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Somite formation and expression of *MyoD*, *myogenin* and *myosin* in Atlantic halibut (*Hippoglossus hippoglossus* L.) embryos incubated at different temperatures: transient asymmetric expression of *MyoD*

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Summary

Genes encoding the myogenic regulating factors MyoD and myogenin and the structural muscle proteins myosin light chain 2 (MyLC2) and myosin heavy chain (MyHC) were isolated from juvenile Atlantic halibut (*Hippoglossus hippoglossus* L.). The impact of temperature on their temporal and spatial expression during somitogenesis were examined by incubating halibut embryos at 4, 6 and 8°C, and regularly sampling for whole-mount *in situ* hybridisation and reverse transcription (RT)–PCR.

There were no significant effects of temperature on the onset of somitogenesis or number of somites at hatching. The rate of somite formation increased with increasing temperature, and the expression of *MyoD*, *myogenin* and *MyHC* followed the cranial-to-caudal somite formation. Hence, no significant effect of temperature on the spatial and temporal expression of the genes studied was found in relation to somite stage. *MyoD*, which has subsequently been shown to encode the MyoD2 isoform, displayed a novel bilaterally asymmetric expression pattern only in white muscle precursor cells during early halibut somitogenesis. The expression of *myogenin* resembled that previously described for other fish species, and preceded the *MyHC* expression by approximately five somites. Two MyLC2 cDNA sequences were for the first time described for a flatfish, probably representing embryonic (MyLC2a) and larval/juvenile (MyLC2b) isoforms.

Factors regulating muscle determination, differentiation and development have so far mostly been studied in vertebrates with external bilateral symmetry. The findings of the present study suggest that more such investigations of flatfish species could provide valuable information on how muscle-regulating mechanisms work in species with different anatomical, physiological and ecological traits.

Key words: Atlantic halibut, *Hippoglossus*, flatfish, muscle, temperature, bilateral asymmetry, MyoD, myogenin, myosin heavy chain, myosin light chain.

Introduction

Activation of the myogenic program in embryonic muscle precursor cells involves a hierarchy of regulatory factors, which lead to the expression of multiple contractile proteins. In response to inductive signals emanating from the adjacent tissues, muscle precursor cells start to progressively express the four myogenic regulating factors (MRFs) MyoD, myf5, myogenin and MRF4 (Weintraub et al., 1991; Sassoon, 1993), which in mammals have been shown to exhibit distinct but overlapping functions. Whereas MyoD and myf-5 play critical roles in the determination of multipotent mesodermal stem cells to a myogenic lineage, myogenin and MRF4 are required for differentiation of myoblasts to myotubes and subsequently to mature muscle cells (Ludolph and Konieczny, 1995; Rudnicki and Jaenisch, 1995). These transcription factors share a highly conserved basic region mediating DNA binding, and a helix-loop-helix (HLH) domain essential for dimerization with the ubiquitously expressed E proteins (Murre et al., 1989; Lassar et al., 1991). The resulting heterodimer has a high affinity for the consensus E-box motif CANNTG located in the promoter or the enhancer of the majority of muscle-specific genes and thereby promotes the transcription of these target genes (Ma et al., 1994; Puri and Sartorelli, 2000). During the embryonic development of most fish species, *MyoD* is first present in adaxial cells of the presomitic mesoderm (Weinberg et al., 1996; Delalande and Rescan, 1999; Temple et al., 2001; Xie et al., 2001; Tan and Du, 2002; Hall et al., 2003; Cole et al., 2004; Zhang et al., 2006), showing that some mesodermal cells are destined to become muscle cells very early in organogenesis. The adaxial cells will later become myoblasts,

migrate and mature into slow superficial red muscle fibres, whereas the lateral cells of the somites form the bulk of fast white muscle fibres (Devoto et al., 1996). *Myogenin* expression succeeds *MyoD* expression, and activates fusion of myoblasts and differentiation to multinucleated myotubes (Rescan, 2001). Subsequently the maturing muscle fibres start to express genes encoding muscle-specific contractile proteins, such as myosin heavy chain (MyHC) and myosin light chain (MyLC) (reviewed in Goldspink et al., 2001).

Since fish are ectothermic animals and most teleost fish eggs are fertilised externally, water temperature is one of the most important external factors regulating developmental rate (Blaxter, 1988), partly by influencing the transcription rate of hundreds of genes (e.g. Malek et al., 2004). Egg incubation temperature affects the size and number of red and white muscle fibres at hatching, as well as muscle growth throughout larval and juvenile life in many fish species (Stickland et al., 1988; Vieira and Johnston, 1992; Brooks and Johnston, 1993; Usher et al., 1994; Johnston et al., 1995; Nathanailides et al., 1995; Johnston and McLay, 1997; Alami-Durante et al., 1997; Johnston et al., 1998; Galloway et al., 1998). By contrast, gene expression of the MRFs is affected by temperature in some fish species (Wilkes et al., 2001; Xie et al., 2001) but not in others (Temple et al., 2001; Hall et al., 2003; Cole et al., 2004).

The Atlantic halibut (*Hippoglossus hippoglossus* L.) is the largest flatfish in the North Atlantic (Haug, 1990), and is of great commercial value both for fisheries and aquaculture, owing to its rapid growth and good flavoured flesh. In Norwegian waters the halibut spawns between December and April, and the mean temperature at which the embryos develop is presumed to be around 5°C (Lønning et al., 1982; Haug et al., 1984; Kjørsvik et al., 1987), with 2 and 9°C being close to the lower and upper thermal tolerance limits, respectively, for these early life stages (Bolla and Holmefjord, 1988; Pittman et al., 1989; Galloway et al., 1999). Incubation of halibut eggs at experimental temperatures within these thermal tolerance limits has been shown to have a positive effect on developmental rate (Pittman et al., 1989; Galloway et al., 1999) but a negative effect on muscle mass size at hatching (Galloway et al., 1999). However, no information is available for any flatfish species about possible effects of temperature on the rate of somite development and gene expression of the underlying regulatory factors. In the present study the muscle-specific genes encoding the regulatory factors MyoD and myogenin and the structural proteins MyLC and MyHC were isolated from Atlantic halibut, and the impact of temperature on their temporal and spatial expression during somitogenesis was examined.

Materials and methods

Rearing and sampling of embryos/larvae

Eggs stripped from one female were fertilised with milt from one male of the Atlantic halibut (*Hippoglossus hippoglossus* L.) broodstock from AKVAFORSK, Sunndalsøra, Norway, kept at 6–7°C. Fertilisation rate was 88%. After fertilisation at 6°C approximately 850 eggs were transferred to each of six 3 l glass bowls; two of these were further incubated at a mean temperature of 4.3±0.2°C (4°C group), two at 5.8±0.6°C (6°C group) and two at 8.8±0.9°C (8°C group). These temperatures were maintained by placing the glass bowls in temperature controlled water baths. The seawater (30–32 p.p.t.) in the bowls had been filtered (0.2 μm), UV treated and 25 p.p.m. of oxytetracycline added. Approximately half of the water was changed every third day.

Embryos/larvae were sampled from each temperature group at regular intervals of day-degrees (d° = days × temperature in degrees Celsius); at 30, 40, 50, 60, 70 and 80 d° after fertilisation and 6 d° after hatching (=day when >50% of the embryos had hatched). Samples for reverse transcription (RT)–PCR analyses (*N*=20 per sampling point) were frozen on dry ice in Nunc cryotubes and stored at –80°C. Samples for *in situ* hybridisation (*N*=25 per sampling point) were fixated in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight and dechorionated before the embryos were rinsed twice in PBS, washed in 50% and 75% methanol in PBS and stored at –80°C in 100% methanol. During dechorionisation the yolk sac was also removed. Samples for examination of somite stage, standard length and myotome height (*N*=10 per sampling point) were fixated in a modified Karnovsky fixative, as described elsewhere (Galloway et al., 1998).

Cloning of muscle specific genes

Total RNA was extracted from whole embryos at different stages and from fast (white) and slow (red) skeletal muscle of a 1-year old Atlantic halibut weighing 45.9 g (14.8 cm in length) using TRIzol (Gibco-BRL, Gaithersburg, MD, USA). Total RNA (100–500 ng) was reverse transcribed into cDNA using the Ready-To-Go T-primed First-Strand Kit (Pharmacia Biotech, Uppsala, Sweden). Initially, partial halibut *MyoD* and *myogenin* cDNA sequences were PCR amplified using degenerate primers against conserved regions. The *MyoD* primers myd1 and myd2 were based on the N- and C-terminal sequences DFYDDPCF and DCLSSIVE, respectively, whereas the *myogenin* primers myg1 and myg2 corresponded to the regions MELFET and TSIVDSIT, respectively. Downstream *MyoD* and *myogenin* cDNA sequences were isolated by 3′RACE (Rapid Amplification of cDNA Ends) combining the *MyoD*-specific forward primers myd16 and myd21, or the *myogenin*-specific primers myg8 and myg13, with the oligo(dT)-tailed primer and the adapter primer described by Frohman et al. (Frohman et al., 1988). Upstream sequences of halibut *MyoD* were successfully PCR amplified from genomic DNA by Genome Walking (Clontech, San Francisco, CA, USA) by including the specific antisense primers myd38 and myd39 according to the manufacturer's protocol. Based on the gene structure of zebrafish *MyoD* (Du et al., 2003) and mouse *myogenin* (Edmondson et al., 1992), the primers sets myd16 + myd23 and myg13 + myg15 were used to PCR amplify the two introns of halibut *MyoD* and *myogenin*, respectively, from genomic DNA (Qiagen DNA

isolation kit). Two partial halibut *MyLC* cDNAs were isolated by running 3′RACE on pooled embryonic cDNA from several developmental stages, using the specific forward primer mlc1 encoding the conserved region MAPKKA. Finally, a partial halibut *MyHC* cDNA was amplified from fast muscle RNA by RT–PCR, using the primers mhc1 and mhc3 encoding the MyHC sequences KNWPWMK and MDLENDK, respectively.

All PCRs were run on a Perkin Elmer 2400 thermal cycler (Cetus, Hayward, CA, USA) by denaturation at 94°C for 2 min followed by 35–40 cycles of amplification at 94°C for 30 s, 58–65°C for 30–50 s depending on the primers, and 72°C for 50 s. The PCR mixture of 12.5 μl contained 0.6 i.u. Taq DNA polymerase (Promega, Southampton, UK) together with 0.2 mmol l^{-1} of each of the dNTPs and 10 pmol of each primer in standard GeneAmp® PCR buffer. PCR amplicons were visualized by agarose gel electrophoresis and ethidium bromide staining, cloned into pGEM-T Easy vector (Pharmacia Biotech), and sequenced in both directions (Applied Biosystems 377A, Foster City, CA, USA).

Spatiotemporal gene expression

Whole-mount in situ hybridization

cRNA probes for whole-mount in situ hybridization (WM-ISH) were prepared from plasmids containing halibut *MyoD*, *myogenin* or *MyHC* cDNAs. The plasmids were linearised using *Sal*I and *Nco*I restriction endonucleases, DIG labelled (Roche Diagnostics, GmBH, Mannheim, Germany), and purified according to the manufacturer's protocol. The labelled sense and antisense probes were electrophoresed together with standards included in the labelling kit to evaluate the probe concentrations. Three or more embryos or larvae per developmental stage from each temperature group were used for WM-ISH with each antisense RNA probe. Corresponding sense probes were used as negative controls on one sample per developmental stage from each temperature group. WM-ISH was performed according to the procedure of Hall et al. (Hall et al., 2003) with modifications as follows. Permeabilisation of all embryonic stages was achieved by digestion in 20 μl ml^{-1} proteinase K for 15 min, and for newly hatched larvae the conditions were 50 μl ml^{-1} for 10 min. Hybridisation with 500 ng ml^{-1} digoxigenin (DIG)-labelled cRNA probe in the hybridisation solution lasted 1–3 days at 70°C. Bound probe was conjugated to an alkaline-phosphatase-labelled anti-DIG antibody (Roche 1093274) at a dilution 1:8000 in the 'Heaven Seven' solution overnight at 4°C. A colour reaction occurred by adding 0.45 mg ml^{-1} nitroblue tetrazolium (NBT) and 0.175 mg ml^{-1} 5-bromo-4-chloro-3-indolylphosphate (BCIP) to the 'Divine Nine' solution and incubating in darkness overnight at 4°C. Photographs were taken on a Leica MZ75 binocular microscope using a Nikon Camera Head DS-5M and a Nikon Camera Control Unit DS-L1. The yolk sac was removed before the embryos/larvae were photographed.

RT–PCR

The temporal expression of MyoD, myogenin and MyLC were also examined by RT–PCR using the intron spanning primers described above for amplifying MyoD and myogenin cDNAs. The MyLC2a and MyLC2b cDNAs, which differed in the length of the 3′ untranslated region (UTR), were coamplified by 3′RACE and separated by gel electrophoresis. From each sampling point, total RNA of five embryos or larvae was mixed before cDNA synthesis. Proper first strand cDNA synthesis was verified by PCR amplifying halibut β-actin (GenBank accession no. AJ630123) using the primers actb1 and actb2. These primers, as well as the 3′RACE primers for MyLC2, spanned intron sequences to exclude possible contamination of genomic DNA.

Primer sequences

myd1 (sense): 5′-gatttctaygaygayccktgytt-3′
myd2 (antisense): 5′-acgatgctggawagrcartc-3′
myd16 (sense): 5′-acagcggcgactcggacg-3′
myd21 (sense): 5′-tggatttcatgtctccgtgt-3′
myd23 (antisense): 5′-acgacggtttagagctg-3′
myd38 (antisense): 5′-tccagtggcgggtagaagctgtcttc
myd39 (antisense): 5′-tcatggcgttgcgcaggatctccact-3′
myg1 (sense): 5′-atggagcttttygagac-3′
myg2 (antisense): 5′-atgctgtccacgatrgacgt-3′
myg8 (sense): 5′-aagaggagcaccctgatgaa
myg13 (sense): 5′-gagcgccatccagtata-3′
myg15 (antisense): 5′-cacgcatgttcccctgct-3′
mlc1 (sense): 5′-aagatggcaccaaagaagg-3′
mhc1 (sense): 5′-aaaaactggccatggatgaa-3′
mhc3 (antisense): 5′-ttgtcattctcmagrtccat-3′
actb1 (sense): 5′-aacggmtccggyatgtgyaa-3′
actb2 (antisense): 5′-tgggaggacgacatggaaaa-3′

Statistics

Significant differences ($P<0.05$) between treatments in somite recruitment rate and number of somites, standard length and myotome height at hatching were determined by a one-way ANOVA followed by a Tukey's B *post-hoc* test.

Results

Cloning of five muscle-specific genes

Five genes were isolated from Atlantic halibut that encoded two muscle regulatory factors and three contractile myosin proteins. The *MyoD* gene was organized as three exons interrupted by two introns (GenBank accession no. AJ630124, Fig. 1). A full-length *MyoD* cDNA (GenBank accession no. AJ630127) was isolated from fast skeletal muscle that encoded a predicted protein of 264 amino acids (aa) including the highly conserved bHLH motif (Fig. 1). Similarly, the splicing of the three exons in the halibut *myogenin* gene (GenBank accession no. AJ487982) resulted in a skeletal muscle *myogenin* cDNA encoding a predicted bHLH-containing protein of 250 aa (Fig. 2). The conserved N-terminal sequence MELFET is encoded by the sense PCR primer and is therefore omitted in Fig. 2. Intron 1 and 2 of the *myogenin* gene contained long dinucleotide repeats of GT and AC, respectively, which might

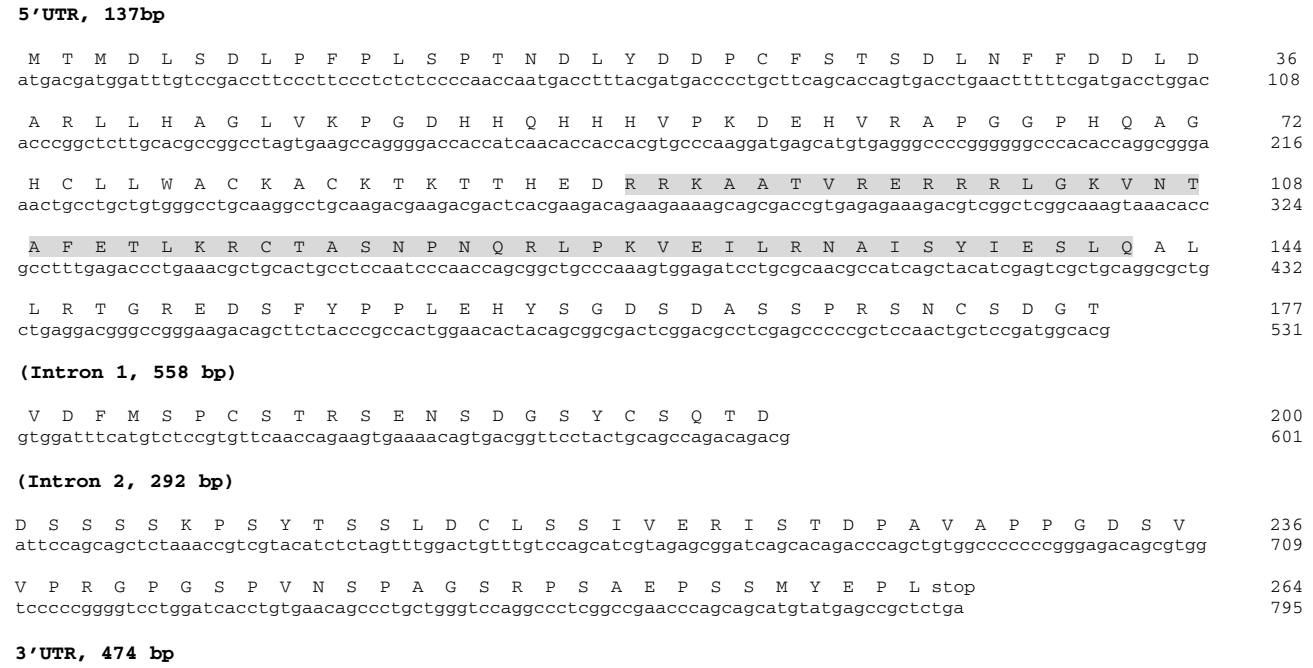

Fig. 1. Atlantic halibut *MyoD* cDNA and predicted amino acid sequences, which are numbered on the right. The bHLH domain is shaded. The locations of the two introns in the genomic sequence are indicated.

hybridize to form a hairpin loop with the short exon 2 in the loop. Accordingly, an alternatively spliced *myogenin* cDNA lacking exon 2 was isolated from skeletal muscle that encoded a predicted C-terminal truncated protein of only 174 aa due to a premature stop codon in the changed reading frame of exon 3 (Fig. 2). Although not quantitatively assayed, the short *myogenin* transcript seemed to be expressed in lower amounts than the intact *myogenin* transcript.

Halibut MyoD and myogenin exhibited the highest overall aa sequence identity to gilthead seabream (*Sparus aurata*) MyoD (71%) and pufferfish (*Tetraodon nigroviridis*) myogenin (82%), respectively. In addition to the conserved bHLH domain and the juxtaposed motif CLP/LWACKA/LCK, vertebrate MyoD and myogenin also showed homology in the C-terminal end (Fujisawa-Sehara et al., 1990) (Fig. 3). This part includes the so-called helix III, which appears to have adopted distinct functions in MyoD and myogenin mainly due to a negatively or positively charged residue in the marked position in Fig. 3 (Bergstrom and Tapscott, 2001). These authors demonstrated that the substitution of the Asp residue at this position in mouse MyoD with His of mouse myogenin disrupted the function of the MyoD helix III. The sequence alignment indicates that the negatively charged Asp, or Glu in cod, is conserved in the vertebrate MyoDs, whereas the positively charged His or Arg are found at this position in the myogenin of mammals and teleosts, respectively (Fig. 3). The exception seems to be the two myogenins of *Xenopus* both containing Asn at this position (Charbonnier et al., 2002).

A partial halibut *MyHC* cDNA of 706 bp was isolated by RT–PCR of juvenile fast muscle RNA (GenBank accession no. DQ302137). The deduced MyHC protein of 235 aa showed highest identity to the orthologue of common carp (92%) and zebrafish (90%). Two genetically distinct *MyLC* cDNAs (GenBank accession no. AJ488286 and AJ488287) were isolated by running 3′RACE on pooled embryonic cDNA. The deduced proteins shared 89% identity and were named halibut MyLC2a and MyLC2b based on the high similarity with the MyLC2 form of other teleosts.

Embryonic development

The embryos in all temperature groups were halfway through epiboly at 30 d° and the first movements of the embryos were observed around 70 d°. Hatching occurred at 84, 88 and 77 d°, or 19, 15 and 9 days after fertilisation at 4, 6 and 8°C, respectively. The first somites were formed between half and complete epiboly in all temperature groups, and at blastopore closure 8–11 somites were formed. Recruitment rate of new somites increased significantly with increasing incubation temperature (Table 1; Fig. 4), and was 4.6±0.3, 5.4±0.1 and 10.5±0.3 somites per day at 4, 6 and 8°C, respectively. The overall number of somites at hatching was 52±2 and was not significantly different among treatments (Table 1). The newly hatched larvae from the 4°C group were significantly shorter than larvae from the other temperature groups, whereas the myotome height of larvae from the 6°C group was significantly larger than that of larvae from the 4°C and 8°C groups (Table 1).

Spatiotemporal expression of muscle genes

WM-ISH with the RNA antisense probes enabled the

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 N  P  Y  F  F  P  D  Q  R  F  Y  E  G  G  D  S  Y  F  P  S  R  L  S  G  G  Y  D  Q  A  G  Y  Q  D  R  N        35
aacccttatttttttccctgaccagcgcttctatgaaggaggggacagctatttcccctctcgcttgtccgggggtacgaccaagccggctaccaggacaggaac      105

 S  M  M  G  L  C  A  S  L  S  G  G  V  G  V  G  V  T  G  T  E  D  K  A  S  P  S  S  M  S  P  H  S  E  A        70
tccatgatgggtttgtgcgcgagtctgtctgggggtgttggagttggggtcacaggaacagaggacaaagcctctccgtccagcatgtcacctcactccgaggcc      210

 H  C  P  G  Q  C  L  P  W  A  C  K  L  C  K  R  K  T  V  T  M  D  R  R  R  A  A  T  M  R  E  K  R  R  L      105
cactgcccaggccagtgcctcccttgggcctgtaagctttgcaaaagaaagacagtgaccatggaccgtcggagagcggccacgatgagggagaagaggcgcctg      315

 K  K  V  N  E  A  F  D  A  L  K  R  S  T  L  M  N  P  N  Q  R  L  P  K  V  E  I  L  R  S  A  I  Q  Y  I      140
aagaaggtgaacgaggcctttgacgctctgaagaggagcaccctgatgaaccccaaccagaggctgcccaaggtggagatcttgcggagcgccatccagtatatc      420

 E  R  L  Q  A  L  V  S  S  L  N  Q  Q  D  T  E  T  G  Q  Q  G  L  H  Y  R  P  S  T  A  Q  A  R              172
gaaaggctgcaggccctggtgtcttccctcaaccagcaggacactgagacaggacagcagggactgcactaccgacccagcacggctcaggccaga             516

(Intron 1, 697 bp)

 V  S  S  S  S  E  P  S  S  G  S  T  C  C  S  S  P  E  W  S  S  T  P  E  Q  C  T  Q  S  Y  S  S  E        205
gtgtcgtcgtcaagcgagcccagttcaggcagcacctgctgcagtagcccggagtggagcagcactccagagcagtgcacacagagctacagcagcgagg          616

(Intron 2, 109 bp)

D  L  L  S  A  S  D  S  P  E  Q  G  N  M  R  A  L  T  S  I  V  E  S  I  S  A  A  D  A  A  V  A  F  P  V      240
atctcctgagtgcttccgactctccagagcaggggaacatgcgtgcgctgacctccatcgtggaaagcatctctgcagcggatgcagccgtggcctttcctgtg      720
 I  S stop

 D  I  P  K stop                                                                                              244
gacattcccaaatag                                                                                               735

3′UTR, 135 bp
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Fig. 2. Atlantic halibut *myogenin* cDNA and predicted amino acid sequences, which are numbered on the right. The bHLH domain is shaded. The locations of the two introns in the genomic sequence are indicated. Alternative splicing by skipping exon 2 (underlined) generated a shorter transcript because of a premature stop codon (bold) in the changed reading frame.

visualisation of a cranial-to-caudal progression in the expression of *MyoD*, *myogenin* and *MyHC* (Fig. 5), whereas sense probes produced no colour reaction. RT–PCR was used to verify the temporal expression of *MyoD* and *myogenin* (not shown). Owing to the difficulty of making specific ISH probes for the two highly similar *MyLC2* genes, their temporal expression was studied by 3′RACE (Fig. 6), which amplified the two cDNAs differing in the length of the 3′UTR. Expression patterns of the muscle genes studied closely followed somite formation, and no significant effects of egg incubation temperature on the timing and spatial expression of *MyoD*, *myogenin* and *MyHC* were found when studied in relation to somite stage. The WH-ISH results shown in Fig. 5 are, therefore, a representative selection from all three temperature groups.

Initial *MyoD* expression was detected prior to somitogenesis at 50% epiboly only by RT–PCR, whereas the first detection of *MyoD* by WM-ISH was first observed very faintly at the 9-somite stage, and only in the lateral cells of somites 7–9 (Fig. 5A). This indicates that somites 1–6 expressed *MyoD* prior to the 9-somite stage, but this could not be detected by WM-ISH. Intriguingly, the *MyoD* signal was shown on only one side of the notochord, or in more somites on one side than on the other. This novel bilaterally asymmetric expression of *MyoD* persisted until 20–30 somites were formed (Fig. 5A–C). As *MyoD* expression progressed caudally in an increasing

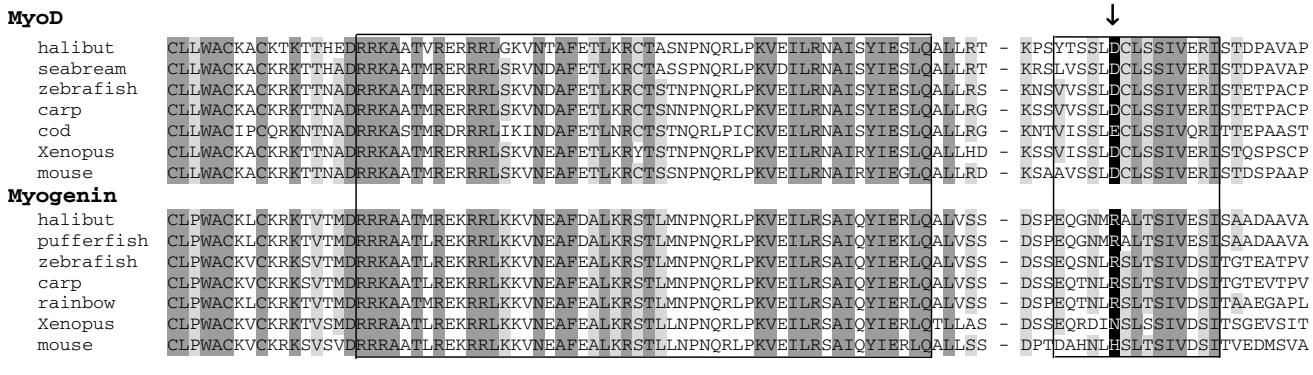

Fig. 3. Alignment of the conserved Cys-rich motif and the boxed bHLH and HIII of Atlantic halibut MyoD and myogenin and their orthologues from gilthead seabream (GenBank accession no. AAL85338), zebrafish (AAK06755, AAM82616), Atlantic cod (AAK17969), pufferfish (AAV70941), common carp (BAA33565, BAA33564), rainbow trout (CAA87010), *Xenopus* (CAA34232, AAL05070) and mouse (NP_112466, NP_034996). Conserved residues of both MyoD and myogenin are shaded in dark grey; distinct residues of the two factors are shaded in light grey. The major determinant of the differential functions of MyoD and myogenin is indicated by an arrow (Bergstrom and Tapscott, 2001).

Table 1. *Somite recruitment rate during somitogenesis and somite numbers, standard length and myotome height of newly hatched halibut larvae*

	4°C	6°C	8°C	*P*-value
Somite recruitment rate (numbers per day)	4.6±0.3[a]	5.4±0.1[b]	10.5±0.3[c]	<0.001
Somite numbers	51±3	52±1	53±1	0.353
SL (mm)	5.42±0.37[a]	6.15±0.12[b]	6.21±0.29[b]	<0.001
MH (mm)	0.39±0.03[a]	0.46±0.03[b]	0.40±0.03[a]	<0.001

SL, standard length; *MH*, myotome height.

Values are mean ± s.d. (*N*=10), and different superscript letters within rows represent significantly different values (one-way ANOVA followed by a Tukey's B *post-hoc* test).

number of somites, the pattern appeared to become more symmetric. From the 25- to 45-somite stage the expression was downregulated in more and more cranial somites (Fig. 5D–F). *MyoD* signals were still present close to hatching (>50 somites), but the individual spatial expression varied from none at all to expression in the caudal half of the newly hatched larvae (Fig. 5G).

Myogenin mRNA was first detected at the 9- to 10-somite stage by the more sensitive RT–PCR method as a heavily stained band. The adaxial cells of somites 3–10 had a faintly bilaterally symmetrical *myogenin* signal at the 9- to 10-somite stage (Fig. 5H), but *myogenin* was not detected in lateral cells of somites 1 and 2. The *myogenin* expression became stronger at the 14- to 15-somite stage as it progressed caudally and was found in adaxial cells of somites 3–15 and in lateral cells of somites 7–15 (Fig. 5I). In 20-somite stage embryos (Fig. 5J) the whole of somites 3–20 expressed *myogenin* mRNA. All somites except number 1 and 2 had a *myogenin* signal in all somitic cells throughout the 25- to 30-somite stages (Fig. 5K,L). Between somite stages 30 to 40 *myogenin* expression was downregulated in more cranial somites so that only somites 25–40 had *myogenin* expression at the 40-somite stage (Fig. 5L,M). Finally, at the 40- to 50-somite stages only the most caudal somites expressed *myogenin* and the staining decreased in intensity (Fig. 5M,N). *Myogenin* expression preceded *MyHC* expression by approximately 5 somites.

Whereas no *MyHC* mRNA was found in the earliest embryonic stages (Fig. 5O), WM-ISH signals were identified adaxially in somites 4–10 at the 17-somite stage (Fig. 5P). By the 20-somite stage, *MyHC* mRNA was also found more laterally at the posterior end of somites 11–20, whereas somites 9–12 showed only adaxial *MyHC* expression (Fig. 5Q). From the 25-somite stage onwards *MyHC* expression gradually progressed caudally (Fig. 5R–T), and close to hatching (>50 somites) the *MyHC* signal was only found in somites 40–50 (Fig. 5U).

The expression of the MyLC2 isoforms was only studied by RT–PCR, hence no spatial information is available. Whereas *MyLC2a* mRNA was detected from the 40-somite stage onwards, expression of *MyLC2b* was initiated just before hatching from the 50-somite stage onwards (Fig. 6). By contrast to *MyLC2a*, the *MyLC-2b* mRNA level increased in intensity throughout embryogenesis, and was the only isoform identified in adult fast and slow skeletal muscle (not shown).

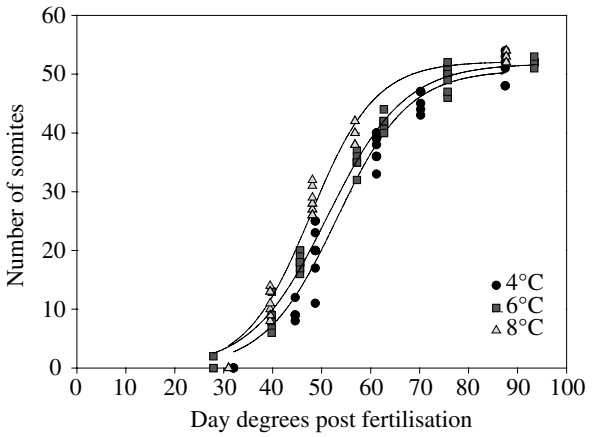

Fig. 4. Somite formation at different egg incubation temperatures. Each point represents one embryo/larva. Sigmoid curves were fitted for each incubation temperature [$y=a/(1+e^{-(x-x0/b)})$, where y refers to number of somites and x to number of d° after fertilisation].

Discussion

The onset of somitogenesis was not significantly affected by incubation temperature and occurred during the latter part of gastrulation in halibut embryos. Hence, the first somites were formed after 30–35 day-degrees (d°) in all three temperature groups. Consistently, the relative timing of both *MyoD* and *myogenin* expression in relation to somite stage was similar in all temperature groups. Furthermore, whereas the rate of somite formation increased with increasing incubation temperature, the relative recruitment rate was approximately one new somite formed per d° in all groups. The final number of somites in newly hatched larvae was therefore similar for all groups and unaffected by the incubation temperature. Hence, halibut embryogenesis, including somite formation is strongly accelerated by temperature, but the relative timing of the different processes seems to be independent of the ambient temperature. However, it should be noted that incubation at 6°C was associated with the longest embryonic period measured in d° and resulted in the greatest larval myotome height. Conversely, incubation at 4°C, which is also close to

the expected natural incubation temperature, produced significantly smaller larvae at hatching. However, whereas the 8°C larvae were of similar length as the 6°C larvae, development of deformities is expected to be higher at elevated incubation temperatures (Lein et al., 1997). Hence, development of the halibut embryo seems to be a fine-tuned process, and the natural temperature regime should be mimicked in aquaculture to produce high-quality larvae with a high growth potential. Temperature has been shown to affect the sequence of organogenesis (Johnston et al., 1995), rate of

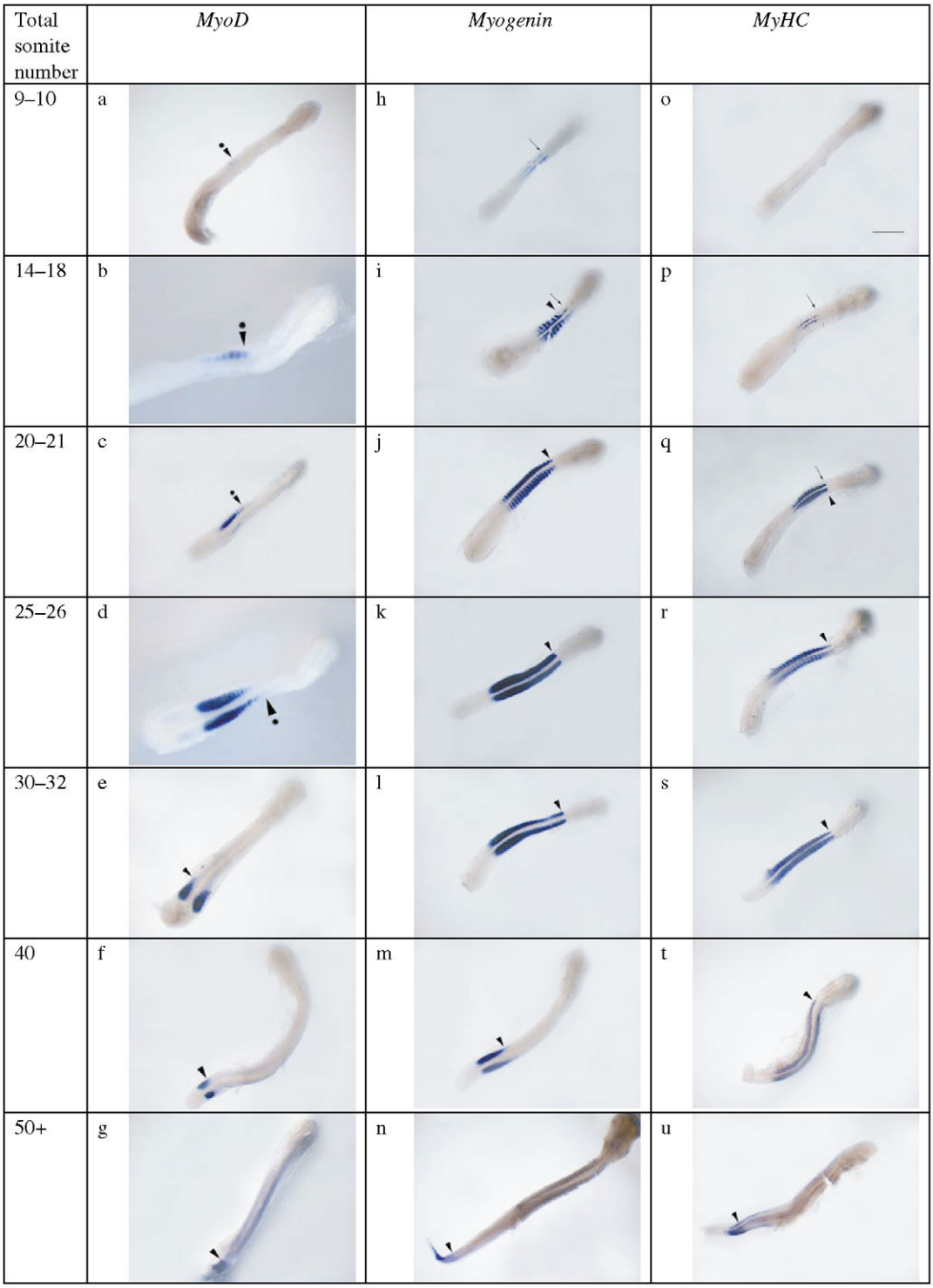

Fig. 5. Spatial and temporal expression of *MyoD*, *myogenin* and *MyHC* mRNA in halibut embryos and larvae with similar numbers of somites. All specimens are dorsal views with the head towards the upper right corner of the picture. Arrowheads indicate the first somite with expression in lateral cells; arrows, the first somite with expression in adaxial cells; dot, asymmetric expression. Scale bar, 0.5 mm for all images.

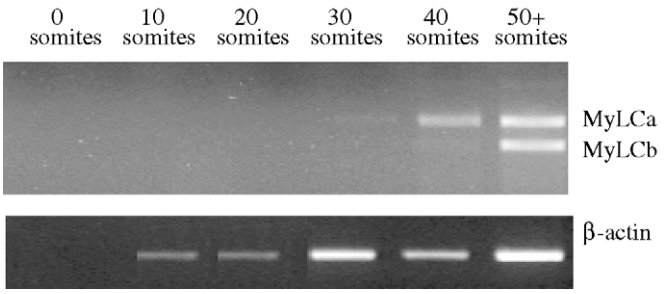

Fig. 6. Temporal expression of MyLC2a and MyLC2b during somitogenesis in Atlantic halibut. The presence of reverse-transcribed cDNAs was verified by amplifying β-actin cDNA.

somitogenesis (Brooks and Johnston, 1994) (present study), number of somites (Brooks and Johnston, 1994), number and size of muscle fibres at hatching (Vieira and Johnston, 1992; Brooks and Johnston, 1993; Usher et al., 1994; Nathanailides et al., 1995; Johnston and McLay, 1997; Alami-Durante et al., 1997; Galloway et al., 1998; Galloway et al., 1999; Hall and Johnston, 2003) and the relative timing of myofibrillogenesis (Johnston et al., 1995) in different fish species. However, in agreement with our halibut study, relative temporal and spatial expression of several muscle genes in Atlantic herring *Clupea harengus* (Temple et al., 2001), Atlantic cod *Gadus morhua* (Hall et al., 2003) and common carp *Cyprinus carpio* (Cole et al., 2004) do not seem to be influenced by temperature. Temple et al. concluded (Temple et al., 2001) that temperature probably affects myofibril synthesis downstream from MyHC transcription, at the translation or assembly stage. By contrast, the relative expression of *MRF* genes was shown to be delayed and prolonged at low egg incubation temperatures in other species such as rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (Wilkes et al., 2001; Xie et al., 2001). These authors suggested that the delayed muscle differentiation at lower temperatures could extend the proliferation phase, enable more myoblast hyperplasia and hence increase muscle fibre numbers at hatching, something that is also seen in fish species with temperature-independent MRF expression.

In most fish species where *MyoD* has been studied to date [zebrafish *Danio rerio* (Weinberg et al., 1996); Atlantic herring (Temple et al., 2001); gilthead seabream (Tan and Du, 2002); common carp (Cole et al., 2004); rainbow trout (Delalande and Rescan, 1999; Xie et al., 2001); flounder *Paralichthys olivaceus* (Zhang et al., 2006)], the gene is expressed in presomitic and somitic adaxial cells, which will later become the superficial slow red muscle fibres (Devoto et al., 1996). Subsequently, *MyoD* is expressed in lateral cells of the somites, which will form the bulk of the fast white muscle mass. In Atlantic halibut, *MyoD* mRNA was detected prior to somitogenesis, but was never observed in adaxial cells of the presomitic or somitic mesoderm at any later developmental stage. This absence of *MyoD* mRNA in the adaxial cells has also been observed in Atlantic cod (Hall et al., 2003). Furthermore, *MyoD* was expressed bilaterally asymmetrically in the earliest stages of halibut somitogenesis. These findings indicate that MyoD may have a different function in halibut and cod than in other fish species, and/or that halibut and cod may have more than one *MyoD* gene. Two MyoD encoding genes have previously been reported in the tetraploid species *Xenopus* (Scales et al., 1990), rainbow trout (Rescan and Gauvry, 1996), gilthead seabream (Tan and Du, 2002) and amphioxus *Branchiostoma floridae* (Schubert et al., 2003). After the present study was performed, another MyoD isoform, referred to as MyoD1, was identified in halibut (Ø. Andersen, S. V. W. Dahle, E. Kjørsvik, T. Bardal, H. Munck and T. F. Galloway, manuscript submitted for publication). The two MyoD isoforms of both halibut and gilthead seabream show about 72% sequence identity, indicating that the two paralogues are the result of a common duplication event about 350 million years ago (Robinson-Rechavi et al., 2001). By contrast to the *MyoD* gene identified in the present study (which should be referred to as *MyoD2*), the halibut *MyoD1* gene was symmetrically expressed in presomitic and somitic adaxial cells from the 5-somite stage onwards, and subsequently spread to lateral cells (Ø. Andersen, S. V. W. Dahle, E. Kjørsvik, T. Bardal, H. Munck and T. F. Galloway, manuscript submitted for publication). The *MyoD* gene isolated from flounder was also initially expressed bilaterally symmetrically in adaxial cells and subsequently in lateral cells (Zhang et al., 2006), but it is not known whether the flounder has two MyoD isoforms. The functional significance of the bilaterally asymmetrical expression pattern observed for halibut *MyoD2* is not known, but we hypothesise that this isoform may have adopted a new function related to the development of external asymmetry in this flatfish species. Alternatively, the asymmetric expression pattern may be related to the development of a greater muscle mass on the ocular side than on the abocular side, as observed in adult halibut (Johnston, 2004). Asymmetry of the muscle mass is not present in halibut yolk sac larvae (Galloway et al., 1995; Galloway et al., 1999), indicating that the early asymmetrical *MyoD2* expression has an effect on gross muscle anatomy during the transformation from larva to juvenile and onwards. During a sizeable part of early vertebrate embryogenesis the Wnt, FGF and Notch signalling pathways convey both symmetric and asymmetric information for bilaterally synchronised somitogenesis and left–right asymmetric patterning of internal organs, respectively (Kawakami et al., 2005). These signals operate upstream from the MRFs, and have so far only been studied in vertebrates with external bilateral symmetry (Levin, 2005). Therefore, there is a need to study more flatfish species with respect to muscle development, in order to elucidate the possible functions of the asymmetrical *MyoD2* expression in embryos of halibut and possibly other species.

Expression of the single halibut *myogenin* gene was initiated at the 9- to 10-somite stage in adaxial cells, possibly correlating with the initiation of myoblast differentiation and indicating that these red muscle precursor cells differentiate prior to the lateral white muscle precursor cells (Devoto et al., 1996;

Rescan, 2001). Subsequently, the halibut *myogenin* signals spread to lateral somitic cells of newly formed somites. When the halibut embryos contained between 30 and 40 somites, the *myogenin* signals began to disappear from the cranial somites in the same manner as the *MyoD2* transcript did. This transient expression pattern of *myogenin* resembles that observed in the Atlantic herring and common carp (Temple et al., 2001; Cole et al., 2004), but is different from that observed in rainbow trout and zebrafish (Delalande and Rescan, 1999; Weinberg et al., 1996). This probably reflects differences in species, final size, muscle fibre types and ecology.

Vertebrate muscle contains many myosin isoforms, depending on developmental stage, muscle fibre type and environmental characteristics (Goldspink et al., 2001). The expression of the myofibrillar contractile proteins MyHC and MyLC has been shown to follow expression of MRFs in all vertebrate species studied to date (Watabe, 2001). The halibut *MyHC* studied here was probably a transient embryonic isoform, and was detected from the 15 somite stage, whereas *MyLC2a* mRNA was first identified at the 40-somite stage. Neither *MyHC* nor *MyLC2a* were expressed post-hatching (results not shown). Different MyLC2 isoforms have been isolated from several teleosts, and a larval MyLC2 was shown to be gradually replaced by an adult isoform in metamorphosing flatfish (Brooks and Johnston, 1993; Yamano et al., 1994; Focant et al., 2000; Focant et al., 2003). Whereas the sequence of flatfish MyLC2 has so far been unknown, the similarity in the expression patterns strongly indicates that the deduced halibut MyLC2a and MyLC2b represent embryonic and larval/juvenile isoforms, respectively.

Factors regulating muscle determination, differentiation and development have so far mostly been studied in vertebrates with external bilateral symmetry. The present study reveals that a *MyoD* isoform, but not the other muscle genes studied, is expressed bilaterally asymmetrically during early somitogenesis of the halibut. This suggests that more such investigations of flatfish species could provide valuable information on how muscle-regulating mechanisms work in species with different anatomical, physiological and ecological traits. The present study also shows that many important events in the determination and differentiation of muscle in halibut occur within a very short time period (10 d° or approximately 2 days) between the formation of the 10th and 20th somite, a period that should be treated with special care in halibut hatcheries.

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