-I isoforms, the turbot appears similar to the catfish *Chrysichthys auratus* and *Heterobranchus longifilis* [4, 21]: appearance of the higher-molecular-mass isoform in the larvae, equal amounts of each isoform in 50-100 mm juveniles, a very low amount of larval TN-I in adults. A distinctive feature of the turbot is the particularly late appearance of turbot TN-T: day-100 juveniles (80 mm) show only the 32.3-kDa isoform and both isoforms are present at fairly equal concentrations in the adult. It would thus seem inappropriate to distinguish a 'larval' and an 'adult' TN-T isoform in the turbot. In *Heterobranchus longifilis*, in contrast, two isoforms appear during the larval stage [21]. The initially predominant larval isoform decreases quite rapidly in proportion to the adult isoform, so that the "half-of-each" mark is reached already at the end of the larval period. Yamano et al. [34] have described the presence of two TN-T isoforms (41.5 and 34.0 kDa) in pre-metamorphic flounder larvae and the appearance of an additional 33.5-kDa isoform during metamorphosis. The two lower-molecular-mass isoforms then become predominant during ulterior growth. They very likely correspond to the TN-T isoforms we have identified in the turbot. We cannot exclude that a TN-T isoform of higher molecular mass might be synthesised in the turbot larva, corresponding to one of the bands observed on SDS-polyacrylamide gels between actin and tropomyosin. Two-dimensional PAGE, however, did not enable us to identify these protein components with certainty. On the other hand, Johnston et al. [22] have described the successive synthesis of embryonic, larval, and adult TN-T and TN-I isoforms in the Atlantic herring *Clupea harengus* L. They found that the adult pattern for these troponins was already established in 11-mm larvae reared at 15°C. All these observations show that the timing of appearance of these basic proteins can vary considerably according to the fish species.

Like the other fish we have studied, the turbot successively produces two classes of parvalbumin isoforms: larval PA II and adult PA V (with PA III and PA IV as by-products). An interesting finding is the late appearance of both classes, from the beginning of metamorphosis for PA II and during the juvenile period for PA V. PA II, moreover, is still the major isoform in young adults (700 g), but a larger specimen (2200 g) used for isolating the parvalbumin isoforms contained only 7.5% PA II. Delayed synthesis and a late isoform transition have already been observed in *Dicentrarchus labrax* [19]. They might be related. As shown by IEF-PAGE,

the larval class is a mixture of an early-appearing PA IIB and a later-appearing PA IIA. The PA IIB-PA IIA transition at the end of the larval stage could reflect physiological modifications of the muscle machinery linked to metamorphosis. Non-denaturing PAGE shows that the total relative parvalbumin content peaks on day 210 (juvenile stage) and slowly decreases in older specimens, as usually found for other fish species. Adult isoforms differ from larval ones by the absence of tyrosine and a higher apparent relative molecular mass despite some reservations about PA IIB. The PA IV isoform has a molecular mass, ultraviolet spectrum, and cysteine content similar to those of PA V, strengthening the conviction that it is a by-product of the latter.

In summary, myofibrillar subunit and parvalbumin profiles vary substantially in the course of turbot development. The changes concern myosin heavy chains, myosin light chain LC2, troponin-T, troponin-I, and the parvalbumins. The timing of the transition from the larval to the adult isoform is always gradual and peculiar to each protein examined. Thus, as concluded for plaice development, metamorphosis of the turbot is not characterised by sudden biochemical changes in the muscles. As the muscle components studied here are involved in the mechanism or regulation of muscular contraction, the sequential appearance of specific isoforms presumably reflects changes in the contractile function of trunk muscle, as the turbot goes through its different stages of development.

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B.F. is a Research Associate of the F.N.R.S. of Belgium.

Address correspondence to:

Dr. Bruno Focant, Université de Liège, Biologie cellulaire et tissulaire, Bât. Anatomie L3, 20, rue de Pitteurs, B-4020 Liège, Belgique.

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Muscle proteins in developing turbot

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