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Expression of myofibrillar proteins and parvalbumin isoforms during the development of a flatfish, the common sole *Solea solea*: comparison with the turbot *Scophthalmus maximus*

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ABSTRACT

Developmental changes in myofibrillar protein and parvalbumin isoform composition were investigated in the myotomal muscle of the flatfish *Solea solea*, characterized by a very brief metamorphic stage. Results were compared with previously obtained data on another pleuronectiform teleost, the turbot (*Scophthalmus maximus*), displaying prolonged metamorphosis. Electrophoretically measurable changes in myofibrillar proteins and parvalbumins were detected late in the sole, after completion of metamorphosis. In the course of development, muscles showed the usual sequential synthesis of isoforms of the myofibrillar proteins myosin light chain LC2, troponin-T, and troponin-I. An adult parvalbumin isoform (PA III) was found to predominate during sole growth. The two flatfish were characterized by highly species-specific parvalbumin isoforms. Compared with turbot, the profiles of the myofibrillar subunits and parvalbumin isoforms varied little in the course of sole development. The early appearance of adult traits might be correlated with the brevity of metamorphosis of this fish.

KEYWORDS*:* Development; Electrophoresis; Flatfish; Myofibrillar proteins; Parvalbumin isoforms; Pleuronectiformes; Sole; Turbot

1.Introduction

From hatching to the adult stage, fish muscle development is associated with sequential synthesis of a range of myofibrillar protein isoforms (for a review, see Huriaux et al., 1999). Likewise, two classes of parvalbumin isoforms (larval and adult) have been found to appear successively during growth of various fish (for a review, see Focant et al., 1999). All these biochemical changes occur gradually during fish growth and do not appear particularly related to any precise step in ontogenesis. In an attempt to gain a more in-depth understanding of relationships between morphological and physiological development and the muscle proteins expressed, it was interesting to investigate biochemical changes in the muscles of fish whose life cycle includes a metamorphosis. This is the case of Pleuronectiformes, flatfish whose metamorphosis involves morphological, functional and behavioural changes (Alhstrom et al., 1984; Calvo and Johnston, 1992; Brooks and Johnston, 1993; Gibson and Johnston, 1995; Wagemans et al., 1998; Wagemans and Vandewalle, 2001) and varies in duration from a few days to

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several weeks, according to the species and environmental conditions.

Table 1 *- Fish samples*

Age (Days) Post-hatch	Total length (mm)	Developmental stage	Number of fish	Sample
4	?	larva	30	whole fish
15	?	larva	30	whole fish
25	10	juvenile	18	whole trunk
30	14	juvenile	10	whole trunk
35	17	juvenile	6	whole trunk
50	26	juvenile	6	whole trunk
88	41	juvenile	2	trunk muscles
100	60	juvenile	2	trunk muscles
125	80	juvenile	1	trunk muscles
200	110	juvenile	1	trunk muscles
450	212	late juvenile	4	trunk muscles
+1100	275	adult	2	trunk muscles

Day 18 to 23: duration of metamorphosis.

Few studies have focused on molecular-level muscle fibre composition in the course of flatfish development. In the plaice *Pleuronectes platessa* L., larval myosin light chains LC2 are gradually replaced by adult LC2 in postmetamorphic fish, while changes in myosin heavy-chain composition appear later (Brooks and Johnston, 1993). The same applies to the Japanese flounder *Paralichthys olivaceus* (Temminck and Schlegel); (Takano-Ohmuro et al., 1991). Differential synthesis of troponin-T isoforms during metamorphosis has also been reported (Yamano et al., 1991). More recently, we described in the developing turbot *Scophthalmus maximus* (L.) sequential expression of different myosin heavy chain, myosin light chain LC2, troponin-T, and troponin-I isoforms (Focant et al., 2000). As in other fish examined, parvalbumins appeared as a succession of larval (PA IIa and PA IIb) and adult (PA V) isoforms (Focant et al., 2000). None of these turbot proteins was detected by electrophoresis before

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the onset of metamorphosis (day 20). Biochemical changes were found to occur gradually, each protein according to its own timing and without any clear link to metamorphosis.

The aim of the present work was to study the distribution of myofibrillar proteins and parvalbumin isoforms from hatching to the adult stage in another flatfish, *Solea solea* (Linne, 1758), and to compare the results obtained for the sole and turbot. This soleid fish presents a dextral and very brief metamorphosis (4 to 5 days) with quick structure modifications (Fukuhara, 1988; Boulhic et al., 1992). In the turbot (Scophthalmidae), on the contrary, metamorphosis lasts 3 to 4 weeks and transformations are slow. Moreover, the sole appears phylogenetically more evolved than the turbot (Chapleau, 1993).

2.Materials and methods

2.1. FISH

Larval, juvenile, and adult soles were reared at the MAFF Fisheries hatchery (Conwy, UK). They were stored dry and frozen until used. Specimens ranging in age from 4 days to the adult stage were dissected and pooled in order to obtain sufficient amounts of muscle material for analysis (Table 1). Concerning the late juveniles (450 days) and adults (q1100 days) several specimens from previous rearings were examined in order to rule out problems of variability. Metamorphosis started on day 18 and ended around day 23. Muscle samples were processed according to Focant et al. (1992).

2.2. PREPARATION OF PROTEINS

Myofibrils were prepared and incubated under various conditions as described in Huriaux et al. (1999). Crude parvalbumin extracts were processed according to Focant et al. (1999).

2.3. ANALYTICAL METHODS

Analytical PAGE separations of myofibrillar proteins and parvalbumin isoforms were performed under various conditions as described in Focant et al. (2000). The different myofibrillar proteins were identified by comparing their mobilities in different gel systems according to their physico-chemical characteristics (Huriaux et al., 1999). Their apparent relative molecular masses (M_r) and isoelectric points (p*I*) were determined from one-dimensional gels as described by the same authors, except for the apparent M_r of the larval myosin light chain LC2, which was evaluated from two-dimensional gels. As troponin-C co-migrated with myosin LC2 in SDS-PAGE, the TN-C band isolated by urea alkali-PAGE in the presence of EGTA was cut out and subjected to SDS-PAGE for determination of the apparent M_r. Parvalbumin isoforms were separated from crude extracts by non-denaturing alkali-PAGE at pH 8.6. Their apparent relative molecular masses and isoelectric points were determined by SDS and IEF-PAGE (pH range 4-6) after isolation of the bands from non-denaturing PAGE (Focant et al., 1999). The alpha- or betatype of tropomyosin was identified by comparison with turbot alpha-tropomyosin.

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Figure 1. *20% acrylamide, pH 8.4, SDS-PAGE of myofibrillar proteins from adult sole (S) and late juvenile turbot (T). 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; *: troponin- C.*

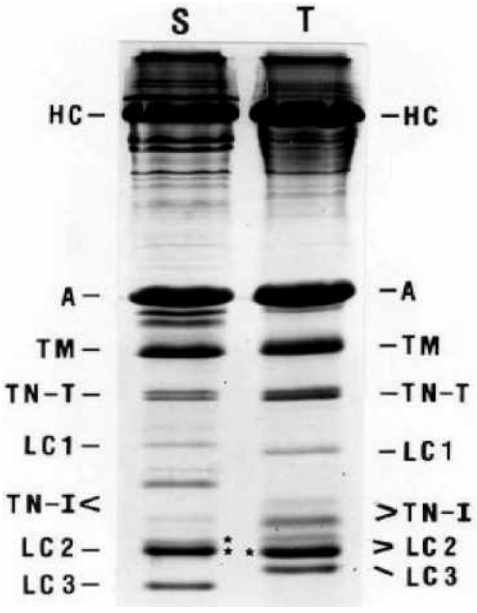

Table 2 *- Apparent relative molecular masses (kDa) of the main myofibrillar proteins from sole and turbot*

Protein	Sole	Turbot*
Actin	44.9	44.5
Tropomyosin	37.5	37.7
Myosin light chain 1	27.8	27.2
Myosin light chain 2 (larval form)	19.9	20.4
Myosin light chain 2 (adult form)	19.1	19.1
Myosin light chain 3	16.1	17.3
Troponin T	33.0	32.9
	32.5	32.3
Troponin I	24.7	23.2
	21.7	21.9
Troponin C	20.0	—
	19.4	19.0

According to Focant et al. (2000).

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***Table 3** - Isoelectric points of myofibrillar proteins from sole and turbot*

Protein	Sole	Turbot*
Tropomyosin	4.97	4.97
Myosin light chain 1	5.09	4.97
Myosin light chain 2 (adult form)	5.09	5.05
Myosin light chain 2 (larval form)	4.92	4.89
Myosin light chain 3	4.59	4.54
Troponin C	4.25	4.30

** According to Focant et al. (2000)*

3. Results

3.1. IDENTIFICATION OF MYOFIBRILLAR PROTEINS

Myofibrillar extract from an adult sole specimen was analysed by one-dimensional SDS-PAGE and compared with a corresponding turbot extract (Fig. 1). The gel displayed, by order of decreasing molecular mass: myosin heavy chains, actin, tropomyosin, a troponin-T (TN-T) doublet, myosin light chain 1 (LC1), two bands of troponin-I (TNI), a doublet corresponding to a mixture of myosin light chain 2 (LC2) and troponin-C (TN-C), and myosin light chain 3 (LC3). Differences between the two flatfish included different apparent relative molecular masses for LC1, the heavier main isoform of TN-I, and LC3 (Table 2). Sole TN-C displayed an additional heavier isoform, as confirmed by two-dimensional PAGE with a urea alkali gel as the first dimension (not shown). The isoelectric points of the myofibrillar components proved similar in the two flatfish, except for myosin LC1. This protein co-focused with adult LC2 in the sole and with tropomyosin in the turbot (Table 3). When subjected to SDS-PAGE, adultsole myosin heavy chains migrated as a single band, somewhat faster than the adult-turbot heavychain band, thus indicating that their molecular mass is lower (Fig. 2).

3.2. EVOLUTION OF MYOFIBRIL COMPOSITION IN THE COURSE OF DEVELOPMENT

One-dimensional SDS-PAGE clearly revealed the presence of actin, tropomyosin, and myosin LC3 from day 30 (length: 1.4 cm) in postmetamorphic specimens. All myofibrillar components were detected from day 35 onward (length: 1.7 cm), but the patterns of the first samples were obscured by a contaminating background of nonmuscle proteins. Throughout sole development, each myofibrillar isoform showed the same apparent relative molecular mass and isoelectric point. So did the myosin heavy-chain band.

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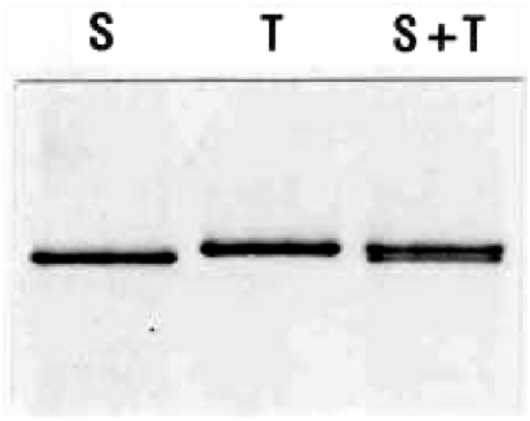
Figure 2. *6% acrylamide, 36% (v/v') glycerol, pH 8.8, SDS- PAGE of myosin heavy chains from adult sole (S) and adult turbot (T).*



A more discriminating technique was then applied to the acidic proteins of sole specimens at different stages of development: two-dimensional PAGE with isoelectric focusing as the first dimension (Fig. 3). As observed previously in the turbot, the same, single isoform of tropomyosin identified as alpha-tropomyosin was found at all developmental stages. All myosin light chains were detectable by day 30, but their relative proportions were found to vary during ulterior growth (Fig. 4). The LC1 content was very low on day 30 and increased until the late juvenile stage. LC3, in contrast, represented 75% of the total light-chain content in 30-day juveniles, but quickly decreased, stabilizing at 26-30%.

Figure 3*. Two-dimensional PAGE of acidic myofibrillar proteins from sole specimens aged: (a) 30 days; (b) 35 days; (c) 88 days, and (d) q 1100 days. IEF-PAGE was used as the first dimension (pH 5.7 on the left side and pH 4.0 on the right side of the window of the electrophoretogram). TM: tropomyosin; LC1: myosin light chain 1; LC2l: larval myosin light chain 2; LC2a: adult myosin light chain 2; LC3: myosin light chain 3. The arrowheads show the position of adult phosphorylated LC2.*

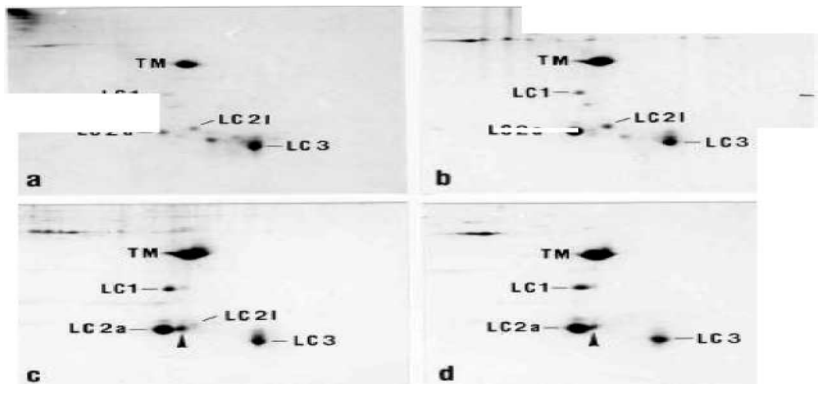


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***Figure 4**. Relative proportions of the myosin light chains as a function of sole age. The data for specimens up to 88 days old were obtained after two-dimensional PAGE. From 100 days onward, the data were calculated by subtracting from the results obtained after one-dimensional SDS-PAGE the percentage of troponin-C estimated after alkali-PAGE in the presence of EGTA. ■, LC1; ◢, larval LC2; ▼, adult LC2; ♦, LC3.*

The proportion of LC2 detected varied in opposite fashion, remaining nearly 60-65% in all, but the first two samples. Its lower content in these samples may reflect the greater instability of this regulatory light chain. After two-dimensional PAGE, the same LC2 spot was observed throughout development, corresponding to adult LC2 (M_r 19.1 kDa; p*I* 5.09) (Fig. 3). A more acidic minor spot, having the same apparent M_r was visible in all samples, except for the first two; it was identified as the phosphorylated form of adult LC2. An additional spot differing from adult LC2 by both its molecular mass and its isoelectric point (M_r 19.9 kDa; p*I* 4.92) was also present in the first four samples, corresponding to the larval LC2 isoform. Its amount decreased from 32% of the total LC2 content in 30-day to only 1% in 88-day juveniles (length: 4.1 cm) (Fig. 4).

Troponin-C appeared identical in all samples, consisting of two isoforms with quite similar M_r values, 20.0 and 19.4 kDa, and the same isoelectric point (4.25). When subjected to SDS-PAGE, the former migrated like larval myosin LC2 and the latter like adult myosin LC2 (Table 2 and Table 3). After urea alkali-PAGE in the presence of EGTA, this calcium-binding protein was estimated to represent 4-5% of the total light-chain content.

One-dimensional SDS-PAGE of the basic proteins showed that two troponin-T and two troponin-I isoforms are synthesised in adult sole muscles (Fig. 1 and Table 2). They were detected from day 50 (juveniles 2.6 cm long). Both TN-T isoforms with close apparent M_r values (33.0 and 32.5 kDa) appeared to be co-expressed during fish development. The 32.5-kDa isoform predominated in all juvenile stages; then the two isoforms appeared in fairly equal content (Fig. 5). As for TN-I, its 24.7-kDa isoform appeared throughout development as the essential component. A 21.7- kDa isoform was detected from day 100 (juveniles 6 cm long); its content increased during later growth, reaching 15% in adult soles (Fig. 5). When subjected to NEIEF-PAGE the 24.7-kDa TN-I split into three spots having the same molecular mass, as observed in the first dimension of Fig. 6. The spot showing an intermediate migration speed at alkaline pH was the main component in all samples. The slower spot (Fig. 6a, left) was present in juveniles. The faster spot (Fig. 6b, right) appeared in fish 100 days old and increased in proportion until the adult stage. Fig. 6b also shows the minor, 21.5-kDa TN-I isoform.

3.3. IDENTIFICATION OF PARVALBUMINS

After non-denaturing PAGE, the trunk muscle of adult fish showed only the PA III isoform. Younger specimens additionally showed traces of isoforms PA I and PA II (poorly defined doublets) (Fig. 7). Molecular mass and isoelectric point values are compared in Table 4 with those previously determined for the turbot (Focant et al., 2000). Both flatfish show a major parvalbumin isoform, PA III in the sole and PA II in the turbot. Unlike the latter, the former displays no PA IV or PA V, but PA III has the high M_r and p*I* typical of adult isoforms. Moreover, PA III appeared homogeneous after IEF-PAGE, contrary to turbot PA II, composed of early and late larval isoforms.

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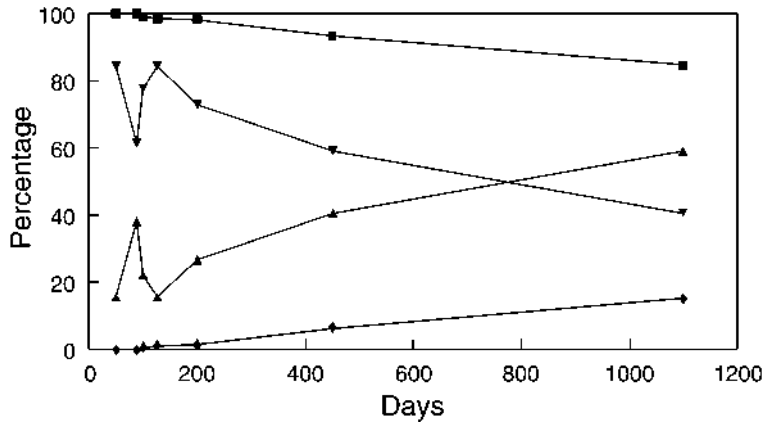
***Figure 5**. Relative proportions of the two troponin-T and two troponin-I isoforms as a function of sole age. The data were obtained after one-dimensional SDS-PAGE, using the usual migration time for troponin-I, but twice the usual migration time for troponin-T. **m**, 33.0 kDa troponin-T; %, 32.5 kDa troponin-T; **j**, 24.7 kDa troponin-I; ♦, 21.7 kDa troponin-I.*



3.4. EVOLUTION OF PARVALBUMIN ISOFORMS IN THE COURSE OF DEVELOPMENT

Fig. 8 shows how the relative proportions of parvalbumin isoforms varied during growth of the sole, as estimated after non-denaturing PAGE. Traces of PA I and PA II were detected only from day 35 (juveniles 1.7 cm long). All three isoforms (PA I, PA II, and PA III) were observed from day 50 (juveniles 2.6 cm long). PA I and PA II decreased rapidly during the juvenile stage. Only PA III was present in adult fish. IEF-PAGE confirmed this variation and also the homogeneity of all three isoforms (not shown).

4. Discussion

The metamorphic transformations of pleuronectiform fishes include the loss of bilateral symmetry of the larval body, with migration of either the left eye (sole) or the right eye (turbot) to the other side of the body. This process is accompanied by major cranial modifications and other metamorphic events and by a transition from a pelagic to a benthic lifestyle (linked to a change in swimming and feeding behaviour)(Marchand, 1992; Wagemans et al., 1998; Wagemans and Vandewalle, 2001). Metamorphosis is a complex biological phenomenon involving hormonal controls regulated by various factors (Hoar, 1957; Youson, 1988; Inui et al., 1995). In this work, developmental changes (from the premetamorphic larval stage to adulthood) in the isoform composition of the various white-muscle myofibrillar proteins and parvalbumins were examined in the sole, a flatfish displaying fast metamorphosis. The patterns were compared with those observed previously in the turbot, whose metamorphosis is slow (Focant et al., 2000).

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***Figure 6**. Two-dimensional PAGE of basic myofibrillar proteins from sole specimens aged: (a) 125 days; (b) 450 days. NEIEF-PAGE was used as the first dimension with proteins migrating to the cathode (right). T: troponin-T; I: troponin-I.*

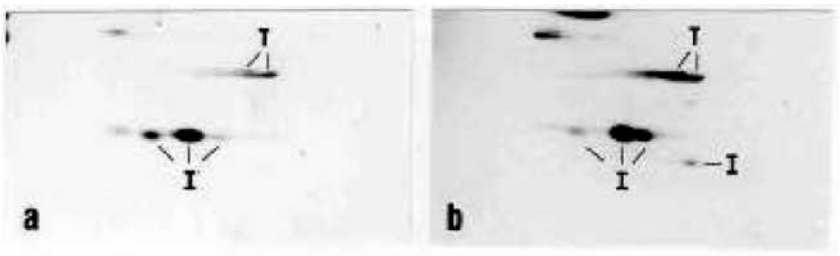
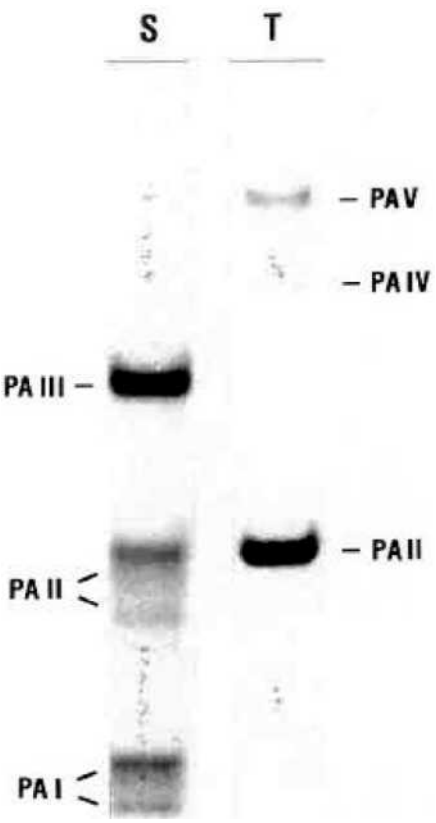


***Figure 7**. Non-denaturing-PAGE (pH 8.6) of parvalbumin isoforms from juvenile sole (S) and adult turbot (T).*



It is worth noting that any myofibrillar components or parvalbumins cannot be detected in the sole until quite late: not until day 30 for the former and day 50 for the latter. By day 30, metamorphosis is already finished in this species. Delayed detection of the turbot proteins was also noted, but the delay was less pronounced: all proteins could be monitored from day 20, corresponding to the beginning of metamorphosis. It is worth noting that any myofibrillar components or parvalbumins cannot be detected in the sole until quite late: not until day 30 for the former and day 50 for the latter. By day 30,

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metamorphosis is already finished in this species. Delayed detection of the turbot proteins was also noted, but the delay was less pronounced: all proteins could be monitored from day 20, corresponding to the beginning of metamorphosis.

Table 4 *- Apparent relative molecular masses (kDa) and isoelectric points of parvalbumin isoforms from sole and turbot*

	Sole		Turbot*	
Isoforms	M_r	p*I*	M_r	pI
PA V			11.75	5.12
PA IV			11.80	4.95
PA III	12.04	5.23		
PA II q	11.36	4.73		
PAIIb			11.40	4.75
PAIIa			11.38	4.58
PAIIa				
PAI+	11.30	4.69		

* According to Focant et al. (2000).

+ Values for PA II and PA I were measured from the doublet cut from non-denaturing gels.

Perhaps the larval isoforms present in premetamorphic soles and during the fast metamorphosis are very labile. Or perhaps they are present in very low amount or not extracted under our experimental conditions. Fresh sampled pre- and post-metamorphic specimens analysed by more discriminative electrophoretic techniques might bring new data in these crucial developmental stages. Delayed detection of myosin subunits and parvalbumins and late transition between isoforms thereof have also been described in the case of a non-metamorphosing fish, *Dicentrarchus labrax* (L.) (Scapolo et al., 1988; Focant et al., 1995; Huriaux et al., 1996).

Sole and turbot myofibrillar components have quite similar apparent relative molecular masses and isoelectric points. Only the myosin heavy chains, myosin light chain LC1, and the heavy form of troponin-I (always the major isoform in the sole) showed slight differences. The higher molecular mass determined for the turbot light chain LC3 is due to a fish-species-dependent migration anomaly at alkaline pH (Huriaux and Focant, 1978, 1985). Sole troponin-C exists as two isoforms having the same isoelectric point but different molecular masses, whereas only one isoform is present in the turbot.

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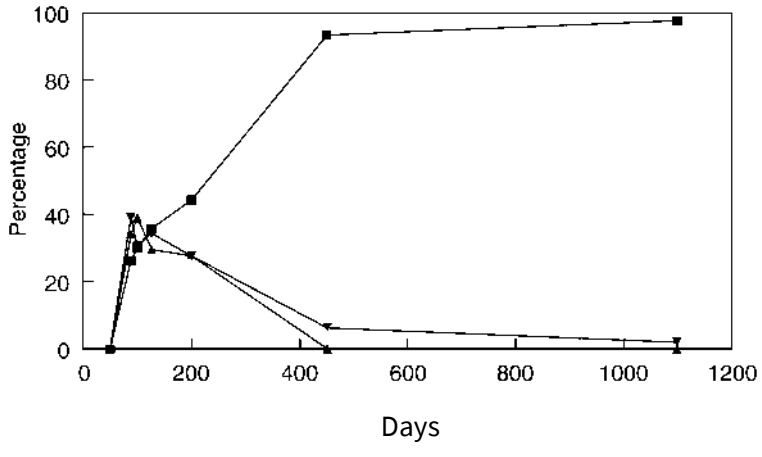
Figure 8. Relative proportions of each parvalbumin isoform as a function of sole age, as determined by non-denaturing-PAGE. ▲, PA I; ▼, PA II; ■, PA III.



Myosin heavy chains, actin, alpha-tropomyosin, and troponin-C appear unchanged during growth of the sole. Myosin alkali light chains are also constant in composition, but the postmetamorphic day 30 sample showed an extremely high ratio of LC3 to LC1. This phenomenon has been observed in the turbot (Focant et al., 2000) and in *Brycon moorei* (Steindachner) (Huriaux et al., 2003), but in these species it was observed in the earlier developmental stages. This is in keeping with the late detection of sole myofibrillar proteins. Two myosin light chains LC2 (larval and adult isoforms) with different molecular masses and isoelectric points are co-expressed in sole in the early postmetamorphic stage (first four samples postmetamorphosis). Synthesis of the minor larval LC2 is very transient as compared to its synthesis in the turbot, where it was detected up to the 210th day (juvenile stage). We cannot rule out the possibility that the larval LC2 might be the main regulatory light chain isoform in younger sole specimens and that it was not detected because of high instability. Transition from the larval to the adult LC2 isoform thus appears earlier in the sole than in the turbot, perhaps due to faster metamorphosis. In several fish two LC2 isoforms have been observed, appearing successively in the course of development (Crockford and Johnston, 1993; Yamano et al., 1994; Johnston et al., 1997; Huriaux et al., 1999; Focant et al., 2000; Huriaux et al., 1999, 2003). In the plaice, two larval and two postmetamorphic LC2 isoforms have been distinguished (Brooks and Johnston, 1993), whereas the sole and turbot produce only one of each. In the sole, two TN-T isoforms and two TN-I isoforms are observed after one-dimensional SDS-PAGE, but their relative proportions vary less markedly in the course of development than in the turbot (Focant et al., 2000). In both fish, the transition from one isoform to the other occurs at different speeds for the two troponins, suggesting that the genes coding for these two thin-filament subunits are differentially regulated. The timing of synthesis of the TN-I isoforms is very peculiar in sole. The 24.7-kDa isoform is practically the only form present in juvenile stages, and even in late juveniles and in adults the 21.7-kDa isoform is present only in low amount.

Troponin-I was detected from day 50 onwards, but presumably younger specimens also express only the 24.7-kDa isoform. Yet, this heavy TN-I isoform appears heterogeneous when subjected to two-dimensional PAGE: three spots are observed, varying in relative proportions during sole growth. This

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suggests sequential expression of these forms. In both flatfish, the lighter TN-T isoform is synthesised before the heavy one, whereas the heavier TN-I isoform is synthesised first. Distinct isoforms of TN-T and/or TN-I have been described in the development of other fish known to undergo metamorphosis or not (Yamano et al., 1991; Crockford and Johnston, 1993; Chikou et al., 1997; Johnston et al., 1997; Huriaux et al., 1999, 2003). The timing of appearance of these basic proteins varies considerably according to the fish species. Isoform changes may affect the regulation of muscle contraction.

Non-denaturing PAGE reveals different parvalbumin isoforms in the sole and turbot: in the former, predominance of a single parvalbumin isoform (PA III), and in the latter, a major larval PA II and a minor adult PA V. To our knowledge, sole is the only fish reported to have a PA III isoform with such a high *pI;* this is usually typical of adult PA IV and PA V. This sole PA III must thus belong to the alpha-class of parvalbumins. Its features confirm its adult character. As in all fish previously examined, the parvalbumin isoforms of these flatfish appear highly species-specific. The parvalbumin pattern could be of value in screening to identify the fish species used in processed foods. The presence of specific parvalbumin isoforms in two fish belonging to the same order (Pleuronectiformes) may reflect the phylogenetic distance between the Scophthalmidae and Soleidae families, the latter being more evolved (Alhstrom et al., 1984).

Our analysis of parvalbumin synthesis during sole growth has yielded some unusual findings. One peculiarity is the early appearance of adult PA III (on day 50), which quickly becomes the predominant component. Larval parvalbumins (PA I and PA II) were detected only in trace amounts, appearing on day 35 and decreasing thereafter. Because of their poor definition and low concentration, we cannot exclude the possibility that we failed to extract them adequately or that these larval isoforms are highly unstable in very small, deep-frozen specimens. Their apparent M_r and p*I* values, characteristic of parvalbumins, and their homogeneous appearance after IEF-PAGE support the assumption that they are indeed parvalbumins. The early predominance of adult PA III in the sole and the fast disappearance of larval PA I and PA II may be related to the brevity of metamorphosis. During the longer turbot metamorphosis, the earlyappearing larval PA IIb isoform and the late- appearing larval PA IIa isoform were successively synthesized, and PAIIa remains a major isoform until the young adult stage. The turbot's late isoform transition is probably involved in metamorphosis-linked physiological modifications of the muscle machinery. No similar correlation was found in the sole, where the adult pattern is established relatively early. Also, noteworthy is the fact that sole parvalbumin isoforms were detected on gels later than the myofibrillar proteins, as previously seen with *D. labrax* (Huriaux et al., 1996), turbot (Focant et al., 2000), and *B. moorei* (Huriaux et al., 2003).

In summary, the profiles of the myofibrillar subunits and parvalbumin isoforms vary little in the course of sole development and display early the adult traits. Among the myofibrillar proteins, changes include only the transient appearance of a larval myosin LC2 and a slight transition among troponin isoforms. The parvalbumin isoform pattern remains fairly unchanged. No conclusions regarding the variation of muscle proteins during metamorphosis could be drawn. The electrophoretic detection of these proteins only after completion of this crucial life stage does not exclude a proportionally more important synthesis of larval isoforms before and during the metamorphosis.

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