**TEMPERATURE AND DEVELOPMENTAL PLASTICITY OF MUSCLE PHENOTYPE IN HERRING LARVAE**

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**Summary**

Myogenesis, the expression of myofibrillar protein isoforms and the development of muscle innervation were investigated in Clyde herring (*Clupea harengus* L.) in two successive spawning seasons at temperatures ranging from 5°C to 15°C. Myotube formation occurred in a rostral to caudal progression at similar somite stages at all temperatures. Superficial mononuclear muscle pioneer fibres were present at the horizontal septum. Myofibrillogenesis was retarded with respect to somite stage at low temperatures; for example, by the 50-somite stage, myofibrils were observed in the muscle pioneers of the first 31 somites at 12°C, but only the first 20 somites at 5°C. In the electron microscope, the earliest stages of myofibril assembly were observed in the muscle pioneer cells and in a proportion of the multinucleated myotubes within the same somite. By the end of somitogenesis, the density of myofibrils in the rostral myotomes was much higher at 15°C than at 5°C. Embryonic isoforms of myosin light chain 2 (LC2), troponin I and troponin T were identified in the presumptive white muscle using two-dimensional gel electrophoresis. Expression of the embryonic isoforms was gradually switched off during the larval stages. The size range over which embryonic isoforms were present was inversely related to rearing temperature. For example, the adult pattern of myosin LC2 expression was established at 11 mm total length (*TL*) at 15°C, but not until 15 mm *TL* at 5°C. Acetylcholinesterase staining was apparent at the myosepta in 31-somite stage embryos at 15°C, but not until approximately the 40-somite stage at 5°C. The red muscle fibres of larvae were initially innervated only at their myoseptal ends. The temperature at which the red muscle fibres became multiply innervated was inversely related to body size, occurring at 12–14 mm at 12°C, but not until 16–19 mm at 5°C. We conclude that the temperature during early development determines the relative timing and degree of expression of the myogenic programme, resulting in significant phenotypic variation in the swimming muscles of the larval stages. Our results highlight a potential mechanism whereby early thermal experience could influence survival and hence the strength of particular year classes of fish.

Key words: fish, larvae, temperature, muscle development, herring, muscle innervation, *Clupea harengus*.

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**Introduction**

The Atlantic herring (*Clupea harengus* L.) is an important component of pelagic ecosystems in the North Atlantic, North Sea and Baltic Sea (Parrish and Saville, 1967). The species has a complex population structure, with numerous races, tribes and stocks that differ in morphology and in ecological and behavioural characteristics (Parrish and Saville, 1967; Jørstad et al. 1991). Herring have transparent eel-shaped larvae that hatch at approximately 6–9 mm in total length, depending on the stock, and swim continuously to avoid sinking. The myotomes contain a single superficial layer of red muscle fibres surrounding an inner mass of approximately 250 larger-diameter white muscle fibres (Batty, 1984; Johnston, 1993). The red muscle fibres of herring larvae express myosin light chains characteristic of adult white muscle (Johnston and Horne, 1994), and the inner fibres contain a distinct larval isoform(s) of myosin heavy chain (Crockford and Johnston, 1993). Mitochondria occupy 46% and 26% of fibre volume in the red and white muscles, respectively, in larvae reared at 15°C (Vieira and Johnston, 1992), which suggests that both fibre types are recruited for continuous swimming. The larvae rely exclusively on cutaneous respiration until the gill filaments start to form at 20 mm, although haemoglobin is not present until towards the end of metamorphosis at 35 mm (De Silva, 1974).

Studies with a wide range of fish larvae have documented numerous changes in muscle structure and function during...
ontogeny. For example, as development proceeds, the aerobic capacity of both muscle fibre types decreases (El-Fiky et al. 1987), and the superficial and inner muscle fibres gradually start to express myofibrillar proteins characteristic of adult red and white muscles, respectively (Rowlerson et al. 1985; Christiansen, 1994; Mascarello et al. 1995). In adult herring, the red muscle fibres are multiply innervated and the white muscle fibres are focally innervated (Bone, 1964). Electromyographic studies with adult Pacific herring (C. pallasi L.) have shown that only the red muscle fibres are used for continuous swimming. Following the recruitment of the white muscle, there is a rapid onset of fatigue, leading to a marked division of labour between the fibre types (Bone et al. 1978).

Temperature has a profound effect on the rate and degree of expression of the developmental programme in fish embryos (Johnston et al. 1996). For example, myofibrils are observed at later somite stages in the rostral myotomes of herring embryos incubated at 5°C than in those incubated at 12°C (Johnston et al. 1995). At hatching, the composition of myofibrillar proteins (Crockford and Johnston, 1993), the volume density and spatial distribution of muscle fibre organelles, and the number and size distributions of muscle fibres (Vieira and Johnston, 1992; Johnston, 1993) have all been shown to vary with rearing temperature. Similar effects of temperature on the cellularity of myotomal muscle in larvae have been observed in such phylogenetically diverse species as Atlantic salmon (Salmo salar L.) (Stickland et al. 1988) and plaice (Pleuronectes platessa L.) (Brooks and Johnston, 1993).

The larvae of Atlantic herring spend up to 3 months in the plankton, depending on the water temperature, and very few survive to the juvenile stage (Heath and MacLachlan, 1987). The aim of the present study was to test the hypothesis that early thermal experience and concomitant changes in the relative timing of myogenesis have an impact on muscle phenotype throughout the larval stages. We therefore investigated the expression patterns of myofibrillar proteins, muscle differentiation and the development of motor innervation in embryos and larvae reared at a range of thermal regimes.

Materials and methods
Production of embryos

Mature Atlantic herring (Clupea harengus L.) were caught in the Firth of Clyde, Scottish West Coast, during March 1995 and 1996. The dissected gonads were returned on ice to the Dunstaffnage Marine Laboratory, Oban. The eggs from six females were attached to individually labelled microscope slides or large glass plates by contact with sea water and were fertilised with the mixed milt from five males. In each year, the eggs were fertilised in batches up to 12 h apart so that all somite stages could be inspected conveniently during the working day. The slides of fertilised eggs were incubated in slide-holders in black-walled tanks at constant temperatures of 5°C, 8°C, 12°C and 15°C (±0.4°C) (photoperiod 10 h:14 h light:dark).

Larval rearing

Larvae were reared in triplicated 501 tanks of circulating sea water at initial temperatures of 5°C, 8°C, 12°C and 15°C (±0.5°C). To improve larval survival, the temperature of each tank was gradually increased following hatching. The temperature records for 1996 are shown in Fig. 1. To avoid confusion, we refer to the four groups by their initial temperatures throughout the paper. All lengths reported are for fresh (unfixed) larvae measured from the snout to the tip of the primordial or caudal fin (total length, TL). Fish were fed to excess daily with live Artemia sp., starting a few days after hatching.

Observations of living embryos

The chorion and tissues of herring embryos are transparent, facilitating direct observations of unstained structures using Nomarski optics. Slides were removed at intervals and examined in Petri dishes containing sea water at the incubation temperature before being returned to the holding tanks (frequent changes of sea water were made during observations to prevent heating). After the 30-somite stage, some of the eggs were scraped off the slides with a razor blade, dechorionated and the embryos anaesthetised in 0.02% bicarbonate-buffered MS 222. The embryos were mounted in glycerol under glass coverslips supported by silicone grease at each corner. The position of the most posterior somite containing myotubes and myofibrils was determined with respect to somite stage at a magnification of 400×. Myofibrils were identified on the basis of the presence of cross-striations.

Fig. 1. The temperature regimes used to rear embryos and larvae of Atlantic herring (Clupea harengus L.) in 1996. The four traces show the temperature regimes for each of the four groups of larvae with initial rearing temperatures of 5°C, 8°C, 12°C and 15°C. The arrows denote the day of hatching.
Electron microscopy of embryos

Dechorionated 55-somite stage embryos were prepared as described above and fixed either for 55 min in 2.5% (v/v) glutaraldehyde, 100 mmol l\(^{-1}\) phosphate buffer, pH 7.4 (at 4°C) (fixation protocol A), or overnight in 2.5% (v/v) glutaraldehyde, 2.5% (m/v) paraformaldehyde, 1% (m/v) sucrose, 2 mmol l\(^{-1}\) CaCl\(_2\), 100 mmol l\(^{-1}\) NaCl, 100 mmol l\(^{-1}\) sodium cacodylate, pH 7.4 (at 4°C). The embryos were washed in the same solution without the fixative, and post-fixed for 2 h in a solution containing 1% (m/v) osmium tetroxide in 100 mmol l\(^{-1}\) sodium cacodylate, pH 7.4 (at 4°C). Samples were subsequently washed in either phosphate buffer (protocol A) or sodium cacodylate buffer (protocol B), dehydrated through alcohol, stained en bloc with uranyl acetate in 70% ethanol and embedded in Araldite resin. Semi-thin (0.5–1.0 μm) sections of whole embryos were cut in an approximately sagittal plane and stained with Toluidine Blue. The orientation of the block was then adjusted in order to cut sections as close as possible to the median plane of the embryo. Ultrathin sections (60–80 nm) of the whole embryo were cut and mounted on pyroxyline-coated slot grids. The somite numbers (counting from the head) of anatomical landmarks were noted; these included the anus, the first somite with myotubes and the first somite containing actin and myosin filaments. In order to obtain increased section quality, the block was pared down in some cases and the somite position re-established with reference to the anus. The approximate length of each of the somites was noted. Following examination of sagittal sections, the block was cut at a point rostral to the anus and remounted in dental wax. Semi-thin transverse sections were cut until the anal pore was reached. From this point, ultrathin sections were taken after cutting 200 or 300 1 μm thick semi-thin sections, enabling the approximate somite number of the sections to be calculated (accuracy ±1 somite). Three embryos each from the 5°C group and the 15°C group were examined.

Acetylcholinesterase staining of embryos and larvae

Eggs were dechorionated with fine forceps and the embryos fixed for 6–12 h in 3% paraformaldehyde in 0.12 mmol l\(^{-1}\) phosphate buffer, pH 7.2 at 4°C. Embryos older than 30 somites were anaesthetised in 0.02% (m/v) bicarbonate-buffered MS 222 prior to fixation. The embryos were washed in phosphate buffer and incubated for 2 h in 1% saponin (w/v) in 0.12 mmol l\(^{-1}\) phosphate buffer, pH 7.2 at 4°C. The detergent was washed out with phosphate buffer (three changes of 40 min each), and the embryos were stained for acetylcholinesterase activity by incubation for 3–5 h in the dark at 4°C in a solution containing (in mmol l\(^{-1}\)): copper sulphate, 3; potassium ferricyanide, 0.5; maleate buffer, 100; acetylthiocholine, 1.7 (Karnovsky and Roots, 1964). The staining reaction was stopped by rinsing several times in phosphate-buffered saline (PBS), and the embryos were mounted in glycerol under glass coverslips supported by silicone grease at each corner. Specimens were photographed using a Leitz DMRB system microscope fitted with Nomarski optics and a polarising filter.

Acetylcholinesterase staining of muscle endplates was also investigated in approximately 35 larvae at each temperature ranging in total length (TL) from 8 to 25 mm. For larvae greater than 15 mm TL, some specimens were split in half longitudinally using fine forceps to give thinner preparations and to aid penetration of the reagents.

Immunocytochemistry of larvae

Ten to twelve larvae per rearing temperature, ranging from 12 to 21 mm TL, were killed by over-anaesthesia as described above and fixed in 4% paraformaldehyde in PBS. Neural processes were stained using an anti-α-acetylated tubulin mouse monoclonal antibody (Sigma, Poole, Dorset). Larvae were incubated in 1% (m/v) saponin in PBS overnight and then subjected to two cycles of freeze–thawing in acetonitrile at −20°C followed by distilled water. Samples were washed for 5 min in PBS, and non-specific binding sites were blocked in a solution containing 10% (m/v) normal sheep serum, 1% (v/v) Triton X-100 and 1% (m/v) bovine serum albumin (STB) (Sigma, Poole, Dorset). The larvae were rinsed in PBS for 5 min and incubated in the primary antibody overnight at a dilution of 1:1000 in STB. The samples were subsequently washed in STB for 2 h with frequent changes of STB and then incubated for 2 h in an anti-mouse IgG Cy3 conjugated secondary antibody (Sigma, Poole, Dorset) at a dilution of 1:200. Unbound secondary antibody was washed out with frequent changes of STB over 3 h, and the larvae were mounted on glass slides in a glycerol mounting medium under coverslips supported by silicone grease at each corner. Larvae were photographed using a fluorescence microscope fitted with a Rhodamine filter (Leitz Ltd, UK).

Electrophoresis of myofibrillar proteins of embryos and larvae

Eggs were dechorionated at the 60-somite stage using jeweller’s forceps and the head and yolk-sac were removed. Two preparations were examined in both 1995 and 1996, each consisting of approximately 100 embryos. Larvae were killed by anaesthesia in a 1:2000 (m/v) solution of bicarbonate-buffered MS 222 in sea water. The yolk-sacs, heads and gut were dissected from 20–30 larvae per sample on a cooled microscope stage (0–4°C). At least two samples per body size class were prepared in 1995 and 1996. The skin was removed from the trunk with fine forceps, taking with it the single layer of superficial muscle fibres (Crockford and Johnston, 1993). The remaining muscle was collected for analysis. Approximately 1–2 g of fast (white) and slow (red) muscle was dissected from the myotomes posterior to the dorsal fin from four adult herring, 30–35 cm TL.

All steps in sample preparation were carried out at 0–4°C in order to minimise proteolytic breakdown. The tissue was homogenised using a chilled hand-held glass homogeniser in 20 vols of ice-cold preparation buffer containing (in mmol l\(^{-1}\)): Tris–HCl, 10; NaCl, 50; EDTA, 1; pH 7.4; and the following proteolytic enzyme inhibitors, 50 μg ml\(^{-1}\): phenylmethylsulphonyl fluoride, 0.5 μg ml\(^{-1}\); leupeptin, 1 μg ml\(^{-1}\); pepstatin A and 0.2 trypsin inhibitor units ml\(^{-1}\).
aprotinin (units as defined by the supplier; Sigma Ltd, Poole, Dorset). The homogenate was centrifuged at 15 000 g for 10 min and the supernatant was discarded. The pellet was rehomogenised, washed and centrifuged a further four times in 20 vols of ice-cold preparation buffer. The final pellet contained washed myofibrils.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described by Laemmli (1970) with the inclusion of 10 mmol l\(^{-1}\) DL-dithiothreitol (DTT) in the sample buffer. Myofibrils were resuspended in a solution containing 60 mmol l\(^{-1}\) Tris–HCl, pH 6.75, 2 % SDS (m/v), 10 % (v/v) glycerol, 10 mmol l\(^{-1}\) DTT and 0.002 % (m/v) Bromophenol Blue to give a final protein concentration of 2 mg ml\(^{-1}\). The samples were heated to 80 °C for 3 min and centrifuged at 5000 g for 5 min prior to use.

Two-dimensional electrophoresis was carried out as described by O’Farrell (1975) using non-equilibrium isoelectric focusing polyacrylamide gel electrophoresis (NEIF–PAGE) as the first dimension. Myofibrils were homogenised in 8 mmol l\(^{-1}\) urea, 1 % (v/v) Ampholyte pH 3–10, 1 % Ampholyte pH 5–7, 5 % (v/v) glycerol, 5 % (m/v) DTT, 2 % (v/v) Nonidet P-40 (NP40) and 0.001 % (m/v) Bromophenol Blue. Samples were warmed to 30 °C for 1 h and centrifuged at 5000 g for 5 min prior to use. NEIF–PAGE pH gradients were prepared in tube gels using the following combination of ampholytes (v/v) (Pharmacia, Uppsala, Sweden): for basic proteins, 1.67 % Pharmalyte pH 3–10 and 3.33 % Pharmalyte pH 8.5–10.5; and for acidic proteins 1.67 % Pharmalyte pH 3–10, 1.67 % Pharmalyte pH 4–6.5 and 1.67 % Pharmalyte pH 2.5–5.0 (Crockford and Johnston, 1993). Gels were fixed for 2 h in 12 % (m/v) trichloroacetic acid and 3 % (v/v) sulphosalicylic acid, and thoroughly rinsed prior to staining. Gels were stained with 0.1 % (m/v) Coomassie Brilliant Blue G-250 in 2 % (v/v) H\(_3\)PO\(_4\), 10 % (m/v) ammonium persulphate plus 20 % (v/v) methanol.

Myosin heavy chain and actin were identified by their relative molecular mass (\(M_r\)) and abundance on SDS–PAGE. Tropomyosin was identified by its anomalous migration on SDS gels in the presence and absence of 8 mmol l\(^{-1}\) urea (Crockford and Johnston, 1993). Troponin C uniquely stains blue with ‘Stains all’ (Campbell et al. 1983). The myosin light chains were identified by their characteristic migration in the neutral to acidic range on two-dimensional PAGE gels (Rowlerson et al. 1985; Martinez et al. 1991) and by purification from adult white muscle using a Sepharose Q column (Pharmacia, Uppsala, Sweden) (Crockford and Johnston, 1995). Herring troponin I and T both have basic isoelectric points and different relative molecular masses (Crockford and Johnston, 1993). In addition, troponin I was purified from white muscle using a stationary-phase rabbit troponin C affinity column (Syska et al. 1974). The apparent \(M_r\) values of the proteins were estimated using standard proteins of known \(M_r\) (Sigma Ltd, Poole, Dorset).

Peptide mapping

Myosin heavy chains were purified using one-dimensional SDS–PAGE on 8 % acrylamide gels. The gels were rapidly stained in Coomassie Blue G-250, and the myosin heavy chain bands were cut out with a razor blade. Peptide maps were run on 15 % SDS–PAGE gels, as described previously, using Staphylococcus aureus V8 and papain (Sigma Chemicals, Poole) (Crockford and Johnston, 1993).

Statistics

The relationships between somite stage (\(S\)) and the number of the most posterior somite containing features of interest (\(Y\)) were fitted using least-squares linear regression (Minitab Inc, USA). Relationships were compared by analysis of deviance using the following models, assuming normal errors: (1) each relationship has a different slope and different intercept: \(Y_{it} = \bar{a}_i + b S_{it};\) (2) there is a common intercept but different slope at each temperature: \(Y_{it} = \bar{a}_i + b S_{it};\) (3) there is a common slope but a different intercept at each temperature: \(Y_{it} = a_i + b S_{it};\) (4) the slope and the intercept are the same at all temperatures: \(Y_{it} = a_i + b S_{it};\) and (5) the mean of the data \(Y_{it} = \bar{a},\) where \(a\) and \(b\) are constants, \(\bar{a}\) and \(\bar{b}\) are the mean values, \(i\) is somite number and \(t\) is rearing temperature. The modelling sequences 1, 2, 4, 5 and 1, 3, 4, 5 were used. Further analysis was by stepwise regression, and the normality of the residuals was tested (Minitab Inc, USA).

Results

Myotube formation

The formation of somites proceeds in a rostral to caudal direction starting at the early neural plate stage. The somite stage of the embryo was used as a reference point throughout this study to provide an indication of how far development had proceeded. Following segregation of the somites, the lateral and ventral portions consisted of a rosette of mesodermal cells with prominent nuclei (Fig. 2A). Numerous mitotic spindles were observed in wax sections stained with haematoxylin–eosin, indicating that these cells were actively dividing (see Fig. 6A in Johnston et al. 1995).

The formation of myotubes can be investigated directly in living embryos using Nomarski optics (Fig. 2B). Myotube formation begins at the horizontal septum and extends rapidly across the whole somite (Fig. 2B). Among the first myotubes to form was a subset with a single prominent nucleus close to the horizontal septum lying just underneath the skin (Fig. 2D). These mononuclear myotubes correspond in position and morphology to the muscle pioneer fibres described in zebrafish (Hatta et al. 1991). The muscle pioneer cells in herring contained large numbers of mitochondria and a more elongated nucleus than in the other myotubes (Fig. 2D,F).

The majority of myotubes are multinucleated and are formed by the fusion of 4–6 myoblasts (Fig. 2C). Initially these myotubes contain numerous mitochondria, large accumulations of electron-dense granules (presumably ribosomes and/or glycogen granules), but no actin or myosin filaments (Fig. 2C,E).

The numbers of somites with complete myotubes and the
somite stage were recorded for living embryos in two consecutive spawning seasons, and the data were fitted by least-squares regression (Fig. 3; Table 1). Myotube construction begins at approximately the 20-somite stage at all temperatures (Fig. 3). Although the regression lines at different temperatures were very similar, analysis of deviance revealed model 1 to be the best fit to the data, i.e. there were significant differences in the slopes and intercepts ($F_{3,370}=4.26; P<0.02$). After approximately the 40-somite stage, there appeared to be little difference in the data collected for embryos reared at each temperature (Fig. 3).

**Myogenesis**

The most posterior somite containing cross-striations (myofibrils) was determined in relation to somite stage in living embryos using Nomarski optics and the data were fitted

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Intercept, $a$</th>
<th>Slope, $b$</th>
<th>Degrees of freedom (error)</th>
<th>$r^2$, adjusted (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>$-25.3\pm1.7$</td>
<td>$1.20\pm0.28$</td>
<td>121</td>
<td>93.9</td>
</tr>
<tr>
<td>8</td>
<td>$-18.1\pm1.2$</td>
<td>$1.08\pm0.025$</td>
<td>106</td>
<td>94.5</td>
</tr>
<tr>
<td>12</td>
<td>$-21.9\pm2.1$</td>
<td>$1.17\pm0.043$</td>
<td>58</td>
<td>92.5</td>
</tr>
<tr>
<td>15</td>
<td>$-22.2\pm1.3$</td>
<td>$1.13\pm0.030$</td>
<td>90</td>
<td>93.9</td>
</tr>
</tbody>
</table>

The data were fitted by least-squares regression to an equation of the form: number of somites with myotubes = $a + b$(somite stage). Values represent mean ± S.D.
by least-squares regression analysis (Fig. 4; Table 2). Myofibrillogenesis was retarded with respect to somite stage as incubation temperature was decreased. For example, by the 50-somite stage, myofibrils were present in the muscle pioneers of the first 31 somites at 12 and 15 °C, but only in the first 20 somites at 5 °C. Analysis of deviance revealed no significant differences in the slope between the temperatures, but highly significant differences in the intercepts ($F_{3,348}=8.28$; $P<0.001$).

Myofibril synthesis was investigated at the electron microscope level in 55-somite stage embryos incubated at 5 °C and 15 °C using serial semi-thin and ultra-thin sections. In 5 °C embryos, the early stages of myofibrils with well-defined Z-lines were visible in somites 30 (two embryos) (Fig. 5) and 32 (one embryo), which is slightly more caudal than the somites at which myofibrils were identified in the light microscope (Fig. 4). In all three embryos, well-defined myofibrils were present in the muscle pioneer cells (Fig. 5B). The muscle pioneer cells were flattened in cross section (Fig. 5A), sending processes between the multinucleated myotubes towards the notochord. The early stages of myofibril assembly were also evident in a proportion of the multinucleated myotubes in the same somite as that in which myofibrils could be first identified in the muscle pioneer cells (Fig. 5C). In 55-somite stage embryos incubated at 15 °C, myofibrils were first observed further back towards the tail than in 5 °C embryos, in somites 44 (one embryo) (Fig. 6A,B) and somite 42 (two embryos). Again, the first stages of myofibrils were observed in the muscle pioneer cells and a proportion of the multinucleated myotubes within the same somite (Fig. 6C,D). Myofibrillar assembly was first observed at the periphery of myotubes with the nascent myofibrils oriented parallel to the longitudinal axis (Fig. 7A). Hexagonally packed thick filaments and thin filaments, and the initial stages of Z-lines and M-lines, were present from the earliest stages (Fig. 7B), although elements of the sarcoplasmic reticulum and T-systems were initially absent (Fig. 7B). At both temperatures, isolated filaments were observed in the one or two somites immediately caudal to the somite in which myofibrils were first observed (not illustrated). They resembled the 'stress-fibre-like structures' described in myotubes from zebrafish Brachydanio rerio (van Raamsdonk et al. 1978) and chick (Fischman, 1967), which are thought to have a role in the assembly of myofibrils (Felsenfeld et al. 1991).

**Table 2. Regression analysis of the relationship between the number of somites with myofibrils and somite stage for embryos of Atlantic herring Clupea harengus.**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Intercept, $a$ (± S.D.)</th>
<th>Slope, $b$ (± S.D.)</th>
<th>Degrees of freedom</th>
<th>$r^2$, adjusted (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>$-49.10±1.82$</td>
<td>$1.20±0.28$</td>
<td>103</td>
<td>93.4</td>
</tr>
<tr>
<td>8</td>
<td>$-40.83±2.72$</td>
<td>$1.27±0.053$</td>
<td>84</td>
<td>87.2</td>
</tr>
<tr>
<td>12</td>
<td>$-37.61±3.26$</td>
<td>$1.37±0.063$</td>
<td>60</td>
<td>88.9</td>
</tr>
<tr>
<td>15</td>
<td>$-30.54±3.14$</td>
<td>$1.24±0.060$</td>
<td>105</td>
<td>80.4</td>
</tr>
</tbody>
</table>

The data were fitted by least-squares regression to an equation of the form: number of somites with myofibrils = $a + b$(somite stage). Values represent mean ± S.D.
The degree of expression of the myogenic programme at any given somite stage was positively correlated with incubation temperature. This is illustrated for the head somites in 56-somite stage embryos (Fig. 8). The number of myofibrils per somite increases for embryos in the series \(5^\circ C > 8^\circ C > 15^\circ C\). The nuclei in the presumptive white muscle fibres were observed to become more elliptical as they matured (Fig. 8).

**Acetylcholinesterase staining in embryos**

In herring embryos, the muscle fibres are focally innervated at the myosepta, giving rise to dark brown lines of acetylcholinesterase staining (Fig. 9A). In many cases, we obtained good staining of the cell bodies of motor neurones in the neural tube (Fig. 9B). Using Nomarski optics, it was possible to correlate the appearance of the primary motor neurone cell bodies with endplate staining by focusing through different focal planes of the section. Each spinal segment contained three large primary motor neurone cell bodies although sometimes a fourth cell body was present (not illustrated), as reported for zebrafish (Westerfield et al. 1986). The cell bodies of the primary motor neurones were observed approximately three or four somites more caudally to the most posterior band of endplate staining (Fig. 9B). After approximately the 45-somite stage, the development of acetylcholinesterase staining at the myosepta was correlated with the appearance of cross-striations (myofibrils) in the pioneer muscle fibres at 12 and 15°C, but lagged one or two somites behind it at 8 and 5°C (Fig. 9A,B). The cell bodies of the smaller, more numerous, secondary motor neurones were also observed (arrowheads in Fig. 9C) in the spinal cord.
approximately 8–10 somites behind the most recently formed primary motor neurone.

The effects of temperature on the development of acetylcholinesterase activity at the myosepta are shown in Fig. 10. At 15 °C, acetylcholinesterase staining at the endplates was first observed in 30-somite stage embryos, with the first 10 somites showing intense staining. The formation of endplates was delayed with respect to somite stage at lower temperatures (Fig. 10). For example, no staining of endplates was observed until the 37–40-somite stage at 5 °C (Fig. 10).

The results of least-squares regression analysis between the number of somites with functional endplates and somite stage was significantly steeper at 5 °C than at 15 °C using stepwise regression analysis (P<0.05) (Fig. 10), consistent with some

Table 3. Regression analysis of the relationship between the number of somites with acetylcholinesterase staining at the myosepta and somite stage for embryos of Atlantic herring Clupea harengus

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Intercept, a</th>
<th>Slope, b</th>
<th>Degrees of freedom</th>
<th>Adjusted R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>−53.55±2.63</td>
<td>1.48±0.051</td>
<td>91</td>
<td>90.3</td>
</tr>
<tr>
<td>8</td>
<td>−37.96±2.50</td>
<td>1.25±0.049</td>
<td>59</td>
<td>91.8</td>
</tr>
<tr>
<td>12</td>
<td>−42.33±7.15</td>
<td>1.42±0.14</td>
<td>27</td>
<td>79.9</td>
</tr>
<tr>
<td>15</td>
<td>−29.60±1.80</td>
<td>1.25±0.034</td>
<td>66</td>
<td>95.3</td>
</tr>
</tbody>
</table>

The data were fitted by least-squares regression to an equation of the form: number of somites with acetylcholinesterase staining = a + b(somite stage).

Values represent mean ± s.d.
Muscle plasticity in herring larvae

Larval growth

The majority of larvae hatched after 27 days at 5 °C, 18 days at 8 °C, 11 days at 12 °C and 8 days at 15 °C. Mean body length at hatching ranged from 8.4 mm total length (TL) at 15 °C to 9.7 mm TL at 8 °C (Fig. 11). Following hatching, there was surprisingly little difference in the rate of growth at the different temperatures (Fig. 11).

Myofibrillar protein composition

We examined the myofibrillar protein composition of the inner presumptive white muscle of embryos and larvae and found a large number of developmental-stage-specific isoforms. Isoforms characteristic of the embryo, larval and adult stages have been given the subscripts e, l and f, respectively (note: subscripts have been omitted in Fig. 12 for

Fig. 7. Transmission electron micrograph of a sagittal section of somite 40 of a 55-somite stage embryo reared at 15 °C. Fixation protocol B. (A) Multinucleated myotubes of the presumptive white muscle. The arrows indicate myofibril assembly at the periphery of the myotubes and the arrowheads indicate the sarcolemmal membrane. (B) Sarcomere of a myofibril in the early stages of assembly showing distinct Z-lines and an M-line. The arrow shows thick myosin filaments and the arrowhead shows thin actin filaments. Note the dense accumulation of electron-dense bodies which are probably ribosomes and glycogen granules (asterisk). The scale bar is 0.25 μm. mt, mitochondria; n, nucleus.

Fig. 8. Extent of myogenesis in the head somites (somites 1–3) of 56-somite stage embryos of Atlantic herring (Clupea harengus L.) reared at (A) 5 °C, (B) 8 °C and (C) 15 °C. These whole-mount embryos were stained for acetylcholinesterase and photographed using Nomarski optics. Note that the abundance of myofibrils in these presumptive white muscle fibres increases with increasing rearing temperature. The arrows indicate myofibrils, which can be distinguished by their cross- striations, and the arrowheads illustrate multinucleated myotubes. ms, myosepta stained for acetylcholinesterase. The scale bar is 20 μm.
clarity). In cases where multiple isoforms are present, they have been given a numbered subscript corresponding to their relative isoelectric point (pI), with 1 representing the most acidic isoform.

SDS–PAGE gels of well-washed myofibrils from 60-somite embryos revealed a large number of unknown minor bands compared with the gels from larvae at hatching (Fig. 12A). There were only relatively minor differences in one-dimensional SDS–PAGE gels between the inner muscle of larvae on the day of hatching and adult white muscle (Fig. 12A). Myofibrillar protein composition was therefore
analysed further using two-dimensional gels and peptide mapping.

**Myosin subunits**

The peptide maps of electrophoretically purified myosin heavy chains (MHCs) produced by digestion with bacterial V8 protease and papain were different from each other (Fig. 13A,B). Peptide digests of MHCs from 60-somite stage embryos and larvae on the day of hatching were different from peptide digests from adult white muscle at all temperatures. Only the peptide maps for herring reared at 8° C are illustrated (Fig. 13A,B). The rightward-facing arrowheads indicate the variation in peptides between embryonic and adult white muscle myosins following digestion with V8 protease (Fig. 13A) and papain (Fig. 13B). Adult red muscle MHC peptide maps were distinct from those of adult white muscle and larval white muscle (leftward-facing arrowheads in Fig. 13A,B).

There was no evidence for developmental isoforms of the alkali light chains of myosin (LC1 and LC3) (Figs 12A,B, 14). LC1 and LC3 from the inner (presumptive white) muscle of 60-somite embryos and larvae had identical relative molecular masses ($M_r$) and isoelectric points to those present in adult white muscle (Figs 12B, 14). The relative molecular masses of LC1 and LC3 were $25 \times 10^3$ and $19 \times 10^3$ respectively. In contrast, adult red muscle contained a single myosin alkali light chain isoform with an $M_r$ of $17 \times 10^3$ (Fig. 12C). Red and white muscle myofilbrils from adult herring had no myosin light chain isoforms in common (Fig. 12D). In two-dimensional gels, myosin from 60-somite embryos had a single myosin light chain 2 (LC2) isoform with an $M_r$ of $19 \times 10^3$ (LC2e) (Fig. 14A,F,J,N). At hatching, an additional LC2 band appeared in larvae reared at 8° C and 15° C, corresponding to the adult isoform (LC2f) (Fig. 14K,O). This isoform was present in trace amounts in larvae reared at 8° C (Fig. 14G), but was absent in larvae reared at 5° C (Fig. 14B). A trace amount of myosin LC2e was present in adult white muscle (Fig. 12B). In 11 mm TL larvae, the ratio of these LC2 isoforms was a function of rearing temperature (Fig. 14C,H,L,P). LC2e was the predominant isoform at 5° C (Fig. 14C), LC2e and LC2f were expressed in approximately equal amounts at 8° C and 12° C (Fig. 14H,L), and LC2f was the major isoform at

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**Fig. 12.** (A) One-dimensional 14 % acrylamide SDS–PAGE gel of myofibrillar proteins from Atlantic herring _Clupea harengus_. Lane M, biotinylated molecular mass marker (kDa); lane 1, presumptive white muscle from a 60-somite embryo incubated at 8° C; lane 2, presumptive white muscle from larvae reared at 8° C on the day of hatching; lane 3, adult white muscle; lane 4, adult red muscle. (B–D) Two-dimensional PAGE gel of acidic myofibrillar proteins from Atlantic herring _Clupea harengus_. (B) Adult white (fast) muscle, (C) adult red (slow) muscle and (D) mixture of red and white muscle myofibrils. Downward-facing arrowheads indicate adult white muscle proteins and upward-facing arrowheads indicate adult red muscle proteins. (E) Two-dimensional PAGE gel of basic myofibrillar proteins from adult white muscle. The gels were stained with Coomassie Brilliant Blue G-250. MHC, myosin heavy chain; Ac, actin; Tm, tropomyosin; TnT, troponin T; LC1, myosin light chain 1; TnI, troponin I; LC2, myosin light chain 2; LC3, myosin light chain 3; IEF, iso-electric focusing.

**Fig. 13.** Peptide maps on one-dimensional 15 % SDS–PAGE gels of purified myosin heavy chains (MHCs) from Atlantic herring _Clupea harengus_. (A) MHCs digested with bacterial V8 protease. Lane 1, presumptive white muscle from 60-somite stage embryos incubated at 8° C; lane 2, presumptive white muscle from larvae reared at 8° C on the day of hatching; lane 3, adult white muscle; lane 4, adult red muscle. (B) MHCs digested with papain. Lane 1, presumptive white muscle from a 60-somite stage embryo incubated at 8° C; lane 2, presumptive white muscle from larvae reared at 8° C on the day of hatching; lane 3, adult white muscle; lane 4, adult red muscle. Rightward-facing arrowheads indicate differences in the gel patterns between embryonic and adult white MHCs. Leftward-facing arrowheads indicate differences in the gel patterns between adult red and adult white muscle MHCs. The gels were silver-stained.
The expression pattern at 15 °C in 11 mm TL larvae was similar to that found in adult white muscle (Fig. 12B). By 13 mm TL, larvae reared at 8 °C and 12 °C also showed the adult pattern of LC2 expression (Fig. 14I,M), whereas LC2e and LC2f were equally expressed in the 5 °C larvae (Fig. 14D). The adult pattern of LC2 expression was not found in the 5 °C larvae until they reached 15 mm TL, 34 days after hatch (Fig. 14E).

Troponin I
There was one minor and one major spot corresponding to troponin I (TnI) isoforms in the inner muscle of 60-somite embryos both with a relative molecular mass of 22×10^3. The major TnI isoform corresponded to one of the adult
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isoforms (TnI1) (Fig. 15A,G,K,O). The other spot is assumed to represent an embryonic isoform (TnIe1) (Fig. 15A,G,K,O).

At hatching, three or four additional TnI isoforms appeared, designated larval isoforms TnI11, TnI12, TnI13 and TnI14, and TnIe was no longer expressed (Fig. 15B,H,L,P). TnI12 and TnI13 were the major isoforms present, and the proportion of TnI12 was greater at 12 °C and 15 °C (Fig. 15L,P) than at 5 °C and 8 °C (Fig. 15B,H). TnI12 was present as a minor component at 12 °C and 15 °C (Fig. 15L,P), but not at lower temperatures (Fig. 15B,H). By 11 mm TL, there were major variations in TnI expression at the different temperatures. The adult pattern of TnI expression was already established at this body size in 15 °C larvae (Fig. 15Q). In 12 °C larvae, there was an increase in TnI12 compared with the level at hatching, and this isoform was now expressed at a similar level to TnI11 (Fig. 15M). At 11 mm TL, TnI12 started to be expressed at lower temperatures, but was more abundant at 8 °C than at 5 °C (Fig. 15C,I). TnI14 was present at hatchling at 12 °C and 15 °C (Fig. 15L,P), but not until 11 mm TL at 5 °C and 8 °C (Fig. 14C,I). 5 °C larvae expressed TnI12, TnI13 and TnI12 and a trace of TnI13 and TnI14 at 13 mm TL (Fig. 15D). The adult pattern of TnI expression was established at 13 mm TL at 8 °C and 12 °C (Fig. 15J,N), whereas at 5 °C some expression of larval isoforms still occurred at 15 mm TL (Fig. 15E). Only at 17 mm TL was the adult pattern of troponin I expression established in larvae reared at 5 °C (Fig. 15F).

Fig. 15. Two-dimensional PAGE gel of basic myofibrillar proteins from presumptive white muscle of Atlantic herring Clupea harengus. (A–F) 5 °C, (G–J) 8 °C, (K–N) 12 °C, (O–Q) 15 °C rearing temperatures. 60-somite embryos (A,G,K,O); larvae on the day of hatching (B,H,L,P); 11 mm total length (TL) larvae (C,I,M,Q); 13 mm TL larvae (D,J,N); 15 mm TL larvae (E), 17 mm TL larvae (F). The gels were stained with Coomassie Brilliant Blue G-250. Tm, troponymosin; TnT1, adult white (fast) muscle troponin T isoform; TnT e1 , TnT e2 , TnT e3 , embryonic troponin T isoforms; TnI f1 , TnI f2 , adult white (fast) muscle troponin I isoforms; TnI e1 , embryonic troponin I isoform; TnI l1 , TnI l2 , TnI l3 , TnI l4 , larval troponin I isoforms. The upward-facing arrowheads show the positions of TnT e1 , TnT e2 and TnT e3 , and the downward-facing arrowheads the positions of TnI e1 and TnI e2 .

Fig. 16. Innervation of the myotomal muscles in herring larvae. Somite 40 of a 12 mm TL larva reared at 12 °C and stained for acetylcholinesterase. The arrows show the ‘finger-like’ neuromuscular junctions at the myoseptal ends of the pioneer/red muscle fibres (pmf). The arrowhead indicates a multi-terminally innervated muscle fibre. The scale bar is 25 μm. ms, myoseptum; rmf, red muscle fibres.
Development of muscle innervation in larvae

Initially acetylcholinesterase activity was only observed at the myosepta. In larvae, the individual endplates to the red and white muscle fibres were shaped like ‘gooseberries’ (Fig. 16). The muscle pioneer fibres were also focally innervated, but with ‘finger-like’ endplates at their myoseptal ends (Fig. 16). At 12 °C, multiple endplates were first observed on the red muscle fibres when the larvae reached 12–14 mm TL (eight larvae examined) (Fig. 16), 12–22 days after hatching (Fig. 11). Multiply innervated red fibres were first observed in a two- or three-fibre-thick band at the horizontal septum along the posterior third of the trunk. Later, the extent of multiply innervated fibres increased considerably and spread to more rostral myotomes. The multiple pattern of innervation developed more slowly with respect to body length at low temperatures. The red muscle fibres in nine out of 13 larvae examined of 16–17 mm TL reared at 5 °C still only had the focal pattern of innervation 50 days after hatching. The four individuals with some multiply innervated fibres had very few endplates (Fig. 17B). In larvae of similar length reared at 12 °C, the majority of red muscle fibres had distributed endplates (Fig. 17D), whereas larvae of the same length reared at 8 °C had an intermediate number of multiply innervated fibres (Fig. 17C). The dorsal fin was relatively well developed in 17 mm TL larvae at 5 °C, whereas the anal fin was only just starting to form, and was more advanced at the higher rearing temperatures (Fig. 17A). Single clusters of endplates were observed at the ventral ends of the anal fin ray muscles, with significantly more muscles innervated in the series 12 °C > 8 °C > 5 °C (Fig. 17B–D). Neurones in 17 mm TL larvae were stained with an antibody to α-acetylated tubulin (Fig. 18). In larvae reared at 5 °C (Fig. 18A) and 8 °C (Fig. 18B), motor nerves were largely confined to the myosepta. However, in 12 °C larvae, an extensive network of branching motor axons was observed innervating muscle fibres in the same superficial focal plane as the pioneer and presumptive red muscle fibres (Fig. 18C,D). Differences in the extent of multiple innervation to the red muscle fibres were still marked in 20 mm TL larvae, increasing in the series 12 °C > 8 °C > 5 °C (Fig. 19B,C,D). Note also that at this
Fig. 18. Neurones in the myotomes of herring larvae, 17 mm TL, stained for anti-α-acetylated tubulin. (A) Larvae reared at 5°C, (B) larvae reared at 8°C, (C,D) larvae reared at 12°C. C is a Nomarski image in the same plane of focus as the fluorescence image in D. The arrowheads indicate branches of neurones innervating the red muscle fibres. The scale bar is 50 μm. ln, lateral line nerve; ms, myoseptum; pmf, pioneer muscle fibres; prf, presumptive red muscle fibres.

Fig. 19. Development of the multi-terminal pattern of innervation to the red muscle fibres in 20 mm TL herring larvae. (A) Scale drawing of a whole-mount preparation stained for acetylcholinesterase activity (colour indicates sites of staining). df, dorsal fin; af, anal fin. (B) Right, myotome 42 (counting from the head) of a larva reared at 5°C. Very few of the red muscle fibres have endplates other than at the myoseptal ends. Left, the development of the anal fin. The colour shows the innervation to the anal fin muscles. (C) Right, myotome 42 (counting from the head) of a larva reared at 8°C. Left, the development of the anal fin, which is more advanced than at 5°C. The fin ray muscles are multiply innervated (colour). (D) Right, myotome 42 (counting from the head) of a larva reared at 12°C. The red muscle fibres are extensively multi-terminally innervated. Left, the development of the anal fin. The anal fin is more developed than at 8°C and all the fin ray muscles are multiply innervated (colour). Right, the scale bar is 50 μm. Left, the scale bar is 500 μm. Arrowheads indicate the multi-terminal pattern of innervation. ms, myoseptum; pmf, pioneer muscle fibres.
body size the anal fin was considerably larger and the fin ray muscles were more extensively multiply innervated at high than at low temperatures (Fig. 19A–D).

**Discussion**

**Temperature and development in herring: the ecological context**

Atlantic herring spawn at a depth of 15–25 m between mid February and early April in the Clyde. Over the last 40 years, the average sea temperature recorded during these months has fluctuated about averages of 5–10°C (based on Jones and Jeffs, 1991). Therefore 15°C is above the normal temperature range experienced by the early life stages of this stock during the recent past, whereas 5–12°C could be considered a natural range. Relatively large changes in the relative timing and the degree of expression of the myogenic programme were apparent for an average temperature difference of only 3°C, which is well within the observed inter-annual variation in sea temperature (Jones and Jeffs, 1991).

**Muscle development in the embryo**

In herring, the first myotubes are formed at the horizontal septum by cells that elongate to span the width of the somite. In zebrafish *Brachydanio rerio*, the resulting mononuclear myotubes are characterised by high levels of *engrailed* expression and have been called muscle pioneers (van Raamsdonk et al. 1978; Felsenfeld et al. 1991; Hatta et al. 1991; Ekker et al. 1992). Myotubes with a similar position and morphology to zebrafish muscle pioneer fibres are present in herring myotomes (Figs 2D,F). The 4D9 antibody recognises *engrailed* gene products in *herring* (Fig. 3; Table 1). The notochord is thought to be involved in somite patterning in zebrafish (Halpern 1992), and the neural tube (Yamada et al. 1993) in birds and mammals. Interactions between the notochord and the paraxial mesoderm have also been shown to be necessary for normal somite patterning in zebrafish (Halpern et al. 1993). The similarity of myotube construction at different temperatures may therefore reflect the highly canalised development of the notochord, which sets the rate at which other axial structures develop.

At the light microscope level, myofibrils were first recognised in the herring muscle pioneer fibres (Fig. 9A), as was reported for zebrafish fibres (Hanneman, 1992). However, we also examined serial sections in 55-somite stage herring embryos to determine the most posterior somite that contained organised contractile filaments. At the electron microscope level, the earliest stages of myofibril synthesis were observed in the muscle pioneer fibres and in a proportion of the multinucleated myotubes within the same somite (Figs 5A, 6A).

In a previous electron microscope study on herring, contractile filaments were first apparent in rostral myotomes (6–10) at the 43-, 38- and 27-somite stages at 12°C, 8°C and 5°C respectively (Johnston et al. 1995). We have now extended these observations to investigate the rostral to caudal progression of myofibril synthesis in relation to somite stage (Fig. 4). Rearing temperature influenced the intercept but not the slope of the relationship between the most posterior somite with myofibrils and somite stage (Table 2; Fig. 4). In addition to starting later, the degree of expression of the myogenic programme with respect to somite stage was reduced at low compared with high temperatures. Thus, towards the end of somitogenesis, the density of myofibrils in the rostral myotomes of embryos decreased in the series 5°C > 8°C > 15°C (Fig. 8).

Muscle differentiation is a downstream consequence of the activation of a family of myogenic basic helix–loop–helix (bHLH) transcription factors (*MyoD*, *myogenin*, *myf-5* and *MRF4*, reviewed in Olsen and Klein, 1994). Gene knock-out experiments in mice have revealed complex and highly redundant control pathways (Rudnicki and Jaenisch, 1995). In zebrafish, *MyoD* is first expressed in the precursor cells to the muscle pioneers prior to somitogenesis. Later, there is a complex pattern of *MyoD* expression in different parts of the somite, with significant variations between rostral and caudal myotomes (Weinberg et al. 1996). Low temperature may delay or reduce the production of the signals required to activate the expression of myogenic transcription factors, and hence muscle-specific genes; alternatively, it may retard the assembly of the contractile proteins into organised myofibrils. Further work is required to distinguish between these competing hypotheses.

The formation of neuromuscular synapses is dependent on complex signalling pathways between muscle fibres and motor neurones (Pike and Eisen, 1990; Sepich et al. 1994). Muscle fibres have a particular role in the synthesis and clustering of acetylcholine receptors (Sepich et al. 1994). In herring embryos, acetylcholinesterase activity was detected at the myosepta three or four somites rostral to the outgrowth of the primary motor neurones from the spinal cord (Fig. 9B). After the 40-somite stage, endplate staining was coincident with the initial synthesis of myofibrils at 15°C, but lagged several somites behind it at 5°C (Tables 2, 3; Figs 4, 5). Our techniques did not allow us to determine whether the pioneer
muscle fibres were the first to be innervated and functional as reported for zebrafish (Myers et al. 1986). Secondary motor neurones were observed approximately 8–10 somites nearer the head than the most recently formed primary motor neurones (Fig. 9C). Studies in zebrafish have shown that both primary and secondary motor neurones are activated in a coordinated manner during a wide range of swimming behaviour (Liu and Westerfield, 1988).

Temperature and the sequential expression of myofibrillar protein isoforms in embryos and larvae

Most myofibrillar contractile proteins exist as multiple isoforms produced by post-translational modification and/or the transcription of multi-gene families. Different combinations of isoforms are expressed in developmental-stage and muscle-fibre-type-specific patterns, producing fibres with different contractile properties (reviewed in Schiaffino and Reggiani, 1996). Previously, we reported that the composition of the myofibrillar proteins varied with rearing temperature in 1-day-old and 7-day-old herring larvae, particularly with respect to troponin T isoforms (Crockford and Johnston, 1993). We have now extended these observations by characterising the embryonic isoforms of myosin light chain 2 (LC2), troponin T (TnT) and troponin I (TnI) and examining their expression patterns during the larval stages.

Myosin LC2 is located at the head–rod junction of the myosin molecule and is thought to be an important determinant of the kinetics of cross-bridge cycling (Lowe et al. 1993; Szczesna et al. 1996). In adult herring, white muscle contains one minor and one major myosin LC2 isoform (Fig. 12), as reported for the plaice (Pleuronectes platessa L.) (Brooks and Johnston, 1993). The minor isoform (LC2e) is the only isoform present in the presumptive white muscle of 60-somite stage herring embryos (Fig. 14A,F,J,N). The presence of a trace amount of LC2e in adult stages may indicate that embryonic isoforms are expressed in newly recruited muscle fibres, as has been reported for myosin heavy chains in fish (Enion et al. 1995). At hatching, larvae reared at 5 °C only expressed LC2e. At higher temperatures, the proportion of LC2e increased in the series 8 °C > 12 °C > 15 °C (Fig. 14B,G,K,O). The amount of LC2e decreased with increasing body size until the adult expression pattern was established at 11 mm at 15 °C, 13 mm at 8 °C and 15 mm at 5 °C (Fig. 14P,I,E). Several other developmental isoforms of myosin LC2 have been identified in plaice white muscle, with complex changes in expression patterns occurring at metamorphosis and during the first year of the juvenile stage (Brooks and Johnston, 1993).

Troponin T (TnT) is a component of the troponin complex on the thin filament together with TnC and TnI. Interactions between tropomyosin (Tm) and the troponin complex mediate the Ca2+-sensitivity of actomyosin ATPase activity. In foetal and neonatal muscle in mammals, several developmental isoforms of TnT have been identified, which arise from the alternative splicing of a single gene transcript (Breitbart et al. 1985; Briggs and Schachat, 1996). Changes in TnT expression during development are correlated with an increased sensitivity to Ca2+ (Briggs et al. 1990). Direct correlations between the pCa2+/tension relationship of single skinned fibres and their content of troponin T isoforms have been demonstrated for rabbit muscles (Greaser et al. 1988). In the presumptive white muscle of herring, we identified up to three embryonic isoforms of TnT on two-dimensional gels (Fig. 15A,G,K,O).

As body length increased, there was a gradual increase in the proportion of TnTf and a decrease in the proportion of embryonic TnT isoforms, with the adult pattern being established at 15 mm at 5 °C, 13 mm at 8 °C and at 11 mm at 15 °C (Fig. 15E,J,Q).

TnI is the inhibitory component of the troponin complex. Separate genes have been identified for TnI in fast and slow skeletal and cardiac muscle in mammals (Koppe et al. 1989). Embryonic and neonatal forms of troponin I have not been described in birds or mammals, although in mice the fast skeletal muscle isoform is a developmental isoform in the early embryonic heart (Zhu et al. 1995). Instead, a mixture of adult fast and slow skeletal muscle isoforms is present in newborn muscles of mammals and the expression of the inappropriate isoform is gradually switched off as development proceeds (Dhoot and Perry, 1980). In contrast, we found evidence for multiple developmental isoforms of TnI in herring. At the 60-somite stage, there was a minor embryonic TnI isoform (TnIe) expressed in addition to one of the two adult isoforms (TnI1) (Fig. 15A,G,K,O). Three out of four larval TnI (TnI1–3) isoforms were identified in 1-day-old larvae (Fig. 15B,H,L,P). The larval TnI4 isoform was not apparent in the inner muscle of 5 °C and 8 °C larvae until they had reached 11 mm TL (Fig. 15C,I). There were complex changes in the expression of TnI isoforms with development. The rate of loss of TnI isoforms and the appearance of the adult fast muscle isoforms with respect to body size were inversely related to rearing temperature (Fig. 15C–F,I,J,L–N,P,Q). The adult pattern of TnI expression was established at 17 mm at 5 °C (Fig. 15F), 13 mm at 8 °C (Fig. 15J) and 11 mm at 15 °C (Fig. 15Q). Whether the multiple developmental isoforms of TnI represent distinct genes or post-translational modification products remains to be determined. Given the lack of similar developmental isoforms in mammals, it is interesting to note that sequencing studies have shown that herring TnI lies outside the tetrapod TnI gene family, showing significant variation in the actin/TnC-binding sequence (Hodgson et al. 1996).

Thus, for all three proteins, we found that the disappearance of embryonic isoforms was delayed relative to larval size as rearing temperature was reduced (Figs 12–15). The expression of individual isoforms is independently regulated, resulting in unique combinations of myofibrillar proteins at different temperatures. The relative persistence of embryonic isoforms in larvae at low temperatures may be some function of the later start of myofibril synthesis with respect to somite stage (Fig. 4).

Development of larval innervation patterns

Initially all the muscle fibres in herring larvae are focally

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innervated, with the muscle pioneers having distinct ‘finger-like’ endplates (Fig. 16). The majority of red muscle fibres had developed distributed endplates by the time larvae had reached 12 mm at 15°C. In contrast, multiply innervated red muscle fibres were still relatively rare in 17 mm larvae reared at 5°C (Fig. 17B). The proportion of red fibres which were multiply innervated in 20 mm larvae increased in the series 12°C > 8°C > 5°C (Fig. 19). There were also differences in the size of the anal fin and the development of the distributed innervation pattern to the anal fin ray muscles at the different temperatures (Figs 17, 19). The consequence of the early development of the adult pattern of red fibre innervation at high temperatures is unknown, but it may reflect a shift in the division of labour between fibre types relative to that in larvae at lower temperatures.

Functional and ecological consequences of variations in larval muscle phenotype

The larval stages of herring are subject to massive mortality of the order of 5–20% per day, primarily as a result of starvation and predation (Heath and MacLachlan, 1987). Swimming performance of the larvae is largely a function of body length and temperature (Batty et al. 1993). In the present study, we have shown that effects of temperature on the relative timing of muscle development observed in the early embryo persist throughout much of the larval stage. The finding that myofibrillar protein composition and muscle innervation patterns vary with respect to body length at different temperatures is important since the size range 12–20 mm coincides with major developments in the fins and changes in swimming style (reviewed in Blaxter, 1988). At 12 mm, the dorsal fin starts to develop as part of the primordial fin, and dorsal fin rays form by 13–14 mm. The anal fin forms as part of the primordial fin at approximately 16 mm, and by 17 mm the primordial fin disappears and the tail begins to turn upwards (Fig. 17). Early larvae employ an anguilliform mode of swimming, in which the amplitude of body movements is a linear function of body length and the lateral acceleration of water along the body is constant. Later, as the unpaired fins are developed, a subcarangiform swimming style is adopted in which the amplitude of the trunk increases markedly towards the tail and reactive forces dominate (Batty, 1984). Our results highlight a potential mechanism whereby early thermal experience could affect the swimming performance and survival of the larvae and hence juvenile recruitment and the strength of particular year classes of fish.

For survivors, we would expect that any developmental differences associated with particular temperature regimes should disappear at some point during the juvenile stage. Studies of enzyme polymorphisms and mitochondrial DNA show that there is relatively little genetic structuring of coastal and oceanic herring populations. The presence of some statistically significant allele frequency differences between stocks indicates, however, that there is not one panmictic population (Smith and Jamieson, 1986; Jørstad et al. 1991).

Batty et al. (1984) reported a lack of correspondence between genetic and morphological variability among different herring populations. Meristic characters such as vertebra and fin ray numbers are commonly used as stock identification factors (Hempel and Blaxter, 1961). In sexually mature fish, the total number of white muscle fibres per myotomal cross section also differs among stocks, varying from 64 400 in Bank (North Sea) herring to 33 900 in Blackwater (estuarine) herring (Greer-Walker et al. 1972). Early experimental studies established that fin ray and vertebra numbers were sensitive to the temperature and salinity at which the eggs developed (Tåning, 1952; Hempel and Blaxter, 1961). More recently, the number of satellite cells, on which all future muscle growth depends, has been shown to vary significantly in larvae reared at different temperatures (Johnston, 1993). Interactions between development and environment therefore remain promising candidates to explain at least some of the physiological (e.g. spawning time) and morphological variation between stocks, including differences in ultimate body size and the total number of myotomal muscle fibres (Johnston et al. 1996).

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