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Developmental changes in the composition of myofibrillar proteins in the swimming muscles of Atlantic herring, *Clupea harengus*

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Abstract. Changes in myofibrillar protein composition during development have been investigated in the swimming muscles of the Atlantic herring *Clupea harengus* L. using a range of electrophoretic techniques. The main muscle-fibre type of larvae, and the fast- and slow-muscle fibres of adult fish were found to contain distinct isoforms of myosin heavy chain (MHC) and myosin light chain 2 (LC2). Larval LC2 was present as a minor component of adult fast-muscle myosin. In contrast, larval and adult fast-muscle myosin appeared to contain identical alkali light chains. Tropomyosin and troponin C were also identical in larval and in adult fast-muscle. All three muscle-fibre types contained unique isoforms of troponin T (TNT) and troponin I (TNI). Larval muscle had multiple isoforms of TNT, some of which may correspond to embryonic forms. It was concluded that although the main muscle-fibre type in larvae shares some myofibrillar proteins with adult fast muscle, it also contains characteristic isoforms of MHC, TNI, TNT and LC2 and therefore represents a distinct fibre type. The particular combination of myofibrillar proteins present at any developmental stage was found to be dependent on the rearing temperature. For example, a higher proportion of embryonic TNT isoforms were present at hatching in larvae reared at 5 °C than at either 10 or 15 °C. Over a period of 7 d, there was a gradual reduction in the number of TNT isoforms, but the pattern in 5 °C larvae after 7 d still did not resemble that in 1 d-old larvae reared at 15 °C.

Introduction

Herring larvae lack gills at hatching and the swimming muscles receive their oxygen supply directly across the skin (Batty 1984). The myotomes of yolk-sac herring larvae contain a single superficial layer of small-diameter muscle fibres which contain high-volume densities of mitochondria ($Vv_{(mt,f)} \geq 46\%$) (Vieira and Johnston 1992). The remaining muscle, which comprises 95% of the myotomal cross-sectional area is composed of larger-diameter fibres that are also aerobic in character ($Vv_{(mt,f)} \geq 26\%$) (Vieira and Johnston 1992). Studies on cyprinid fish have shown that the main lactate dehydrogenase (LDH) isoenzyme present in larval muscle is the aerobic H_4-form (El-Fiky et al. 1987). Later, as the gills and capillary circulation develop, the activities of aerobic enzymes in muscle decrease and the anaerobic M_4-form of LDH becomes the pre-dominant isoenzyme (El-Fiky et al. 1987). In herring, the transition from larval to adult muscle-fibre types is complete at ~29 mm (Batty 1984) and coincides with the development of functional gills (de Silva 1974), and pronounced changes in swimming style (Batty 1984, Blaxter 1988).

It is known from studies of birds and mammals that muscle development is associated with the sequential expression of a range of myofibrillar protein isoforms (Bandman et al. 1982, Crow and Stockdale 1986, Briggs et al. 1990). Isoforms of myosin heavy chains (MHC) and light chains (LC) which are specific to particular stages of development have been identified in skeletal muscle (Whalen et al. 1979, Barton et al. 1985). In some cases, isoforms which are present in specific fibre types in the adult are transiently expressed in a non-specific manner in the embryo (Dhoot and Perry 1980, Barton and Buckingham 1985). Myofibrillar protein isoforms are either the products of different genes (Periasamy et al. 1984) or are produced by an alternative splicing mechanism and/or differential transcription (Barton and Buckingham 1985, Breitbart et al. 1985). For example, in the rat, the alternative splicing of a single fast-muscle troponin T gene could theoretically result in 64 TNT isoforms (Breitbart et al. 1985), of which 13 have been identified (Sabry and Dhoot 1991). In the case of tropomyosin (Tm), different isoforms are generated from the alternative splicing and differential transcription of several genes (Montarras et al. 1981).

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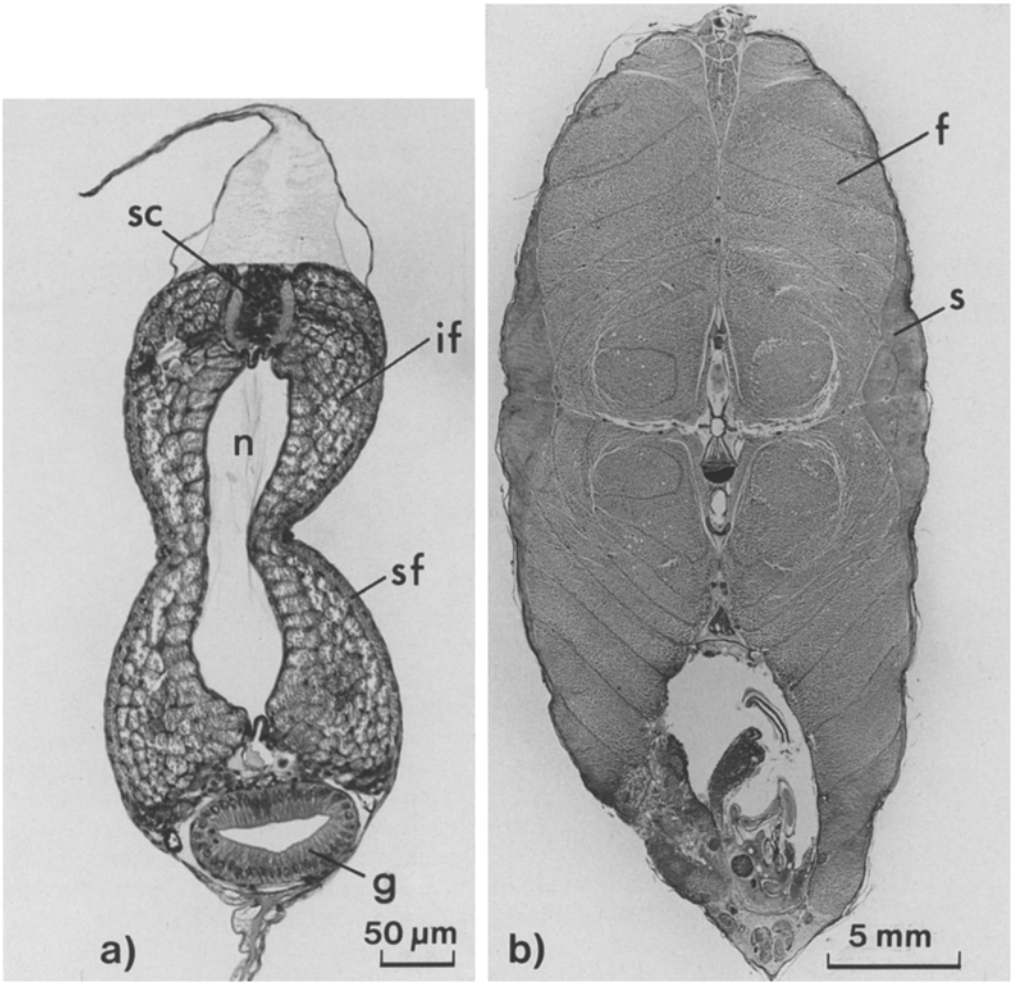

Fig. 1. *Clupea harengus*. Transverse wax sections of a 1 d-old larvae (a) and herring of 26 cm standard length (b) stained with haemotoxylin-eosin. if: inner muscle fibres; sf: superficial muscle fibres; f: adult fast-muscle fibres; s: adult slow-muscle fibres; sc: spinal cord; n: notochord; g: gut

In contrast, relatively little is known about the expression of myofibrillar protein isoforms in developing fish muscle. In an immuno-histochemical study of zebrafish larvae, van Raamsdonk et al. (1978) found that anti-sera raised against red and white muscle proteins from adult carp cross-reacted against the superficial and inner-muscle fibres, respectively. Using polyclonal antibodies to mullet proteins, four myosin isoforms were identified in the muscle of larval *Dicentrarchus labrax* (Scapolo et al. 1988). These were referred to as early white-muscle myosin (hatching to Days 10 to 28), early red-muscle myosin (hatching to Day 28), late white-muscle myosin (Day 28 to 20 mo), and late red-muscle myosin (Days 28 to 80). In the barbel (*Barbus barbus* L.), different myosin isoforms were identified in embryonic, larval and adult fast-muscle, and the ratios of LC1:LC3 and parvalbumin isoforms were also found to change during development (Focant et al. 1992).

The present study examines developmental changes in the myofibrillar protein composition of myotomal muscles in the Atlantic herring *Clupea harengus* L. Since temperature is known to have a profound effect on development in fish (Blaxter 1988), the influence of rearing temperature on the expression of myofibrillar proteins in larvae was also investigated.

Materials and methods

Fish

Spring-spawning Atlantic herring (*Clupea harengus* L.) were caught in the Firth of Clyde, Scotland, in March 1990. Samples of fast and slow muscles were isolated from the myotomes of adult fish (32 to 35 cm standard length) and stored in liquid nitrogen until used. The gonads of ripe herring were transported to the Dunstaffnage Marine Research Laboratory, Oban, Argyll. Eggs were fertilised and attached to glass plates which were incubated in black-walled tanks supplied with flowing seawater (Blaxter 1968). The initial characterisation of contractile proteins in larvae was carried out using fish reared at ambient temperatures (8 to 10 °C). In order to investigate the effect of temperature on myofibrillar protein composition, eggs from a single female were fertilized with milt from several males and incubated at either 5, 10 or 15 °C in temperature-controlled rooms. 1d- and 7d-old larvae were killed by anaesthesia in a 1:5000 (v/v) solution of benzocaine in seawater. The yolk sacs and heads of individual larvae were removed under a binocular microscope on a cooled stage (4 °C), and the trunks were skinned and gutted using jewellers' forceps. This procedure removed the single layer of superficial muscle fibres which remained attached to the skin. The arrangement of muscle fibres in larvae and in adult fish is shown in Fig. 1.

Muscle samples for electrophoresis were suspended in a small volume of preparation buffer and stored in liquid nitrogen (−159 °C) until required. The preparation buffer contained (in

mmol/l): Tris-HCl, 10; NaCl, 50; ethylene-diaminetetraacetic acid, 1; pH 7.4; and the following proteolytic enzyme inhibitors, 50 μg/ml n-tosyl-L-phenylalanine chloromethyl ketone, 50 μg/ml phenylmethylsulphonyl fluoride, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin A and 0.2 U/ml aprotinin.

Gel electrophoresis

Myofibrils were prepared on ice from muscle samples as described by Focant et al. (1976). To prepare samples for electrophoresis, aliquots of myofibrils of known protein concentration (Lowry et al. 1951) were collected by centrifugation. Sodium-dodecyl-sulphate polyacrylamide-gel electrophoresis (SDS PAGE) was carried out as described by Laemmli (1970) with the inclusion of 10 m*M* DL-dithiothreitol (DTT) in the sample buffer. Alkali-urea PAGE (AU PAGE) and sample preparation was performed using the method of Focant et al. (1976). Two-dimensional electrophoresis was carried out as described by O'Farrell (1975) using the following combination of ampholines (Pharmacia, Uppsala, Sweden): basic proteins, 1.67% Pharmalyte 3–10 and 3.33% Pharmalyte 9–11; acidic proteins, 1.67% Pharmalyte 3–10, 1.67% Pharmalyte 4–6.5 and 1.67% Pharmalyte 2.5–5. Peptide maps of electrophoretically purified myosin heavy chains were prepared using the methods in Crockford et al. (1991).

Gels were stained with either Coomassie Brilliant Blue G-250 (Neuhoff et al. 1988), 1-ethyl-2-[3-(ethylnaphtho[1,2-d]-thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-d]-thiazolium bromide ("Stains-all") (Campbell et al. 1983) or silver (Bloom et al. 1987).

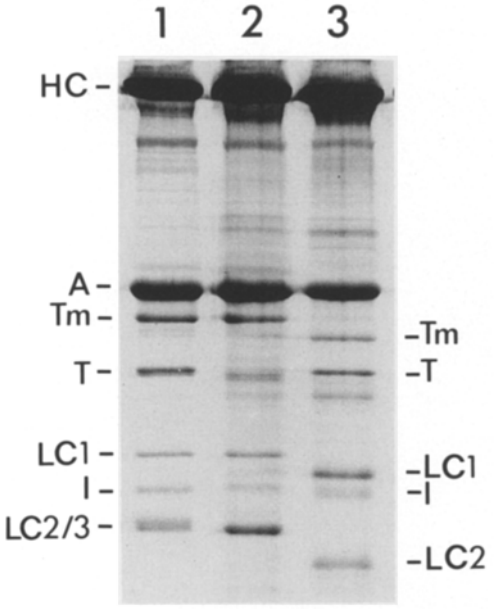

Fig. 2. *Clupea harengus*. 14% acrylamide SDS PAGE gel of myofibrillar proteins from adult fast-muscle (Lane 1), larval inner-muscle (Lane 2), and adult slow-muscle (Lane 3) stained with Coomassie Brilliant Blue G-250. HC: myosin heavy chain; A: actin; Tm: tropomyosin; T: troponin T; LC1: myosin light chain 1; I: troponin I; LC2: myosin ligth chain 2; LC3: myosin light chain 3

Identification of myofibrillar proteins

Myosin heavy chains and actin were identified by their relative abundance and their apparent moleculer mass on SDS PAGE. The position of tropomyosin on 2-D PAGE was indicated by its anomalous migration on SDS PAGE in the presence and absence of 8 *M* urea (Sender 1971, Crockford and Johnston 1990). Troponin C was identified on 1-D PAGE and alkali urea gels using "Stains all" which stains Ca^{2+}-binding proteins blue (Campbell et al. 1983). The light chains of myosin were identified by their characteristic migration in the neutral to acid pH range on 2-D PAGE gels (Rowlerson et al. 1985, Martinez et al. 1990). Isoforms of troponin T and troponin I are well separated from the other myofibrillar proteins on IEF gels. Both proteins have basic isoelectric points; however, troponin I migrates with a significantly lower apparent relative molecular mass (M_r) on SDS PAGE than troponin T isoforms (Wilkinson et al. 1984, Imai et al. 1986). The apparent M_r of the myofibrillar proteins were estimated using protein standard kits covering the range 14.2×10^3 to 205×10^3 (MW-SDS-200, MW-SDS-70L, Sigma).

Results

One-dimensional SDS PAGE gels revealed protein bands which were specific to either adult fast muscle, adult slow muscle or the inner muscle fibres of the larvae of *Clupea harengus* (Fig. 2). The only proteins which had similar

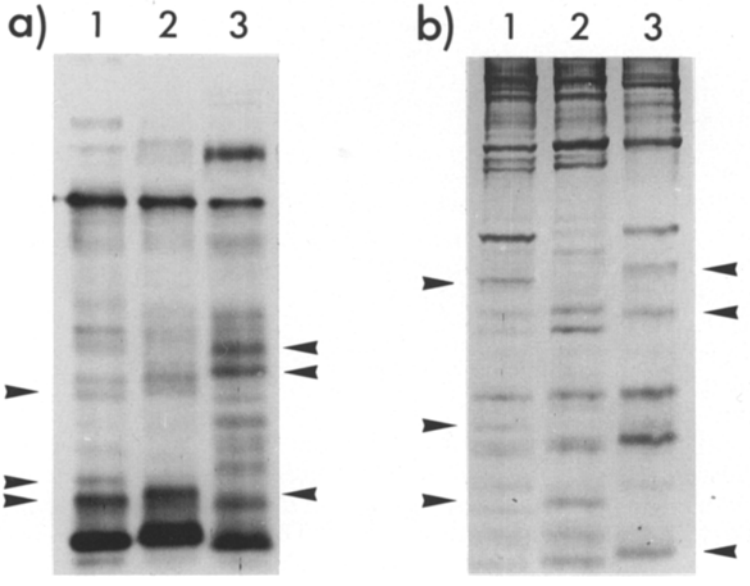

Fig. 3. *Clupea harengus*. Peptide maps of myosin heavy chains from adult fast-muscle (Lane 1), larval inner-muscle (Lane 2), and adult slow-muscle (Lane 3), digested with α-chymotrypsin (a), or *Staphylococcus aureus* V8 protease (b). Peptides were separated on 15% acrylamide SDS PAGE gels and stained with silver. Arrows on left of lanes indicate differences between larval inner-muscle and adult fast-muscle, those on right indicate differences between larval inner-muscle and adult slow-muscle

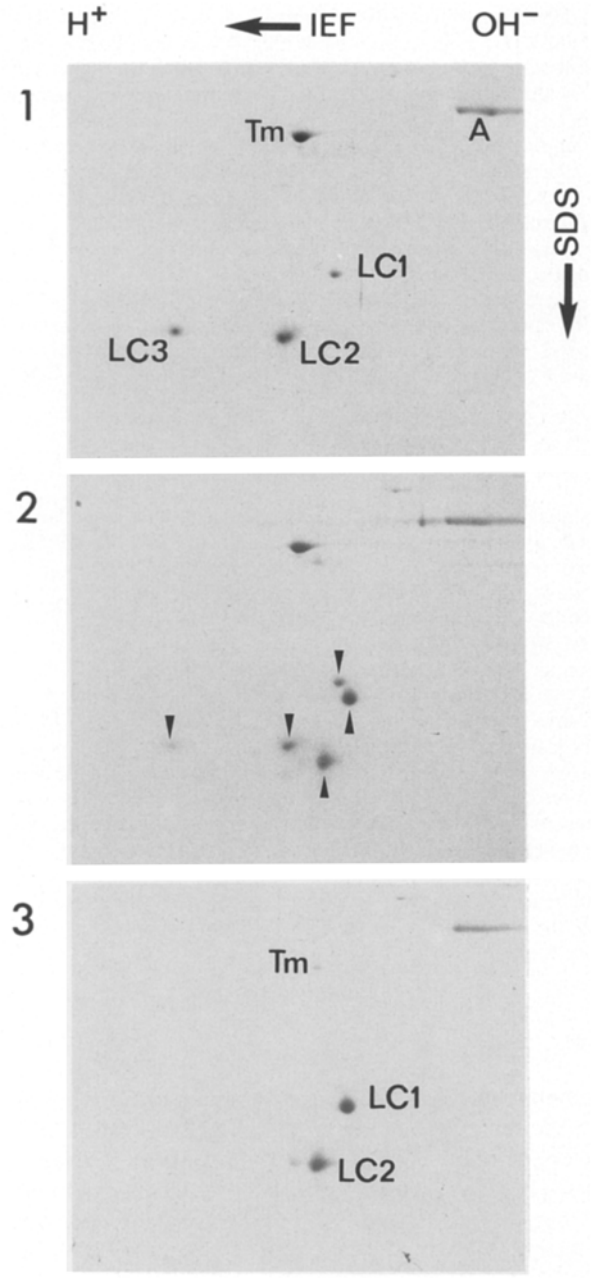

Fig. 4. *Clupea harengus.* Two-dimensional PAGE of acidic myofibrillar proteins from larval inner-muscle (1), mixture of larval inner-muscle and adult slow-muscle (2) and adult slow-muscle (3) stained with Coomassie Brilliant Blue G-250. Isoelectric focusing (IEF) in first-dimension and 15% acrylamide SDS PAGE in second dimension. Abbreviations as in Fig. 2. Larval muscle proteins indicated by downward-facing arrowheads, adults slow-muscle proteins by upward-facing arrowheads

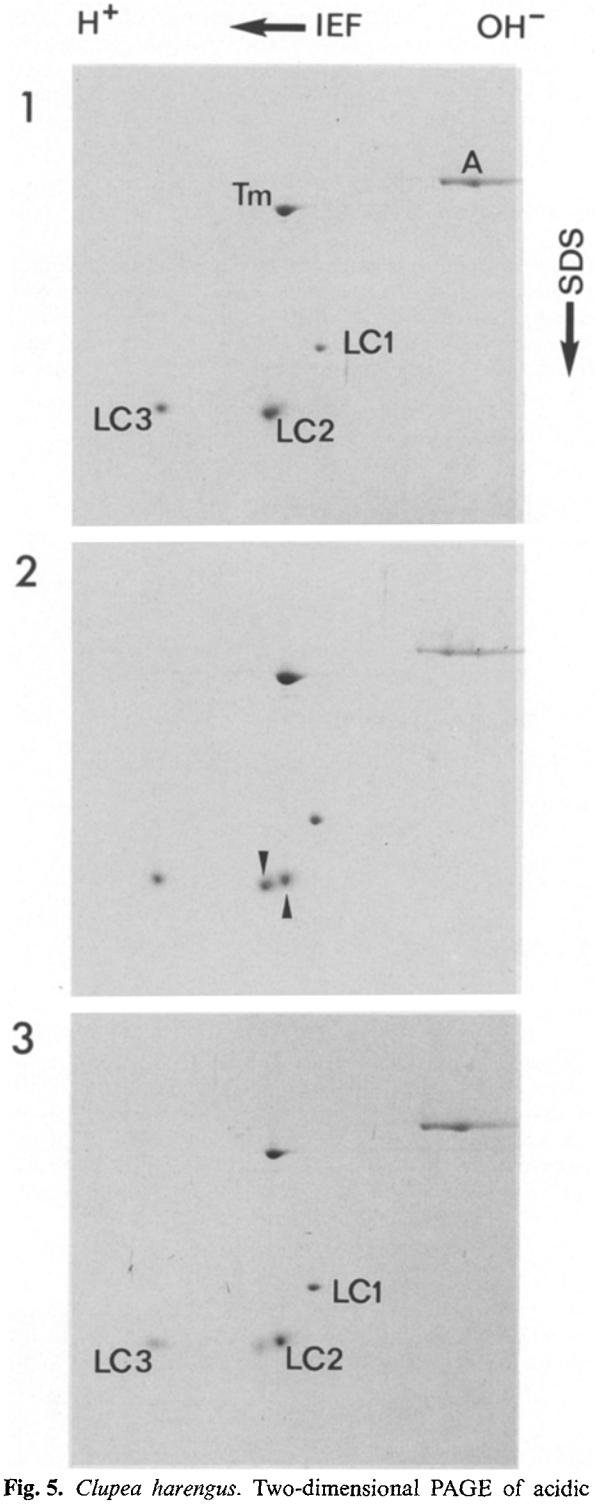

Fig. 5. *Clupea harengus.* Two-dimensional PAGE of acidic myofibrillar proteins from larval inner-muscle (1), mixture of larval inner-muscle and adult fast-muscle (2), and adult fast-muscle (3) stained with Coomassie Brillant Blue G-250. Larval muscle LC2 is indicated by a downward-facing arrowhead, adult fast-muscle LC2 by an upward-facing arrowhead. Further details as in Fig. 4

apparent relative molecular masses (M_r) in all fibre types were actin and the heavy chains of myosin (Table 1; Fig. 2). However, peptide-mapping revealed that distinct isoforms of myosin heavy chains were present in all three muscle types (Fig. 3). The apparent M_r of the myofibrillar proteins are shown in Table 1.

Two-dimensional (2-D) PAGE of the acidic proteins show that larval muscle has no bands in common with adult slow-muscle (Fig. 4). Myosin alkali light chain 1 (LC1), myosin alkali light chain 3 (LC3) and tropomyosin (Tm) had the same relative mobility on 2-D gels in larval and adult fast-muscles (Fig. 5). A major and a minor spot for myosin light chain 2 (LC2) were found in adult fast-muscle (Fig. 5). The minor spot, which had a slightly lower isoelectric point (pI) and apparent M_r

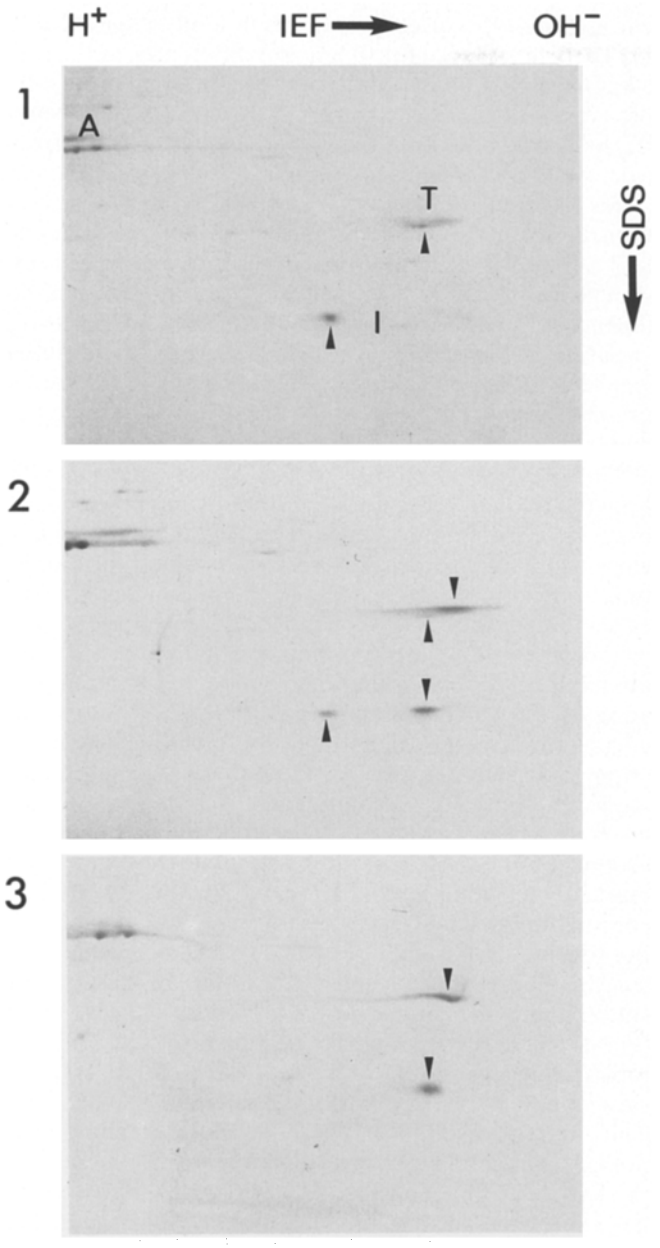

Fig. 6. *Clupea harengus.* Two-dimensional PAGE of basic myofibrillar proteins from larval inner-muscle (1), mixture of larval inner-muscle and adult fast-muscle (2), and adult fast-muscle (3) stained with Coomassie Brillant Blue G-250. A: actin; T: troponin T; I: troponin I. Larval muscle proteins are indicated by upward-facing arrowheads and adult fast-muscle proteins by downward-facing arrowheads (note that, for clarity, direction of arrowheads is opposite to that in Fig. 4)

corresponded to the single LC2 present in larval muscle (Fig. 5). One- and two-dimensional PAGE of the basic proteins showed that different isoforms of troponin T (TNT) and troponin I (TNI) were present in larval muscle and adult fast-muscle, differing in both pI and apparent M_r (Figs. 2 and 6). Using "Stains-all", troponin C (TNC) from larval muscle and adult fast-muscle was found to have the same apparent M_r and relative mobility on AU PAGE. TNC had a higher apparent M_r and lower relative mobility on AU PAGE in slow adult muscle than in the other fibre types (gels not shown).

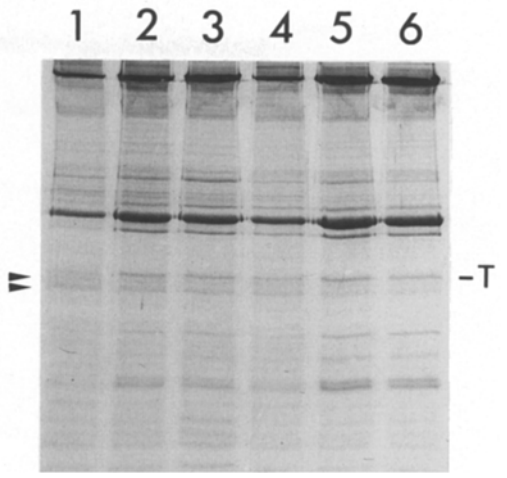

Fig. 7. *Clupea harengus.* 14% acrylamide SDS PAGE gel of myofibrillar proteins from larval inner-muscle fibres stained with Coomassie Brilliant Blue G-250. 1, 2, 3; 1 d-old at 5, 10 and 15 °C, respectively; 4, 5, 6: 7 d-old at 5, 10 and 15 °C, respectively. T: larval troponin T; arrowheads indicate position of embryonic isoforms of troponin T

Table 1. *Clupea harengus.* Apparent relative molecular masses of the main myofibrillar proteins in herring muscle

Proteins	Larval inner	Adult	
		fast	slow
Actin (A)	44.2	44.2	44.2
Myosin heavy chain (MHC)	210	201	210
Myosin light chain 1 (LC1)	24.7	24.7	23.5
Myosin light chain 2 (LC2)	≈19.1	≈19.1	17.1
Myosin light chain 3 (LC3)	≈19.1	≈19.1	–
Tropomyosin (Tm)	39.8	39.8	37.8
Troponin T (TNT)	32.2	32.6	33.1
Troponin I (TNI)	22.4	21.8	22.2
Troponin C (TNC)	15.8	15.8	16.3

The composition of larval myofibrillar proteins was influenced by rearing temperature, particularly with respect to TNT isoforms (Fig. 7). In larvae reared at ambient seawater temperatures, one major and several faint spots of TNT were visible after staining with Coomassie Blue (Fig. 6). These minor TNT isoforms were the major TNT components in 1 d-old larvae reared at 5 °C (Figs. 7 and 8). Seven days after hatching, the minor TNT isoforms had almost disappeared at 10 to 15 °C, but were still prominent at 5 °C (Fig. 7).

Discussion

White and red muscles in teleosts are composed of fast- and slow-twitch fibres, respectively (Altringham and Johnston 1988). Electromyographical studies with adult Pacific herring (*Clupea harengus pallasi*) have shown that there is a distinct division of labour between white and red muscles. It was found that fish (15 to 17.5 cm fork length) were able able to swim at 4 body lengths/s for at

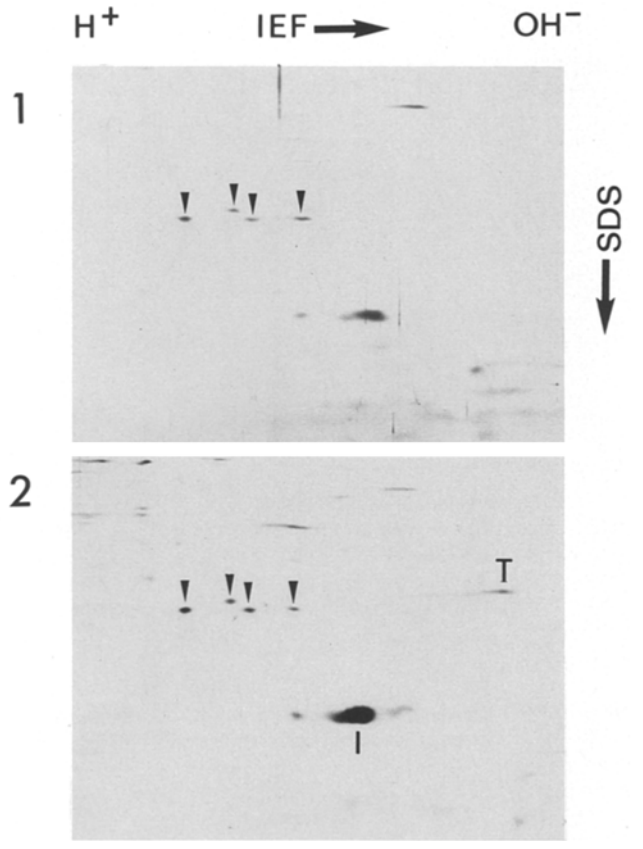

Fig. 8. *Clupea harengus*. Two-dimensional PAGE of basic myofibrillar proteins from inner-muscle of larvae reared at 5 °C (1) and at 10 °C (2) stained with silver. Further details as in Fig. 4. T: troponin T; I: troponin I; arrowheads indicate embryonic isoforms of troponin T

least 5 h using their red muscle alone. At higher speeds, fast-muscle fibres were progressively recruited and speeds above 5 body lengths/s could only be sustained for 1 to 2 min (Bone et al. 1978). In tuna, maximum contraction velocities (V_{max}) are 2 to 4 times higher in fast than in slow myotomal muscle fibres (Johnston and Brill 1984). Experiments with isolated muscle fibres have shown that the contraction times and shortening speeds (V) of slow-muscle fibres are too slow to generate net positive work at the high tail-beat frequencies required for fast swimming (Altringham and Johnston 1990, Johnston 1991). Fast-muscle fibres are recruited when the V/V_{max} for slow-muscle fibres exceeds 0.17 to 0.36, which corresponds to the speeds predicted for maximum power and efficiency from the force-velocity relationship (Rome et al. 1988, 1992). Myosin heavy chains are thought to be the major determinant of V_{max} in amphibian and mammalian muscles (Lännergren 1987, Reiser et al. 1988), with the myosin light chains playing a modulatory role (Sweeney et al. 1986, Greaser et al. 1988). Fast- and slow-muscle fibres in adult herring contain distinct isoforms of both myosin heavy and light chains, as has been reported for several other teleost species (Rowlerson et al. 1985, Scapolo and Rowlerson 1987, Karasinski and Kilarski 1989). The present study on *Clupea harengus* has shown that all the components of the tropomyosin–troponin complex occur as fibre-type-specific isoforms in adult stages (Table 1). There is evidence for functional differences in the calcium sensitivity of myofibrils between different muscle-fibre types in fish. For example, in the cod *Gadus morhua* L., half-maximal force generation requires higher free-calcium concentrations (pCa) in fast than in slow-muscle fibres (Altringham and Johnston 1982). Direct correlations between the pCa-tension relationship and the content of tropomyosin and troponin T isoforms have been shown in single-skinned fibres from rabbit muscle (Schachat et al. 1987, Greaser et al. 1988). Therefore, variation in the mechanical properties of muscle fibres can be achieved by changing some or all of the isoforms present, giving rise to a whole spectrum of fibre types between pure fast and pure slow.

The Reynolds numbers of fish larvae increase with growth and development, resulting in pronounced changes in swimming style (Batty 1984). At hatching larvae swim with a serpentine motion, in which the amplitude of swimming movements of the body increases linearly towards the tail. Following the disappearance of the primordial fin-fold and development of the caudal and dorsal fins, a sub-carangiform mode of swimming is adopted, in which the amplitude of the body wave increases towards the tail and inertial forces become more important (Batty 1984). As the fish grow, there is an increase in both stride length, and swimming speed for a given tail-beat frequency. The tail-beat frequency is around 27 Hz at 10 °C in yolk-sac larvae and decreases markedly in adult fish (Batty and Blaxter 1992). The contraction speed (V) required for maximum power output during swimming decreases with increasing fish length (Anderson and Johnston 1992). In the present study, the inner-muscle fibres of herring larvae were found to contain myosin heavy chain TNT and TNI isoforms which were not present in either adult fast- or slow-muscle fibres. In contrast, larval Tm, LC1, LC3 and TNC were indistinguishable from those of adult fast-muscle. Larval LC2 was present as a minor component of the LC2 found in adult fast-muscle (Fig. 5). The sequential expression of development-specific isoforms of the myofibrillar proteins almost certainly reflects the changing functional requirements of the muscle as the fish grows.

The existence of a larval isoform of TNI in herring is of particular interest since developmental isoforms of troponin I have not previously been identified in mammalian and avian skeletal muscle. Embryonic muscles in mammals and birds express both adult TNI isoforms, which gradually become exclusively the fast or slow isoform, depending on the muscle type (Dhoot and Perry 1980, Toyota and Shimada 1981).

Optimal temperature ranges exist for the development of each species, with abnormalities and mortality increasing markedly at the extremes (Polo et al. 1991). Most aspects of development in fish larvae are temperature-sensitive (Blaxter and Hempel 1963, Herzig and Winkler 1986, Blaxter 1988). Temperature not only affects the rate of development, but also vertebrae number (Tåning 1952), the sequence in which external features and organs appear (Fukuhara 1990), muscle cellularity and ultra-

structure (Stickland et al. 1988, Vieira and Johnston 1992), and the developmental stage at which hatching takes place (Herzig and Winkler 1986). In the present study, it was found that the particular combination of myofibrillar protein isoforms present at any stage in development was dependent on the rearing temperature. This suggests that temperature alters the sequential expression of individual myofibrillar components to different extents, which can result in potentially unique combinations of isoforms. The consequences of altered isoform expression on the locomotory capacity of larvae remains to be determined.

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