

Embryonic temperature and the relative timing of muscle-specific genes during development in herring (*Clupea harengus* L.)

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Accepted 13 August 2001

Summary

Temperature influences many aspects of muscle development in herring (*Clupea harengus*). In Clyde herring, myofibril synthesis occurred later with respect to somite stage in embryos reared at 5 °C compared with 12 °C. The aim of the present study was to test the hypothesis that the relative timing of expression of myogenic regulatory factors (MRFs) and myosin heavy chain (MyHC) transcripts changes with developmental temperature. Reverse transcriptase/polymerase chain reaction (RT-PCR) was used to clone partial coding regions of MyoD, myogenin and MyHC from juvenile Clyde herring. Embryos were reared at 5, 8 and 12 °C, and the spatial and temporal expression patterns of transcripts were investigated using cRNA probes and *in situ* hybridisation. Antisense probes revealed a

rostral–caudal progression of all three transcripts. MyoD transcription initially took place in the adaxial cells of the unsegmented, presomitic mesoderm, whereas myogenin transcription first occurred in newly formed somites. The MyHC gene transcript was not detected until approximately nine somites had formed. Since the somite stage at which the MRFs and MyHC were first expressed was independent of temperature, the hypothesis was rejected. We suggest that the effects of temperature on myofibril synthesis must occur downstream from MyHC transcription either at the level of translation or at the assembly stage.

Key words: herring, *Clupea harengus*, temperature, muscle, MyoD, myogenin, myosin heavy chain.

Introduction

In vertebrates, the development of trunk musculature occurs within the somites, which are formed from the paraxial mesoderm and progress in a rostral–caudal direction (Christ and Ordahl, 1995). However, the formation of embryonic muscle fibres in teleost fish differs in a number of respects from that described in other vertebrates (Currie and Ingham, 1998). Cells of the paraxial mesoderm become committed to a muscle fate towards the end of gastrulation, which is much earlier than in amniotes (Kimmel et al., 1990). The sclerotome is also much reduced compared with that in higher vertebrates, and most of the somite differentiates into myotomal musculature (Morin-Kensicki and Eisen, 1997). Restriction of mesodermal cells to a muscle lineage is thought to be associated with the expression of MyoD, a basic helix–loop–helix transcription factor (Weinberg et al., 1996). Studies in zebrafish have shown that MyoD is first expressed prior to segmentation in the adaxial cells lying either side of the notochord and occurs in response to axial hedgehog signalling (Weinberg et al., 1996; Devoto et al., 1996; Blagden et al., 1997). Zebrafish adaxial cells migrate through the lateral mesoderm to the surface of the myotome to form a superficial layer of slow muscle (Devoto et al., 1996;

Blagden et al., 1997). In contrast, MyoD expression in birds and mammals is first detected after the formation of somites, and no analogues of the adaxial cells have been detected (Rudnicki and Jaenisch, 1995).

There are four members of the MyoD gene family of myogenic regulatory factors (MRFs) in vertebrates. Gene ‘knock-out’ experiments in the mouse have established that MyoD proteins are components of a highly complex regulatory network that play a pivotal role in myogenesis (Rudnicki et al., 1993; Hasty et al., 1993). The primary MRFs, MyoD and myf-5, are involved in muscle lineage determination whereas the secondary MRFs, myogenin and MRF4, play a role in initiating and stabilising the differentiation programme (Perry and Rudnicki, 2000). MRFs share a highly conserved basic region, which mediates DNA binding, and a helix–loop–helix domain that regulates dimerisation with the universal proteins E12 and E47 coded by the E2A gene (Ma et al., 1994). The resulting heterodimer has a high affinity for the E-box motif present in the promoter region of the majority of muscle-specific genes (Watabe, 2001). Accessory proteins involved in muscle differentiation include a family of cysteine-rich proteins

(CRPs) with a LIM domain that form a complex with the MyoD/E protein complex (Kong et al., 1997). The cloning and expression of MRFs, E-proteins and CRP isoforms have been reported in a very restricted number of fish species [for a review, see Watabe (Watabe, 2001)].

Fertilisation of the eggs is external in the majority of teleosts, and the rate of development is therefore strongly influenced by the prevailing temperature. There is also evidence that temperature affects the relative timing of various aspects of the muscle developmental programme (Johnston et al., 1996). We have studied myogenesis in a spring-spawning stock of Atlantic herring (*Clupea harengus* L.) from the Firth of Clyde, Scotland (Johnston et al., 1995; Johnston et al., 1997; Johnston et al., 1998). Historical records indicate that sea temperatures during deposition of the eggs range from 5 to 10°C and increase by several degrees during the period of embryonic development (Jones and Jeffs, 1991). At 5–15°C, the cranial-to-caudal progression of multinucleated myotube formation occurred at similar somite stages (Johnston et al., 1995). In contrast, subsequent aspects of differentiation, as demonstrated by the appearance of myofibrils and the development of acetylcholinesterase staining at the neuromuscular junctions, occurred at later somite stages as the incubation temperature was reduced (Johnston et al., 1995; Johnston et al., 1997).

Recent studies indicate that the degree of developmental plasticity of myogenesis associated with temperature variation differs between herring populations spawning at different times of the year (Johnston et al., 2001). Although transitory in nature, changes in the relative timing of differentiation with temperature may be of considerable ecological importance. Clyde herring larvae were found to exhibit more advanced developmental characters at shorter body lengths, including expression of adult myofibrillar protein isoforms (Johnston et al., 1997) and the formation of fin rays and associated muscles (Johnston et al., 1998; Johnston et al., 2001), when hatching from eggs incubated at 12°C compared with 5°C. Over the length range 12.5–18.0 mm, larvae hatching from eggs incubated at 12°C had a more advanced carangiform style of swimming and superior fast-start performance than fish hatching from eggs incubated at 5°C, in spite of the fish having the same thermal experience following hatching (Johnston et al., 2001).

The objective of the present study was to test the hypothesis that temperature influences the relative timing of transcription of the MRFs and myosin heavy chain gene, thereby providing a potential mechanism for the delay in the appearance of myofibrils at low temperatures.

Materials and methods

Embryos

Mature herring (*Clupea harengus* L.) were caught in the Firth of Clyde during March 2000. The gonads were removed and transported on ice to the Gatty Marine Laboratory. Half the eggs from six females were scattered over glass microscope slides in sea water and fertilised with half the milt from six

males. The slides were placed in racks in flow-through aquaria at 5, 8 or 12°C. To sample all developing stages during the working day, the remainder of the eggs were fertilised 12 h later and also incubated at 5, 8 or 12°C.

Embryos were sampled approximately every five somites by removing the chorion with fine forceps and fixing overnight at 4°C in 4% (m/v) paraformaldehyde in phosphate-buffered saline (PBS) in 0.1% (v/v) dimethyl pyrocarbonate (DMPC). The embryos were then washed twice in DMPC PBS, once in 50% DMPC PBS/50% methanol (v/v) and stored at –75°C in 100% methanol.

cDNA cloning, probe synthesis and in situ hybridisation

Total RNA was isolated from juvenile herring fast (MyoD and MyHC probe synthesis) and slow (myogenin probe synthesis) skeletal muscle using an RNeasy Midi kit (Qiagen). First-strand cDNA synthesis was carried out using a 3' rapid amplification of cDNA ends (Race) system (Gibco BRL Life Technologies) in which synthesis was initiated at the poly(A) tail using Adapter Primer. PCR amplification was carried out using Abridged Universal Amplification Primer (AUAP) and gene-specific primers designed from various vertebrate species (see Table 1). A 100 µl polymerase chain reaction (PCR) contained 0.5 µg of first-strand cDNA, 10 µl of 10× *Taq* polymerase buffer (500 mmol l⁻¹ KCl, 100 mmol l⁻¹ Tris-HCl, pH 9.0, and 1.0% Triton X-100) (Promega), 1.5 mmol l⁻¹ MgCl₂, 0.2 mmol l⁻¹ dNTP mix, 0.2 µmol l⁻¹ each of forward and reverse primer and 1 unit of *Taq* polymerase (Promega). Using a DNA thermal cycler (Techne, Cambridge, UK), reaction conditions were 94°C for 2 min followed by 30 cycles, for MyoD and MyHC, of 1 min of denaturation at 94°C, 1 min of annealing at 59°C and 1 min of extension at 72°C. Reaction conditions for myogenin involved a 30-cycle touchdown PCR protocol of 94°C for 30 s, 63°C for 30 s reducing by 0.3°C with every cycle and 72°C for 30 s. After the last cycle, extension of incomplete products was carried out by holding the samples at 72°C for 7 min.

PCR products were analysed on 1% agarose gels in TAE buffer (4 mmol l⁻¹ Tris-HCl, 4 mmol l⁻¹ sodium acetate, 2 mmol l⁻¹ EDTA). Products were isolated from gels using a QIAquick Gel Extraction kit (Qiagen) and cloned into pCR[®]4-TOPO[®] (Invitrogen). DNA sequencing was performed by The Sequencing Service (School of Life Sciences, University of Dundee, Scotland; <http://DNASEQ.bioch.dundee.ac.uk>) using DYEnamic ET terminator chemistry (Amersham Pharmacia Biotech) on Applied Biosystems automated DNA sequencers.

Plasmids were linearised using the restriction enzymes *Not*I and *Spe*I (Promega). Probes were synthesised using digoxigenin-UTP (DIG) and bacteriophage T7/T3 RNA polymerases (Boehringer Mannheim). Antisense probes were produced with *Not*I linearisation and T3 RNA polymerase.

Embryos were processed for *in situ* hybridisation following standard procedures (Schulte-Merker et al., 1992; Joly et al., 1993). MyoD and MyHC antisense probes were used on 20, 22 and 15 embryos from the 5°C, 8°C and 12°C groups,

Table 1. Primer sequences used in PCR amplification of MyoD, myogenin and myosin heavy chain

Primer name	Sequence	Gene
AUAP	5'-GGCCACGCGTGCACACTATGAC-3'	MyoD and MHC
1	5'-TGCCTACTGTGGGCATGCAA-3'	MyoD
2	5'-CCACTTTGGGCAGCCTCTGG-3'	MyoD
3	5'-GATGCACGTCTACAAATCCGAACCA-3'	MyoD
4	5'-CTTTYGAGACCAACCCCTACTT-3'	Myogenin
5	5'-CCACTCTGGRCTGCTRCAGCA-3'	Myogenin
6	5'-CGATCGGGGAGAAGCGGCGCTGAAGAA-3'	MyHC

respectively. Myogenin antisense probes were used on 18, 14 and 16 embryos from the 5 °C, 8 °C and 12 °C groups, respectively. Sense probes were tested on three embryos of varying ages from each group. Treatment in 50 µg ml⁻¹ proteinase K was carried out for 2–3 min. Hybridised transcripts were detected using a DIG nucleic acid detection kit (Boehringer Mannheim). Embryos were photographed on a Leica MZ8 microscope with a RS Photometrics Coolsnap digital camera using Openlab (Improvision).

Two 15-somite stage embryos reared at 8 °C and stained for MyoD and two 46-somite stage embryos reared at 8 °C and stained for myogenin were embedded in wax and cut to produce 10 µm transverse and sagittal sections.

Results

cDNA cloning was achieved for MyoD, myogenin and MyHC. A partial coding and non-coding nucleotide sequence of MyoD was cloned from juvenile herring fast muscle through several rounds of PCR amplification. Primers 1 and 2 gave a 146-nucleotide sequence which was used to design primer 3 (Table 1). This primer was used in combination with AUAP to give a 1192-nucleotide sequence, including the poly(A) tail, which was used to make the DIG-labelled probe. Combining the two sequences gave a 1322-nucleotide sequence (GenBank accession number AF265553). The deduced amino acid sequence showed 76 % identity to zebrafish (*Danio rerio*), 79 % identity to both trout (*Oncorhynchus mykiss*) 'TMyod2' and carp (*Cyprinus carpio*) and 80 % identity to trout 'TMyod' (Fig. 1). The basic helix–loop–helix (bHLH) region showed 93 % identity to carp and 95 % identity to both trout (TMyoD and TMyoD2) and zebrafish.

A 552 partial coding, nucleotide sequence of myogenin from juvenile herring slow muscle (GenBank accession number AF367622) was cloned using forward primer 4 and reverse primer 5 (Table 1). The amino acids for myogenin showed 82 %, 79 % and 84 % identity to zebrafish, trout and carp myogenin, respectively (Fig. 2). The bHLH region showed 98 % identity to zebrafish, trout and carp.

Herring	ACKRKTTSNDRRKAATMRERRRLSKVNDAPESLKRCTSTNPNQRLPKVEILRNAISYIES	60
Zebrafish	-----A-----T-----	60
TroutMyoD2	-----G-----N-----	60
TroutMyoD	-----A-----T-----D-----	60
Carp	-----A-----T-----N-----	60
Herring	LQSLRRGGQEDNYYPVIDHYSGSDSASSPRSNCSNCGMADPTRPSCSTRRRNNYDSAYFS	120
Zebrafish	---A---SQE.D.---ME-----M--MG-T-QT---S--S--N	118
TroutMyoD2	-----QDG-.-LE-----Q-----M-YNA-T-A-S--S-A	119
TroutMyoD	---G---A---G---M-----M--NGQ--PP---K---T--N	120
Carp	---A---QE.-.-LE-----M--MG-T-Q---S--S--N	118
Herring	ETPN.DSRNKNNTVISSLDLSSIVERITTETSPTCV..QEGSEG.SPCSPQEGSVLTE	176
Zebrafish	DA--A-A-----S-V-----S--PA--VLSVP-AH---H---SD	177
TroutMyoD2	---A---S---AAV-----N---S-D--A--TVLSG-----I-SR	178
TroutMyoD	-A---H-K-S-----N---D--A-A-.-D---S---GD--IAS-	177
Carp	D---A-A--T-SS-V-----S--PA--VLSVP--H--.-----S-	177
Herring	PVAPLPSPTSCIPSSHDNPYQVL	201
Zebrafish	TGTTA-----PQQQAQET.----	201
TroutMyoD2	NGGTV---N-PQP--.DP.----	201
TroutMyoD	NG--I---IN-V-AL---T---	202
Carp	TG--A-----T-PQQQARDP.----	201

Fig. 1. Comparison of the deduced partial amino acid sequence of herring MyoD with comparable sequences for zebrafish (Weinberg et al., 1996), trout TMyoD2 (Rescan and Gauvry, 1996), trout TMyoD (Rescan et al., 1994) and carp (Kobiyama et al., 1998). Dashes represent amino acid residues identical to those of herring; dots represent gaps. The boxed area indicates the basic helix–loop–helix domain.

Herring	DQRFYEGGDNYFPGARMPGGFDQGGYQER.GSMMGLCGDGRLLSGMVGLEDKASPSAGL	59
Zebrafish	-----A-F-QSRIN.---E-A---D-.N-----M-TTT-.---P---SS-	57
Trout	-----FYQSRL.---Y-----G-----G...-G-VLGGGMEDKAT	56
Carp	-----F-QSRLT.-----T--D-.S-----NG---V---P---SS-	57
Herring	GVPLSPHQEQPHCPGQCLPWACKVCKRKSVTMDRRKAATMRERRLKVKVNEAFEALKRST	119
Zebrafish	-LSM---Q-----L-----	117
Trout	PSG---P-.-----L---T-----	115
Carp	-LS---Q-----L-----	117
Herring	LMNPNQRLPKVEILRSATQYIERLQALVSSLNQQDHDQTG.GLHYR...AAQP.PRVSSS	174
Zebrafish	-----E-E-GN...ATA--PH.TG---	174
Trout	-----EN--GTQ--Q...TGPAQ---	172
Carp	-----E-E-GN...STA-.QA---	171
Herring	SDQSGGST	182
Zebrafish	-----	182
Trout	-E-----	180
Carp	-----	179

Fig. 2. Comparison of the deduced partial amino acid sequence of herring myogenin with comparable sequences for zebrafish (Chen et al., 2000), trout (Rescan et al., 1995) and carp (Kobiyama et al., 1998). Dashes represent amino acid residues identical to those of herring; dots represent gaps. The boxed area indicates the basic helix–loop–helix domain.

We also cloned a 612-nucleotide sequence of MyHC partially encoding L-meromyosin, including the poly(A) tail, from juvenile herring fast muscle (GenBank accession number AF367621) using forward primer 6 and AUAP. The herring MyHC sequence showed 91 %, 90 % and 88 % amino acid

identity to zebrafish, trout and 10 °C-acclimated carp MyHC, respectively (Fig. 3).

Herring larvae hatched after 10 days at 12 °C, 14 days at 8 °C and 24 days at 5 °C. The first somites were formed 42, 66 and 110 h after fertilisation at 12, 8 and 5 °C, respectively. During embryonic development, herring were sampled for *in situ* hybridisation. Sense probes for MyoD, myogenin and MyHC produced no staining (not shown). *In situ* hybridisation using antisense probes showed a rostral–caudal progression of all three RNA transcripts. Initial MyoD transcript expression occurred in the adaxial cells of the unsegmented, presomitic mesoderm (Fig. 4A,B). The MyoD transcript was then expressed in the adaxial cells of newly formed somites and in the lateral, posterior regions of the somites themselves, with expression increasing until the entire myotome was stained (Fig. 4C–G; Fig. 5A–E). As MyoD expression passed along the body, so it started to fade from the most anterior somites (Fig. 5E), leaving expression in the adaxial cells and the centres of the somites (not shown). Temperature had no effect on the timing of MyoD expression with respect to somite stage (Fig. 6A–D).

Myogenin expression also occurred in a rostral–caudal direction, closely following that of MyoD. However, it was never detected in the adaxial cells of the presomitic mesoderm, but occurred initially within the adaxial cells of newly formed somites. Expression within these cells appeared low compared with the staining intensity of MyoD transcript (Fig. 5F). Myogenin transcript expression within the somite then

Herring	EQDTSSHLERMKKNLEVTVKDLQHRLDEAENLAMKGGKKQLQKLESRVRELEGEVEGEQR	60
Zebrafish	---A-----I-----T-I-A--	60
Trout	-----I-----T--A--	60
Carp	---A-----M-L-----S-----H--A--A--	60
Herring	RGVDAVKGVRYKERRVXLYTQTEEDKKNVTRLQDLVDLQKLVKAYKRAEEAEQANT	120
Zebrafish	---A-----E-----N-----S-----S	120
Trout	-----E-----G-----M-----HS-----A--Q	120
Carp	--A-----E-----N-----	120
Herring	HLSKCRKVQHELEEAERADIAESQVKNLRAKGRDGGKGEAA	163
Zebrafish	---L-----S-----S-----S	163
Trout	-M--F-----T-----T--S-----V-	163
Carp	--RY-----SH-----S-EA--T-VEE	163

Fig. 3. Comparison of the deduced partial amino acid sequence of herring light meromyosin with comparable sequences for zebrafish (Xu et al., 2000), trout (Gauvry and Fauconneau, 1996) and 10 °C-acclimated carp (Hirayama and Watabe, 1997). Dashes represent amino acid residues identical to those of herring; dots represent gaps.

expanded laterally before progressing rostrally (Fig. 5F). When embryos reached approximately 34 somites, the myogenin transcript began to disappear from the more rostral somites, leaving staining only in the outer regions of those somites (Fig. 5H–J; Fig. 7). Expression of the myogenin transcript was very transient; it disappeared from the rostral somites before MyoD transcript expression (Fig. 5C–E, H–J). Incubation temperature had no effect on the timing of myogenin transcript expression with respect to somite stage (Fig. 6E, F).

MyHC transcript expression was also found only in the somites. However, it lagged behind MyoD and myogenin transcript expression, such that there were approximately nine somites between the most recently formed somite and the most

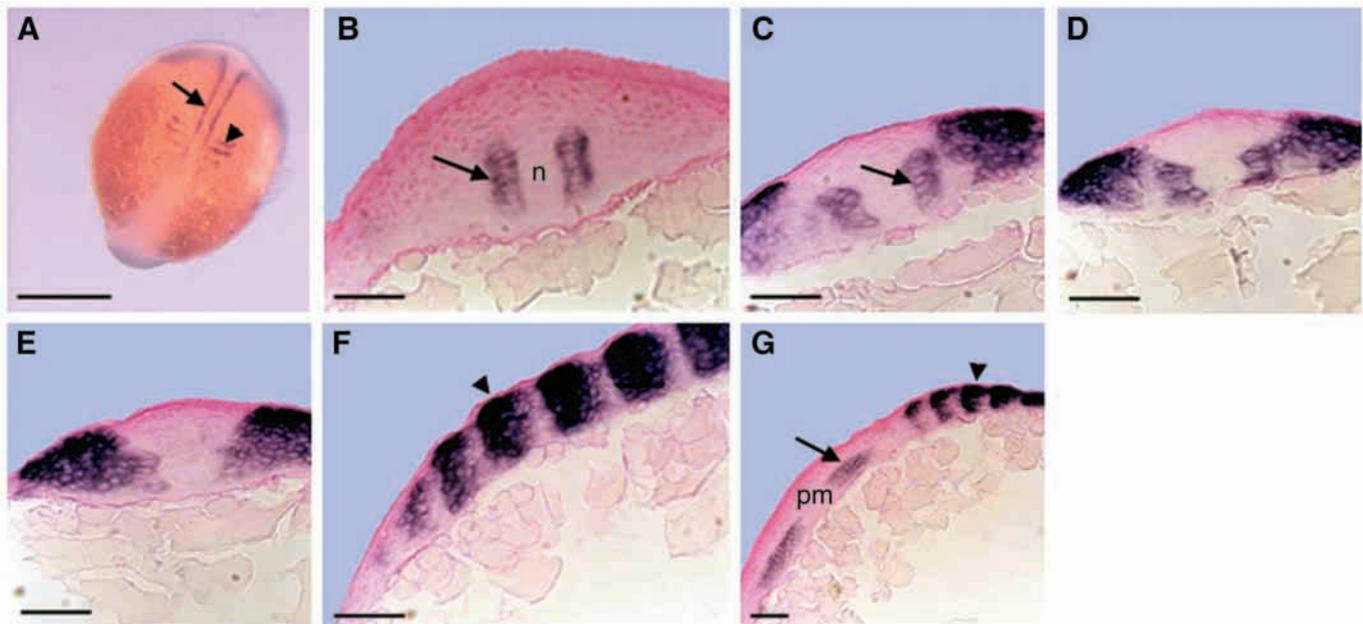


Fig. 4. MyoD expression in herring embryos reared at 8 °C. (A) Three-somite embryo; head positioned towards the bottom of the figure. The MyoD transcript accumulates in the adaxial cells prior to expanding into the lateral posterior regions of newly formed somites. (B–G) 15-somite herring embryo; (B–E) transverse sections, arranged most posterior first, showing the accumulation of MyoD transcript in (B) the adaxial cells only and (C–E) the adaxial cells and the somites. (F, G) Sagittal sections, head towards the top of the figure, showing the accumulation of MyoD transcript in the somites and (G) in the adaxial cells of the presomitic mesoderm. n, notochord; pm, presomitic mesoderm. Arrows indicate adaxial cells; arrowheads indicate somites. Scale bars, 50 µm.

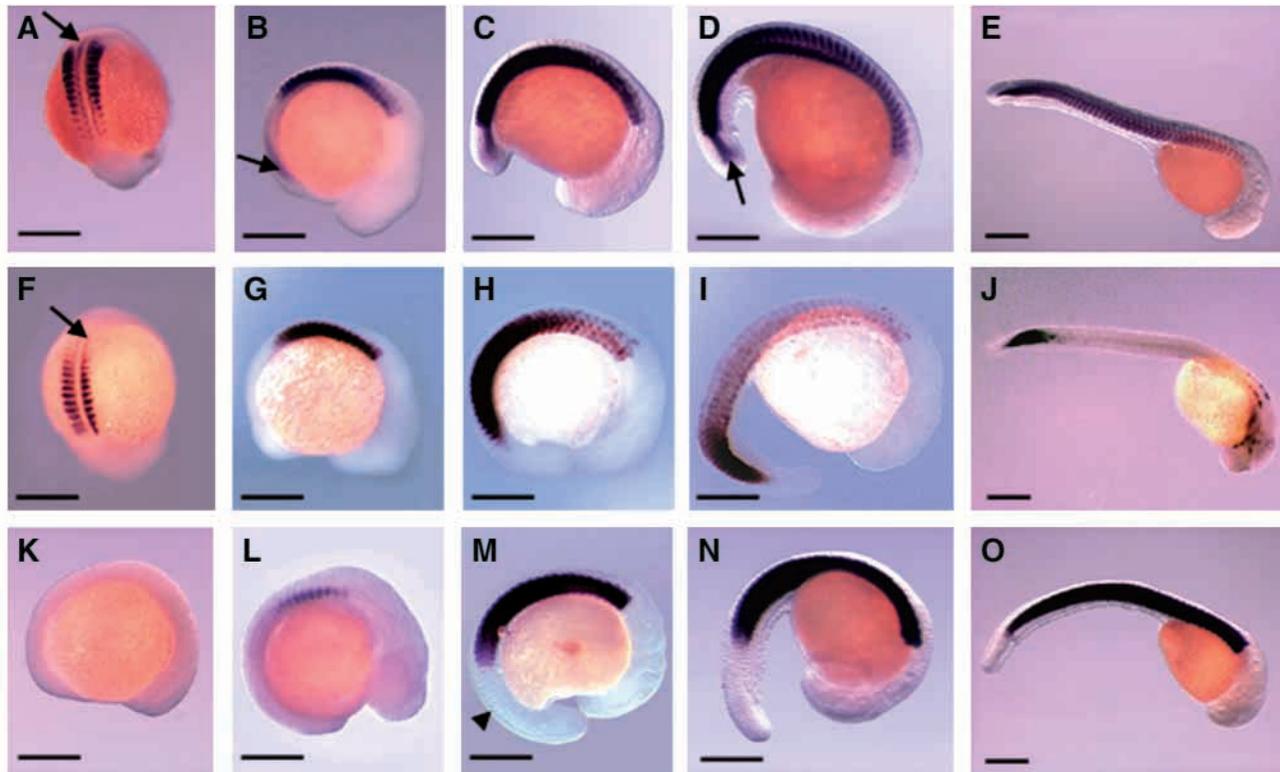


Fig. 5. Comparison of MyoD, myogenin and MyHC RNA transcript expression in herring embryos with similar numbers of somites and at a variety of rearing temperatures. (A–E) Expression of MyoD, (F–J) expression of myogenin, (K–O) expression of myosin heavy chain. (A,F) Embryos, dorsal view, head positioned towards the bottom of the figure. All other embryos are positioned anterior to the right. (A,F,K) 15-somite embryos. (B,G,L), 18-, 17- and 18-somite embryos, respectively. (C,H,M), 35-, 34- and 34-somite embryos, respectively. (D,I,N), 49-, 46- and 48-somite embryos, respectively. (E,J,O) Embryos with almost complete segmentation. Arrows indicate adaxial cells; the arrowhead indicates unstained somites. Scale bars, 300 μm .

posterior somite to express MyHC (Fig. 5K–O). This pattern occurred irrespective of developmental temperature (Fig. 8).

Discussion

Herring is an important species in terms of both fisheries and pelagic ecosystems (Overholtz et al., 2000). Successful recruitment to the adult population is a highly variable process dependent on numerous factors including food availability, predation pressure and environmental conditions (Cushing, 1990; Gallego et al., 1996). Temperature is a probable source of variability in muscle phenotype in natural populations, which can impact on swimming performance with potential consequences for survival (Temple et al., 2000; Johnston et al., 2001). Previous laboratory experiments have shown that the appearance of myofibrils in the myotubes of Clyde herring embryos was progressively delayed with respect to somite stage as developmental temperature was decreased (Johnston et al., 2001). In the present study, we tested the hypothesis that differences in the relative timing of myofibril formation are related to changes in the timing of MRF expression and/or transcription of the myosin heavy chain (MyHC) gene.

We cloned and sequenced partial-length cDNAs of MyoD, myogenin and fast muscle MyHC from herring. When

comparing herring and the few fish species studied to date, the bHLH regions of MyoD and myogenin were highly conserved, with percentage identities comparable to those reported between carp and other vertebrates (Kobiyama et al., 1998). Using cRNA probes and *in situ* hybridisation, we found that expression of the MyoD transcript in herring embryos occurred initially within the adaxial cells of the presomitic mesoderm before progressing to the adaxial cells and the posterior regions of newly formed somites (Fig. 4). Similar expression patterns have been reported in zebrafish and trout (Weinberg et al., 1996; Delalande and Rescan, 1999). However, in herring, we detected initial MyoD transcript in as few as three somites (Fig. 4A), whereas in zebrafish it was simultaneously observed in the first 5–7 somites (Weinberg et al., 1996). In trout, Delalande and Rescan (Delalande and Rescan, 1999) found that the expression patterns were fulfilled by two nonallelic genes, TMyoD and TMyoD2. Increased gene copy number is a common feature in fish and is thought to have arisen from multiple duplications of the fish genome (Meyer and Schartl, 1999). Further study would be required to determine whether more than one MyoD gene existed in herring.

Myogenin expression in herring embryos followed that of MyoD, as has also been found in zebrafish, carp and trout (Weinberg et al., 1996; Kobiyama et al., 1998; Delalande and

Rescan, 1999). As in the present study, myogenin transcript expression in trout and zebrafish embryos was never detected in the adaxial cells of the presomitic mesoderm but extended from the adaxial cells of newly formed somites into the posterior compartment of the somite before extending anteriorly into the somite (Weinberg et al., 1996; Delalande and Rescan, 1999). In herring embryos with completed segmentation, the myogenin transcript was also detected in non-myotomal muscle in the head region, as found in trout (Delalande and Rescan, 1999). Weinberg et al. (Weinberg et al., 1996) reported that myogenin transcript levels remained high after those of MyoD had decreased in the more rostral somites of the zebrafish. In contrast, herring myogenin transcript levels showed a very transient expression pattern, decreasing before the transcript levels of MyoD (Fig. 5). Variations in the transcriptional regulation of the MRFs have been noted between fish and other vertebrates (Watabe, 1999). The reasons for differences in MRF expression between fish species are not clear, but may depend on muscle fibre types and/or evolutionary considerations including duplicated genes which may not yet have been recognised.

MyHC transcript expression in the somites of herring embryos lagged behind myogenin expression by approximately nine somites (Fig. 5). Staining produced a characteristic chevron pattern thought to occur from the accumulation of message at the myoseptal ends of the fibres (Ennion et al., 1999). Recent work by Rescan and colleagues (Rescan et al., 2001) has challenged the general conception of muscle differentiation in fish based on that of the zebrafish. Using cRNA probes for slow and fast MyHC in the trout, they found that adaxial cells gave rise not only to slow muscle but also to fast muscle, which differentiated prior to the lateral migration of the slow muscle progenitors. In carp embryos, two MyHC genes, EGG22 and EGG24, were found each to exhibit an identical expression pattern within the fast muscle of the somites until 2 weeks post-hatching, when they were replaced by gene transcripts for more developmentally mature isoforms (Ennion et al., 1999). The carboxyl-terminal region of the herring juvenile, fast muscle MyHC showed higher identity (92%) to the equivalent region of EGG24 than to that of EGG22 (86%).

The patterns of MRF and MyHC transcript expression in herring embryos provided no support for our hypothesis that temperature would affect the timing of transcription with respect to somite stage. In contrast, using reverse transcription and competitive polymerase chain reaction techniques, Yamane et al. (Yamane et al., 2000) found a delayed expression of MRFs in the masseter muscle of the mouse when compared with the tongue, which correlated with

the retarded differentiation of the masseter. Although the concentration of MRFs within the somites may impact upon rates of myofibril synthesis, any semi-quantitative analyses of embryonic muscle samples in the present study would be difficult to interpret because of the transient nature and complex patterns of expression observed. No differences in the staining intensity of the MyHC transcript in *in situ* hybridisations were observed between embryos reared at different temperatures. We suggest, therefore, that the effects of temperature on delayed myofibril synthesis in herring embryos must impact downstream from MyHC transcription either at the level of translation or at the assembly stage.

Studies with human cultured cells have shown that, although myosin heavy chain is one of the first myofibrillar proteins to be expressed, its characteristic A-band structure appears much later in differentiation and requires a cytoskeletal scaffold (van der Ven et al., 1999). Myosin is thought to polymerise directly into thick filaments 1.6 μm long since shorter precursors are not observed in differentiating myotubes (Fischman, 1970). Thin filaments, the other major component of the myofibril, are arranged in an anti-parallel polarised manner in each sarcomere

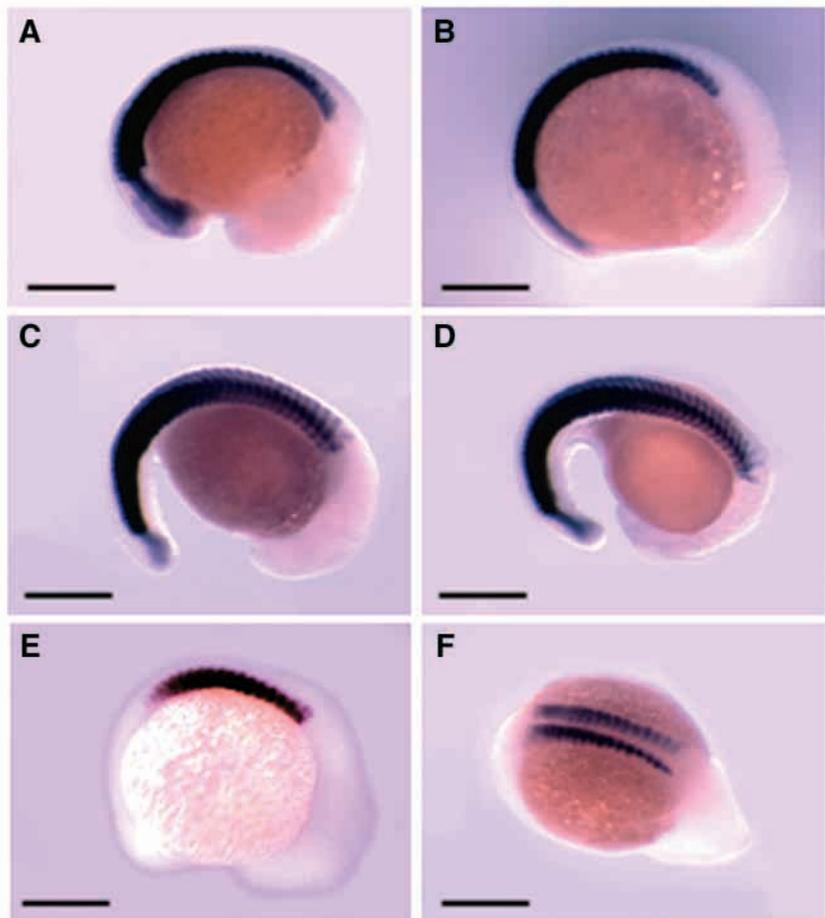


Fig. 6. MyoD (A–D) and myogenin (E,F) expression in embryos reared at different temperatures. (A) 30-somite embryo reared at 5 °C, (B) 30-somite embryo reared at 12 °C, (C) 42-somite embryo reared at 5 °C, (D) 42-somite embryo reared at 8 °C, (E) 17-somite embryo reared at 8 °C and (F) 17-somite embryo reared at 12 °C. Scale bars, 300 μm .

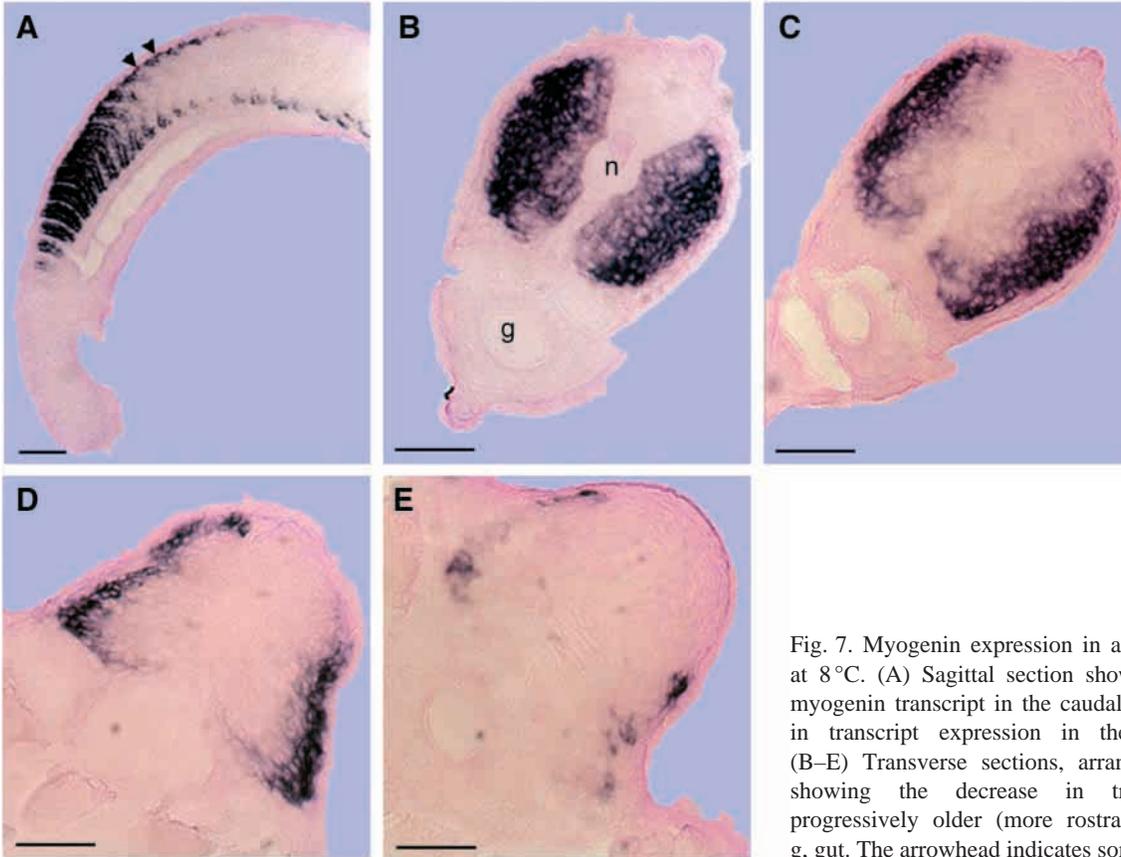


Fig. 7. Myogenin expression in a 46-somite embryo reared at 8 °C. (A) Sagittal section showing the accumulation of myogenin transcript in the caudal somites and the decrease in transcript expression in the more rostral somites. (B–E) Transverse sections, arranged most posterior first, showing the decrease in transcript expression in progressively older (more rostral) somites. n, notochord; g, gut. The arrowhead indicates somites. Scale bars, 300 µm.

with the barbed ends at the Z-line and the pointed ends in the middle of the sarcomere (Huxley, 1960). The barbed capping protein (CapZ) is thought to function early in myofibril assembly to nucleate actin filament assembly and to establish filament polarity by aligning the barbed ends of the filaments with the Z-line (Schafer et al., 1995). Tropomodulin is associated with the capped ends of actin filaments and is also expressed early in differentiation prior to the cross-linking of the filaments to the Z-line by α -actinin (Almenar-Queralt et al., 1999).

Another key protein involved in filament assembly and alignment is the giant sarcomeric protein titin that extends from the Z-line to the M-line (Gregorio et al., 1999). Titin has a role in directing the assembly of sarcomeres and maintaining sarcomere integrity by providing mechanical linkages with other sarcomeric proteins. Cell culture studies to characterise a functional knock-out of titin resulted in a failure of thick filament formation and the absence of ordered actin/myosin arrays, although the sarcomeric proteins were expressed (van der Ven et al., 2000). There is also evidence that the cytoskeletal scaffolding protein nebulin has an important role in the accessibility/exchangeability of actin into nascent myofibrils (Nwe and Shimada, 2000). Thus, there are numerous potential targets for temperature to impact on the assembly of myofibrils downstream from the transcription of the myosin heavy chain gene, involving either the transcription of other genes and/or movement of the cytoskeletal

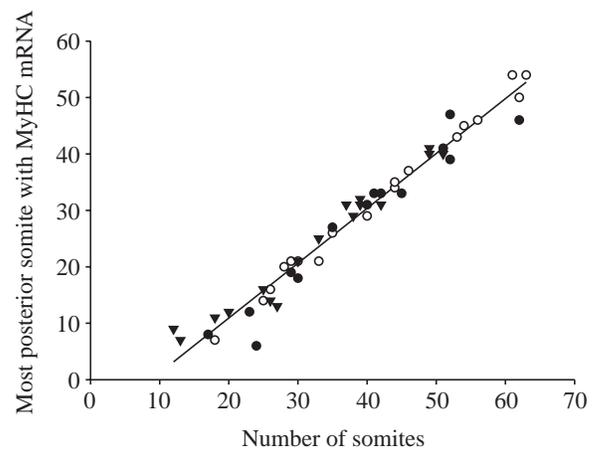


Fig. 8. The relationship between the most posterior somite expressing myosin heavy chain (MyHC) transcript and the number of somites in herring embryos incubated at different temperatures. Open circles represent embryos reared at 5 °C, filled circles represent embryos reared at 8 °C and filled triangles represent embryos reared at 12 °C. A common least-squares regression line is shown; slope $b=0.97\pm 0.03$, mean \pm S.E.M., $N=49$; $r^2=0.96$.

scaffold/sarcomeric proteins. The complexity of the assembly mechanism may make it relatively more susceptible to disruption by temperature change than earlier stages in muscle differentiation.

This work was supported by a grant from the Natural Environment Research Council awarded under the Developmental Ecology of Marine Animals programme. We wish to thank Professor Shugo Watabe, Yasushi Hirayama and Atsushi Kobiyama for their advice and hospitality. We are also grateful for the technical expertise of Mr Ron Stuart.

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