TEMPERATURE AND MUSCLE DEVELOPMENT IN TELEOST FISH

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by

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Abstract

The early development of the Atlantic cod *Gadus morhua* L. was studied from fertilization of the egg until first feeding at 4, 7 and 10°C. A developmental staging series was prepared, using morphological landmarks visible with the light microscope. The relative timing of developmental events and expression of muscle-specific protein (MSP) genes were compared between temperature groups. No differences were found in the time of hatching or timing of appearance of the otic placode, unpaired median fin fold, gut lumen, otic vesicle, lens of the eye, otoliths, first muscular contractions, swimbladder, and hindgut, or in the rate of development of somites, myotubes, myofibrils and acetylcholinesterase activity over the temperature range studied. Similarly, no differences were found between the timing of expression of *MyoD*, *α-actin, myosin heavy chain, troponin T, troponin I, troponin C* or *muscle creatine kinase*. However, closure of the blastopore occurred late with respect to segmentation at higher temperatures, at the 3-somite, 10-somite and 12-somite stages at 4, 7 and 10°C respectively. Larvae reared at 10°C also had significantly more (+14%) deep white fibres at hatch, whereas numbers of superficial red fibres remained unchanged. These differences in muscle cellularity might be related to changes in the relative timing of epiboly, through differential proliferation of presomitic myogenic cells and/or their relative exposure to inductive signals. To investigate the effect of temperature on expression of the myogenic regulatory factor (MRF) genes, embryos of the common carp, *Cyprinus carpio* L. were reared from fertilization of the eggs to inflation of the swim bladder in the larval stage at 18 and 25°C. No differences were seen in the timing of expression of *MyoD*, *Myf-5* and *myogenin*, or in five *myosin heavy chain* isoforms (2 embryonic and 3 adult-type transcripts) during development. It was concluded that muscle development is highly canalized within the normal developmental range for both species.

Chapter 1: General introduction

Temperature is the most influential environmental factor impacting upon biological systems, because of its intrinsic effects on the kinetics of chemical reactions and molecular stability. Organisms inhabit a thermally broad range of habitats, ranging from just over 100°C in geothermal hot-springs to around -80°C in high latitude and high altitude terrestrial habitats, and in order to survive, a huge variety of adaptations and strategies have evolved. The spatial distribution of organisms commonly reflects gradients in environmental temperature, with species replacement occurring in similar niches from equatorial to polar regions and from low to high elevations (Hochachka and Somero, 2002). Within the Animalia, organisms tend to adopt one of two strategies, depending upon their source of body heat. Endotherms produce heat at high rates in order to decouple changes in ambient temperature from internal body temperature, whereas ectotherms produce heat at comparatively low rates, and heat exchange with the environment is much more important (Eckert *et al.*, 1988). An example of where the boundaries of these categories blur however, is in the Scombrids (tunas) and the Laminids (mackerel sharks). Although fish are generally strict ectotherms, convergent evolution between these two families has resulted in a suite of morphological, physiological and biochemical specializations which can maintain *in-vivo* body temperatures up to 12°C above ambient water temperatures (Bernal *et al.*, 2001), a phenomenon known as "regional endothermy".

Aside from these specialized examples, fish conform to the principles of ectothermy, and their metabolic rate is dependant upon the temperature of their external environment. Different species inhabit waters ranging from -1°C in the polar oceans to at least 43°C in tropical hot springs, and exhibit marked differences in their

enzyme activities to maintain physiological functionality (Somero, 1975; Johnston and Altringham, 1985; Fields and Somero, 1998; Fields *et al.*, 2002). Temperature also has profound effects on embryonic development, and in variable environments, can result in considerable phenotypic plasticity. Variations in meristic (repeated) characters such as the number of fin rays, somites, and vertebrae (reviewed in Tåning, 1953) have been documented for a number of species exposed to different temperature regimes during embryogenesis. Such variation is most likely the product of upstream (intraspecific[1]) heterochronies in development, since heterochrony is thought to be *the* developmental phenomenon producing all morphological change (Gould, 1977; Gould, 1992; Reilly *et al.*, 1997). Myotomal muscle is a particularly plastic tissue, which has been shown to exhibit differences in the number of fibres at hatch (Table 1), the timing and development of myofibrils and innervation (Johnston *et al.*, 1995; Johnston *et al.*,1997), and the volume density of mitochondria (Johnston, 1993) between conspecifics reared at different temperatures.

This thesis reports the results of investigations into the effects of temperature on muscle development and growth in the Atlantic cod *Gadus morhua* and the common carp *Cyprinus carpio*. Both species are of major economic importance with a long history of human exploitation and consumption. The cod has been fished in the North Atlantic since at least the 8th century, when dried saltcod fuelled the voyages of the Vikings to Great Britain, Greenland and even as far as Newfoundland (Kurlansky, 1997). More recently, cod has formed the bulk of North American and European fishery landings and been the subject of intense political controversy culminating in the European "cod wars" of the 1970s and 1980s. Currently, the farming of cod in

[1] It is important at this early stage to emphasize differences between intra- and inter-specific heterochrony. Developmental heterochrony is most often studied on a phylogenetic (interspecific) level, where it is used to infer evolutionary relationships. However, on a tokogenetic (intraspecific) level, heterochrony plays a central role in producing the morphological variation observed among individuals within populations (Hall, 1992; Reilly, 1994).

Table 1. Trends in muscle fibre number and cross-sectional area in teleost embryos reared at different temperatures
Values are calculated from original data or figures. *Rising temperature used to simulate seasonal warming, average value shown. ND - no significant difference between groups. s – superficial slow muscle fibres. d – deep fast muscle fibres

Species	Temperature range between treatments (°C)	Difference in fibre number (low temp-high temp)	Difference in fibre cross sectional area (low temp-high temp)	Stage compared	Duration of Incubation at different temperatures	Reference
Atlantic salmon (*Salmo salar*)	1.6-10.0	↓33% d	↑40%	Hatch	Fertilisation-Hatch	Stickland *et al.* (1988)
	5.0*-11.0	↓40% d	↑38%	Hatch	Fertilisation-Hatch	Nathanailides *et al.* (1995)
	5.0-10.0	↓17% d	↓18%	Hatch	Fertilisation-Hatch	Johnston & McLay (1997)
Rainbow trout (*Oncorynchus mykiss*)	5.0-15.0	↓20% d	↓22%	Hatch	Eyed stage-Hatch	Matschak *et al.* (1998)
Atlantic herring (*Clupea harengus*)	5.0-15.0	↑66% d	↓43%	Hatch	Fertilisation-Hatch	Vieira & Johnston (1992)
Atlantic cod (*Gadus morhua*)	1.0-8.0	↑25% d	↑9%	Hatch	Fertilisation-Hatch	Galloway *et al.* (1998)
Atlantic halibut (*Hippoglossus hippoglossus*)	5.0-8.0	ND d	↓20%	Hatch	Blastopore closure-Hatch	Galloway *et al.* (1999)
Turbot (*Scopthalmus maximus*)	17.0-22.0	↓43% s	ND	Juvenile	Fertilisation-Juvenile	Calvo & Johnston (1992)
Whitefish (*Coregonus laveretus*)	17.0-22.0	↓21% d	↑19%	Hatch	Fertilisation-Hatch	Hanel *et al.* (1996)
Plaice (*Pleuronectes platessa*)	5.0-15.0	↑20% d	↑10%	Hatch	Fertilisation-Hatch	Brookes and Johnston (1993)

commercial aquaculture operations has become economically viable due to breakthroughs in larval rearing technology and the high retail value of market-sized fish. However, there is still a dearth of literature concerning their early development and growth, which is essential to the developing industry and comparative studies of teleost development.

The common carp originates from the region of the Caspian Sea. From there it is believed to have been brought west by Roman soldiers to the Black Sea and the river Danube. It also spread eastwards to China, probably introduced by the Chinese imperial army. From as early as the eighth century, carp were farmed extensively in earthen ponds in the grounds of European monasteries. Today, table-carp farming accounts for approximately 90% of world freshwater fish culture (Micheals, 1988). A great deal is known about carp physiology, and many molecular genetic markers have already been isolated, making them an ideal model for fish species with indeterminate patterns of growth (unlike the zebrafish, which exhibits a determinate growth pattern).

This introduction summarizes what is currently known about the mechanisms of muscle patterning and growth in fish embryos and larvae and, where information is available, their relationships with temperature.

Mechanisms of patterning of embryonic muscle

The early cellular movements contributing to somitogenesis are highly conserved within the vertebrates (Pouquié, 2001). In teleosts, cells of the embryonic shield (functionally equivalent to the Spemann organizer of amphibians and Henson's node in the chick; Oppenheimer, 1936; Stickney, 2000) first give rise to axial mesoderm of the notochord and pre-caudal plate (Verma, 1971; Hill and Johnston, 1997a). The paraxial mesoderm then condenses in adjacent bilateral strips, from cells

initially situated at the margin of the germ ring, which migrate dorsally (Kimmel *et al.*, 1990). Further diversification of the paraxial mesoderm, and its segmentation into somites, are highly dependant on the axial structures (Lassar and Munsterberg, 1996).

The onset of somitogenesis varies with respect to epiboly between species, beginning at approximately 45% in the cod (Fridgiersson, 1978), and in the salmonids (Ballard, 1973; Gorodilov 1996), but only after completion of epiboly in carp (Verma, 1970), mummichog *Fundulus heteroclitus* (Armstrong and Child, 1965), medaka *Ozyrias latipes* (Iwamatsu, 1994), herring *Clupea harengus* (Hill and Johnston, 1997a), and zebrafish (Kimmel *et al.*, 1995). Somite development proceeds in a rostral-caudal wave as the paraxial mesoderm is segmented into bilaterally symmetrical, epithelial-bound blocks of cells. Final somite number is extremely variable within the teleosts, ranging from 18 in the ocean sunfish *Mola mola* (Brainerd *et al.*, 2001) to over 200 in some eels (Richardson *et al.*, 1997). A variety of genes have been implicated in teleost mesodermal segmentation and these are reviewed elsewhere (Currie and Ingham, 1998; Stickney *et al.*, 2000). Within the teleost somite, the ventral sclerotome, which will form the axial bone and cartilage of the embryo is greatly reduced compared with that of amniotes. This may reflect a reduced demand for the supporting skeleton, and an increased locomotory requirement for axial, rather than appendicular muscle associated with life in a supportive aqueous medium (Bone, 1966). As a result, the positional relationships between tissues are altered, and the sclerotome, instead of forming adjacent to the underside of the notochord, arises at some distance from it (Morin-Kensicki and Eisen, 1997). The notochord and neural tube are thus embedded entirely within the myotome, which constitutes the bulk of the somite (Currie and Ingham, 1998).

The onset of the myogenic program requires the expression of members of the myogenic regulatory factor (MRF) family of basic-Helix-Loop-Helix (bHLH) transcription factors (MyoD, Myf-5, myogenin, MRF4). Knockout studies in mice have established that MyoD and Myf-5 are required for muscle lineage specification whereas myogenin and MRF4 are involved in the initiation and maintenance of the differentiation program (Rudnicki and Jaenisch, 1995). They are components of a complex and partially redundant control network which includes the MEF-2 gene family of MADS box transcription factors (Ticho *et al.*, 1996). The promoter regions of most, if not all, muscle-specific proteins contain MyoD and/or MEF2 binding sites (Watabe, 1999; Watabe, 2001). Studies in zebrafish have shown that expression of MyoD and some muscle proteins including tropomyosin occur prior to the formation of somites (Weinberg *et al.*, 1996; Devoto *et al.*, 1996; Tan and Du, 2002). In zebrafish, a 4 x 5 array of *MyoD* expressing cuboidal cells can be identified either side of the notochord prior to segmentation at the level of the major horizontal septum (Fig. 1a, b, c). These so-called adaxial cells are surrounded by smaller more numerous and irregularly shaped cells of the presomitic mesoderm (Fig. 1b). The adaxial cells can be stained by the S58 antibody, which identifies slow muscle myosin (Crow and Stockdale, 1986; Devoto *et al.*, 1996; Blagden *et al.*, 1997; Roy *et al.*, 2001). In an elegant series of experiments, Devoto and co-workers injected vital dyes into single cells in the segmental plate of zebrafish and examined their subsequent development (Devoto *et al.*, 1996). The adaxial cells were found to elongate to span the whole somite longitudinally, before migrating laterally through the somite to form the single superficial layer of slow muscle fibres characteristic of fish larvae (Fig. 1c-f). This radial migration coincides with the expression of the homeobox gene *Prox1*, which is not present in fast muscle (Roy *et al.*, 2001).

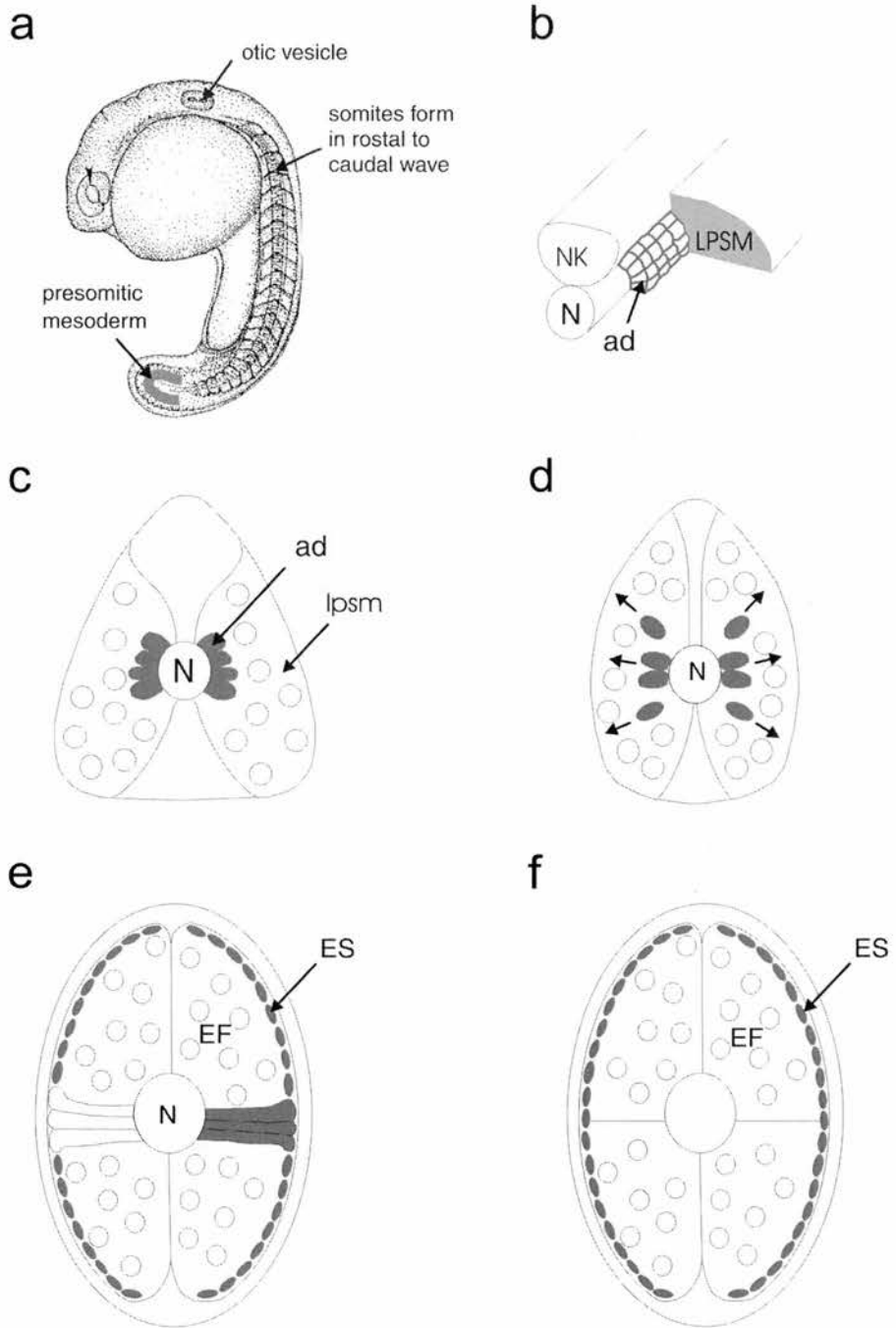


Fig. 1. Stages of embryonic myogenesis in the zebrafish (*Danio rerio*).

a. 21-somite stage embryo showing the presomitic mesoderm towards the tail bud.
b. Diagram illustrating the presence of a 4 x 5 array of cuboidal adaxial cells (ad) present on either side the notochord (N) in the most posterior somite of the embryo shown in (a). These adaxial cells can be identified in the presomitic mesoderm on the basis of histology and gene expression patterns.
c. Diagram showing a transverse section through the somite illustrated in (b) and (c).

(d-f) show later stages of development of the same somite illustrated in (c).

d. The adaxial cells elongate to span the somite width and migrate through the somite to a superficial position.
e. The adaxial derived slow muscle fibres retain connection with the notochord at the level of the major horizontal septum. This subset of fibres termed muscle pioneers can be recognised by their expression of engrailed proteins.
f. The situation at the end of the embryonic stage of myogenesis. A superficial layer of embryonic slow muscle fibres (ES) surrounds a larger number of embryonic fast muscle fibres (EF) .

Other abbreviations: NK, neural keel; LPSM, lateral presomitic mesoderm. Based on Devoto *et al.* (1996).

The adaxial cells closest to the horizontal septum have long processes that maintain a connection with the notochord for some time after the migration of the other adaxial cells is complete (Devoto *et al.*, 1996; Blagden *et al.*, 1997). This sub-population of "muscle pioneer" cells express high levels of the Engrailed (Eng) protein family and are the first fibres to become innervated in the zebrafish (Hatta *et al.*, 1991; Felsenfeld *et al.*, 1991; Ekker *et al.*, 1992; Halpern *et al.*, 1993). The initial slow muscle layer is also formed by adaxial cell migration in a broadly similar manner in the pearlfish *Rutilus frisii meidingeri* (Stoiber *et al.*, 1998), rainbow trout *Oncorhynchus mykiss* (Rescan *et al.*, 2001), Atlantic herring (Temple *et al.*, 2001) and seabream *Sparus aurata* (Tan and Du, 2002). In zebrafish, myosin expression is observed in the adaxial cells before its expression in the more lateral, presumptive fast muscle cells (Devoto *et al.*, 1996). In contrast, during myogenesis in rainbow trout, differentiation of the fast muscle and the appearance of myosin heavy chain transcripts begins in the medial fast fibre domain prior to the differentiation and migration of the slow muscle precursors (Rescan *et al.*, 2001). Myofibril assembly also occurs as a rostral to caudal wave, mirroring the initial pattern of somite formation. In an electron microscopy study, Johnston *et al.* (1995) observed the initial assembly of myosin and actin filaments into myofibrils at the 38-somite stage in Clyde herring reared at 10°C. Myofibril assembly was co-incident in the muscle pioneers and a proportion of the multinucleate fast muscle fibres within the same somite. The limited information available is therefore compatible with some inter-specific variation in the timing of embryonic slow and fast muscle fibre formation. In zebrafish, the elongation and striation of the muscle pioneers occurs at the same time as the myotomes transform from the simple block-like form to a characteristic

chevron shape, suggesting their involvement in myotomal patterning (van Raamsdonk *et al.*, 1978; Halpern *et al.*, 1993).

The hedgehog (Hh) family of glycoproteins secreted from the notochord and floorplate are thought to play a major role in the patterning of the embryonic slow muscle layer (Fig. 2). Zebrafish mutants that lack a differentiated notochord show defects in their paraxial mesoderm, including the lack of a horizontal septum, muscle pioneer cells and abnormalities in myotome shape. Injection of *Sonic hedgehog* (*Shh*) sense mRNA into eggs results in the activation of *MyoD* expression throughout the presomitic paraxial mesoderm and the subsequent differentiation of the entire myotome into embryonic slow muscle (Hammerschmidt *et al.*, 1996; Barresi *et al.*, 2000). Analysis of the *sonic you* (*syu*) mutant, which is homozygous for a disruption in the *Shh* gene, show a drastic reduction in the number of slow fibres (Schauerte *et al.*, 1998). Analysis of other notochord deficient mutants (*ntl* and *flh*) that lack muscle pioneers has led to the suggestion that another member of the hedgehog family of proteins, Echidna hedgehog (Ehh), may be required for muscle pioneer cell formation (Currie and Ingham, 1996; Barresi *et al.*, 2000). Whereas *Shh* is expressed in both the notochord and floorplate, *Ehh* is confined exclusively to the notochord. A third hedgehog family transcript *Tiggywinkle hedgehog* (*Twhh*) is expressed in the floorplate early in development (Ekker *et al.*, 1995, Currie and Ingham, 1996).

Recent evidence suggests several midline signals, probably hedgehog glcoproteins, collaborate to maintain adaxial slow muscle myogenesis (Coutelle *et al.*, 2001). In the *cyclops* mutant, which lacks floor plate cells, slow muscle myogenesis is unaffected. Similarly, in the *syu* mutant the lack of a notochord delays but does not completely abolish slow adaxial myogenesis. However, *cyclops;sonic you* double mutants, which lack both tissues do not develop any slow muscle derived from the

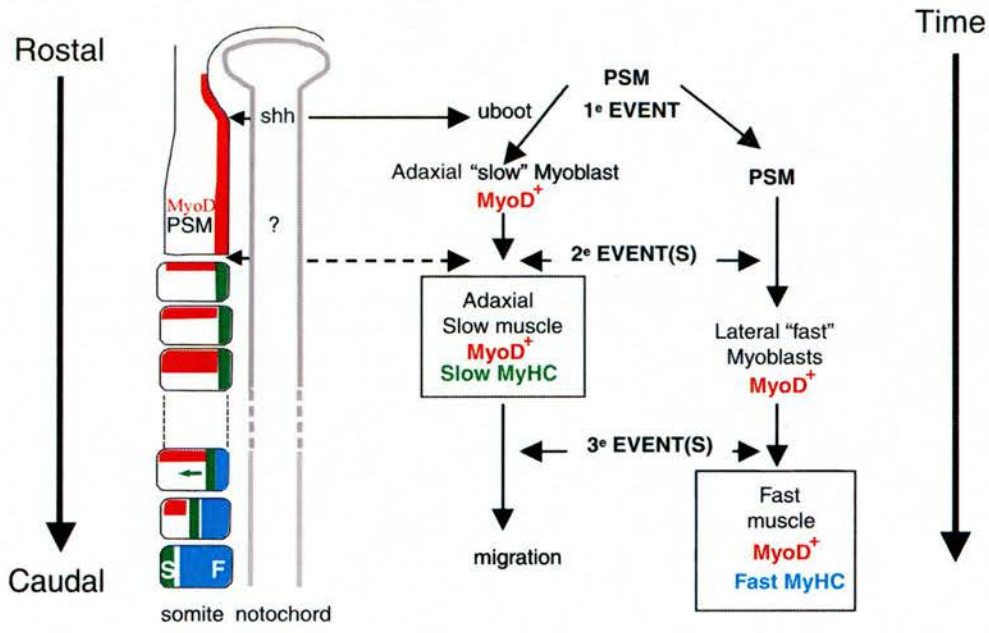


Fig. 2. Mechanisms thought to be involved in the patterning of the embryonic slow and fast muscle fibres in zebrafish. PSM - presomitic mesoderm, S - slow muscle, F - fast muscle. Based on Blagden *et al.* (1997).

adaxials. *In-situ* hybridization and ectopic expression studies have demonstrated that Shh is necessary for normal expression of *MyoD* and *Myf-5* in the adaxial slow muscle precursors, but not in the lateral paraxial mesoderm (Coutelle *et al.*, 2001). The primary inductive mechanism for fast muscle is unknown at present (Fig. 2). However, it has recently been shown that mutations in the zebrafish *u-boot* (*ubo*) gene cause the adaxial cells to abort their developmental program, fail to migrate or express *Prox1*, and transform into muscle cells with fast fibre properties (Roy *et al.*, 2001). It is argued that the *ubo* gene acts as a Hh-dependant myogenic switch, selectively propelling naïve myoblasts away from the "default" fast muscle pathway, into a slow muscle lineage.

Myogenic genes and genome duplication

Two non-allelic MyoD encoding genes have been reported in the rainbow trout, and named *TmyoD* and *TmyoD2* (Rescan and Gauvry, 1996). *TMyoD* mRNA expression occurs in the adaxial cells of the unsegmented mesodermal plate and in the forming somites (Delalande and Rescan, 1999). Once the myotomes have acquired their chevron shape, *TMyoD* expression extends to the fast muscle precursors. In contrast, *TMyoD2* expression is limited to the posterior compartment of the somites (Delalande and Rescan, 1999; Rescan *et al.*, 1999). The two *MyoD* paralogues are thought to originate from a recent tetraploidization of the salmonid genome (Allendorf and Thorgaard, 1984), followed by a diversion of function, as suggested by the differing expression patterns. This mechanism of functional diversification by gene duplication is a significant theme in evolutionary developmental biology (Wagner, 2001). Indeed, gene duplication events are thought to have produced all four members of the *MyoD* family from a single common ancestor (Atchley, 1994). This hypothesis

might account for some of the redundancy in function (Wang and Jaenisch, 1997) although it is equally possible that more detailed studies of knockouts will reveal further, subtle differences in phenotype (e.g. Ordahl and Williams, 1998; Sabourin and Rudnicki, 2000)

Recently however, the picture has become more complex with the isolation of a second *MyoD* paralogue from the (non-salmonid) gilthead seabream. (Tan and Du, 2002). In this case, both *MyoD1* and *MyoD2* transcripts are expressed in the adaxial cells prior to somitogenesis, but *MyoD2* becomes restricted to the somites as segmentation begins. The seabream paralogues are much more divergent in sequence identity than the trout paralogues (Tan and Du, 2002) suggesting an earlier duplication.

Analysis of *Hox* gene clusters and the advent of whole genome sequencing projects have provided evidence for at least one, and probably two genome duplication events during early chordate evolution (Lundin, 1999; Taylor and Brinkmann, 2001; Spring, 2002). Evidence for a third genome duplication, which apparently took place after the sarcopterygian radiation is hotly disputed (Taylor *et al.*, 2001a; Tayor *et al.*, 2001b; Robinson-Rechavi *et al.*, 2001a; Robinson-Rechavi *et al.*, 2001b; Robinson-Rechavi *et al.*, 2001c). Under such a scheme the sarcopterygian lineage, which includes the coelacanths, lungfishes and all land vertebrates would have only half the number of genes of actinopterygian fish (Meyer and Schartl, 1999). Although many of the duplicated genes would have been lost, others would have evolved new functions, and this may explain the presence of the second *MyoD* paralogue in the seabream. Furthermore, in view of the recent salmonid tetraploidization, up to 4 paralogues might be expected in these species.

Mechanisms of muscle expansion in embryos and larvae

The number of adaxial cells per somite in zebrafish is substantially less than the number of superficial muscle fibres present in the embryo at hatching (van Raamsdonk *et al.*, 1978; Barresi *et al.*, 2001). In zebrafish embryos, 24h post-fertilization at 28.5°C, the number of slow muscle fibres begins to increase from germinal zones at the dorso-ventral extremities of the slow muscle layer by a process of stratified hyperplasia (Barresi *et al.*, 2001). Similar germinal zones have been described in the myotomes of marine teleost larvae including the sea bass *Dicentrarchus labrax* (L.) (Veggetti *et al.*, 1990; Ramírez-Zarzosa *et al.*, 1998), seabream (Rowlerson *et al.*, 1995; Patruno *et al.*, 1998); Clyde herring (Johnston, 1993; Johnston *et al.*, 1998) and plaice *Pleuronectes platessa* (Brooks and Johnston, 1993).

In seabream, a germinal zone lying under the embryonic slow muscle layer is thought to give rise to additional slow fibres from hatch to the mid-point of larval life. The same germinal zone also gives rise to intermediate (pink) muscle fibres, which have a distinct pattern of myosin expression from the slow fibres (Mascarello *et al.*, 1995). Labelling experiments with the thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU), have provided evidence that other germinal zones produce additional fast muscle fibres. These fast muscle germinal zones become exhausted towards the end of larval life (Rowlerson *et al.*, 1995). Following metamorphosis, between 46 and 60d post-hatch, the number of fast fibres in the seabream remains relatively constant and muscle expansion occurs via fibre hypertrophy. Between 60 and 90d post-hatch, new muscle fibres are added from myogenic precursor cells scattered throughout the myotome (Rowlerson *et al.*, 1995), a process termed "mosaic hyperplasia" (Rowlerson and Veggetti, 2001). In contrast, stratified hyperplasia of slow muscle

fibres from the deep surface of the superficial germinal layer continued until at least 90d post-hatch (Rowlerson *et al.*, 1995). Mosaic hyperplasia involves the proliferation of a population of precursor cells which subsequently migrate and fuse to form myotubes before maturing into new muscle fibres of around 4-5μm diameter. These three phases of myogenesis have also been also observed in a variety of other teleost species (reviewed in Koumans and Akster, 1995; Rowlerson and Veggetti, 2001).

There is evidence that the relative timing and importance of the three phases of myogenesis and the location of the zones of stratified hyperplasia varies considerably between species. In species with determinate growth that have a small maximum body size including zebrafish (van Raamsdonk *et al.*, 1979) and male guppies *Poecilia reticulata* (Veggetti *et al.*, 1993), the first two phases of myogenesis are the primary means of increasing fibre number. On the other hand, mosaic hyperplasia is the major means of muscle expansion in species which show indeterminate growth and reach a large body size (Johnston, 2001).

In Clyde herring, the embryos hatch as transparent larvae at around 8mm total length (TL). The number of slow, but not fast muscle fibres, increases between the end of the segmentation period and hatching (Johnston, 1993). Fibre number then remains constant until 12-15mm TL for fast muscle and 20-22mm TL for slow muscle, depending on the temperature regime (Johnston *et al.*, 1998). At this stage stratified hyperplasia is observed from germinal zones beneath the slow muscle layer and at the lateral apices of the myotomes (Johnston *et al.*, 1998). However, in contrast to seabream, new slow muscle fibres in herring are added to the outside of the superficial monolayer of cells present at hatch (Johnston and Horne, 1994; Johnston *et al.*, 1998).

As muscle fibres increase in diameter they absorb additional nuclei which partially compensates for the fall in nuclear:cytoplasmic ratio with growth (see Koumans *et al.*, 1993). The turnover of nuclei in adult rat *extensor digitorum longus* muscle is of the order of 2% per week (Schmalbruch and Lewis, 2000). The turnover of nuclei in teleost muscle has not been investigated. However a population of myogenic cells that is activated to repair injury has been identified in zebrafish and seabream (Rowlerson *et al.*, 1997). It seems likely that a pluripotent embryonic stem cell population gives rise to the various precursor cells involved in stratified and mosaic hyperplasia, and the supply of nuclei for turnover and repair of slow, intermediate and fast muscle fibre types. However, the embryological origin and level of commitment of these various precursor populations remains to be determined

Mechanism of patterning of larval and juvenile muscle fibre types

Stratified hyperplasia of slow muscle fibres in embryonic and larval zebrafish occurs in the absence of hedgehog signalling. Studies with the slow-muscle-omitted gene (*smu*) mutant which are defective in Shh signalling have shown that slow fibres are added from germinal zones at the correct time and place in the absence of a scaffold of adaxial cell derived slow fibres (Barresi *et al.*, 2000). The primary inductive mechanism for the patterning of fibres produced from germinal zones (stratified hyperplasia) or scattered myogenic precursors (mosaic hyperplasia) is unknown as is the influence of motor innervation on muscle phenotype.

Developmental changes in myofibrillar protein expression in larvae

The myofibrillar proteins, with the possible exception of α-actin, exist as different isoforms, which are differentially expressed according to functional demand

and ontogenetic stage. Different protein isoforms can arise from gene families, alternate splicing mechanisms and/or post-translational modifications. Myosin comprises six polypeptides encoded by 3 to 5 genes comprising heavy (200,000 Daltons) and light chains (15,000-26,000 Daltons) (Fig. 3a). The composition of heavy (MyHC) and light chains (MyLC) determines the contractile properties of the muscle fibres, including their ATPase activity, maximum shortening speeds and force-velocity relationships (Bottinelli *et al.*, 1994; Crockford *et al.*, 1995). There are two heavy chains per myosin molecule, each of which consists of a structural α-helical rod portion and a globular head (Fig. 3a). Each muscle fibre type expresses a distinct isoform of heavy chain (carp, Rowlerson *et al.*, 1985; Atlantic herring, Crockford and Johnston, 1993). The myosin heads contain one essential (alkali) light chain and one regulatory (phosphorylatable) light chain that are encoded by different genes. The two alkali light chains arise from an alternate mRNA splicing mechanism in mammals, but are encoded by distinct genes in teleosts (Hirayama *et al.*, 1997). Different isoforms of essential and regulatory light chains are associated with slow and fast muscle fibre types, and with different stages of development

The developmental-stage specific expression of myofibrillar isoforms show considerable variation amongst the species studied to date. Using polyclonal antibodies, four myosin isoforms were identified in the myotomes of larval sea bass *Dicentrarchus labrax* (Scapolo *et al.*, 1988). They were referred to as "early white muscle myosin" (hatching to 10-29d post-hatch), "late white muscle myosin" (28d to 20 months post-hatch), "early red muscle myosin (hatch to 28d post-hatch) and "late red muscle myosin" (28-80d post-hatch). The composition of the component polypeptides has also been investigated by 1- and 2-dimensional polyacrylamide electrophoresis and peptide mapping, although such studies require the isolation of

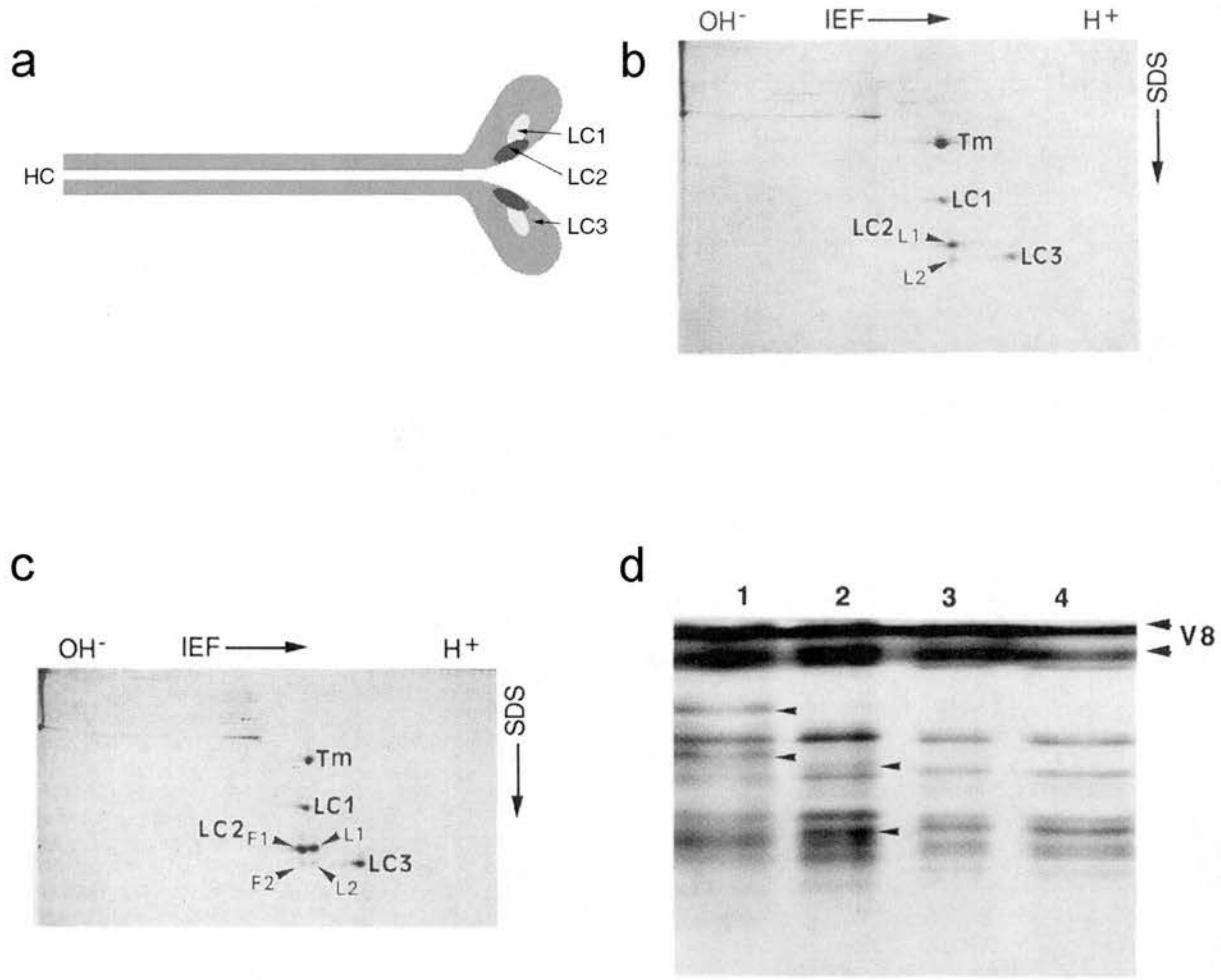


Fig. 3. Developmental changes in myosin expression in the fast myotomal muscle of the plaice, *Pleuronectes platessa*.

a. Sub-unit structure of myosin. Each myosin molecule comprises 6 polypeptide chains, 2 heavy chains (HC) and 4 light chains (LC). The heavy chains comprise a structural rod portion and a globular head containing the light chains, the actin binding site and ATPase active site (not illustrated). The essential or alkali light chains exist as 2 isoforms (LC1 and LC3). Each head contains 1 essential light chain (LC1 or LC3) and 1 regulatory or phosphorylatable light chain (LC2).
b & c. Two-dimensional polyacrylamide gel electrophoresis (PAGE) of the acidic myofibrillar proteins from fast muscle. b. 1d-old larva, c. a 10 week-old juvenile that had completed metamorphosis. IEF: isoelectric focusing; SDS: sodium dodecyl sulphate; LC1 myosin light chain-1; LC2: myosin light chain 2 (subscript L represents larval isoforms and subscript F adult isoforms); LC3: myosin light chain 3.
d. Peptide maps of myosin heavy chains digested with *Staphylococcus aureus* V8 protease and run on 15% SDS PAGE gels which were silver stained. Lane 1: adult fast, Lane 2 wild-caught 1-group juvenile; 10 week old laboratory reared juvenile that had recently completed metamorphosis; Lane 4: 6 week old laboratory reared larvae. Arrowheads indicate differences in the peptides obtained from digests of the different myosin heavy chains. From Brooks and Johnston (1993).

pure population of fibres, and are largely restricted to fast muscle. In the barbel (*Barbus barbus* L.) different isoforms of myosin were identified in embryonic, larval and adult fast muscle and the ratios of the light chains LC1:LC3 were also found to change during development (Focant *et al.*, 1992).

Myosin from the inner fast muscle layer of hatched plaice embryos (*Pleuronectes platessa*) contains two larval forms of the phosphorylatable light- chain 2 ($LC2_{L1}$ and $LC2_{L2}$) (Fig. 3b). Brookes and Johnston (1993) showed that following the completion of metamorphosis (10 weeks at 8°C) fast muscle fibres co-expressed LC2 isoforms characteristic of fast muscle in adult plaice in addition to the larval isoforms (Fig. 3c). However, expression of the larval isoforms was much reduced compared to the start of metamorphosis. The two adult LC2 isoforms consisted of a major ($LC2_{F1}$) and a minor ($LC2_{F2}$) component. The latter is thought to correspond to an isoform initially expressed in myotubes and immature muscle fibres. The larval LC2 isoforms were still detectable in 0-group, but not 1-group wild caught plaice. In contrast, peptide maps of myosin heavy chains from larvae pre- and post-metamorphosis were identical, but distinct from those on both 0-group and 1-group plaice (Fig. 3d). Williams and Brown (1992) found that metamorphosis in another species from the same genus, the American flounder (*Pleuronectes americanus*) was not associated with any change in the average speed or duration of escape responses made in response to predatory amphipods. This observation is consistent with the observation that heavy and essential light chains of myosin show no change in expression associated with metamorphosis (Brooks and Johnston, 1993). The function of the different myosin LC2 isoforms is unknown.

Larvae of Atlantic herring undergo marked changes in swimming style between 12 and 22-25mm as the unpaired fins (Batty, 1984) and adult patterns of

innervation to the slow muscle layer develop (Johnston *et al.*, 1997). Prior to the development of the dorsal and anal fins the larvae swim with an anguilliform motion, in which the amplitude of body movements is a linear function of body length and the lateral acceleration along the body is constant (Batty, 1984). Once the primordial fin is absorbed, and the unpaired fins develop rays, a subcarangiform style of swimming is adopted in which the amplitude of the trunk increases markedly towards the tail, and reactive forces dominate (Batty, 1984). The myofibrillar protein isoform composition of fast muscle gradually changes from one that is characteristic of larvae, to that of adult fish over the length range 11 – 17mm (Johnston *et al.*, 1998). However, not all proteins show the larval-adult transition at the same body length. Johnston *et al.* (1997) found no evidence for a change in LC1 and LC3 isoforms during development. However, newly hatched embryos expressed a single embryonic isoform; $LC2_e$, which became co-expressed with the adult form $LC2_F$ as a minor component at 11mm TL. An embryonic form of troponin I (TnI) was expressed in 60-somite stage embryos to be replaced by 3 or 4 larval TnI isoforms between hatching and 15mm TL. The adult form of TnI started to be expressed at 15mm TL, and at 17mm TL was the only TnI isoform present. Troponin T (TnT) isoforms are thought to arise from alternative splicing of a single gene. Johnston *et al.* (1998) found multiple embryonic, larval and adult isoforms of TnT in the fast muscle of herring, with the adult pattern only established at 17mm TL. Peptide mapping studies revealed a change in myosin heavy chain composition in fast muscle fibres between 20 and 25mm TL. The initial superficial monolayer of slow muscle fibres stained with a monoclonal antibody to adult fast muscle myosin light chain 3 ($LC3_f$) until the larvae had reached 22 to 24mm TL. Expression started to be switched off first in the superficial slow fibres adjacent to the major horizontal septum, and then extended

towards the lateral line (Johnston *et al.*, 1998). The slow fibres added externally to this superficial layer were unstained for $LC3_f$, suggesting they already expressed the slow muscle LC3 isoform.

The functional significance of these changes in myofibrillar protein composition during the larval stages is unclear, but probably reflects a decrease in contraction time with increasing body size due to scaling considerations.

Myogenesis of the fin muscles

Early morphological and ultrastructural studies suggested that the pectoral fin muscles were derived from the direct extension of undifferentiated cells of the myotome into the developing fin buds in both selacians (Dohrn, 1884) and teleosts (Corning, 1842; Harrison, 1895; Grimm, 1973). In contrast, amniotes have a small number of myogenic precursors specified by their position in the lateral hypaxial myotome, and identified by their expression of the transcription factor *Pax 3,* which migrate to the limb bud and are the source of all appendicular muscles (Amthor *et al.*, 1998). Recent observations in zebrafish have failed to confirm the earlier morphological studies in teleosts, although it has been shown that fin muscles in the dogfish *Scyliorhinus canicula* are derived from direct extensions of the epithelial somites (Neyt *et al.*, 2000).

In zebrafish, expression of *MyoD* in the pectoral fins is initiated at 29h post-fertilization, 5h after the differentiation of the myotomal muscle is complete, and differentiation of muscle fibres starts 12h later around a condensing chondrocyte core (Weinberg *et al.*, 1996). The origin of myogenic precursors to the pectoral fin muscles was investigated by Neyt *et al.* (2000) who injected the lipophylic tracer DiI into individual somites of a transgenic strain of zebrafish that expressed green fluorescent protein (GFP) under the control of the muscle-specific *α-actin* promoter. Coincident

DiI (red) and GFP (green) fluorescence indicated a contribution of the injected cells to the developing muscles in the fin bud. Somitic cells were also labelled by the uncaging of caged fluorescent dextran within specific somites in conjunction with immunostaining for myosin heavy chains. These elegant experiments showed that the fin musculature was derived from cells present in the ventral portion of somites 2-4. Similarities with the amniote pattern of limb development were further strengthened by the finding that orthologues of the mouse *lbx1* and *mox2* homeobox-containing genes were expressed in specific regions of somites 2-4 and in presumptive migratory myoblasts within the developing fin bud.

There is much less information available about myogenesis in the unpaired fins. The muscles stiffening the dorsal and anal fins arise relatively late in larval life as the primordial fin is absorbed. The embryological origin and patterning of the myogenic precursors involved is entirely unknown.

Temperature and early myogenesis

The embryos of each species have a defined temperature tolerance, which is usually less than that of juvenile or adult stages. Towards the lethal limits for normal development, abnormalities and developmental failure become relatively common (Stockard, 1921). There are a number of reports of heterochronic development in teleost embryos and larvae, particularly with respect to temperature. For example, the developmental sequence of pectoral fin formation, mouth opening and eye pigmentation has been shown to vary with temperature for various tropical fish larvae including red seabream *Pagrus major* and Japanese flounder *Engraulis japonica* (Fukuhara, 1990). The most detailed and ecologically relevant studies have been carried out in Atlantic herring and Atlantic salmon. In Clyde herring, Johnston *et al.*

(1995) showed that the formation of the initial superficial monolayer of slow muscle fibres was only apparent at the end of segmentation at 5°C, but occurred at the 48-somite stage at 8°C and the 40-somite stage at 12°C. The first signs of myofibril assembly and the appearance of acetylcholinesterase (AchE) activity at the myosepta also occurred at later somite stages at 5°C, than 8, 10 or 12°C (Johnston *et al.*, 1995; Johnston *et al.*, 1997). Thus, the timing of key events in early myogenesis varied with respect to other developmental landmarks. There is conflicting evidence concerning the involvement of the MRF family in the plasticity of muscle phenotype. Temple *et al.* (2001) found that *MyoD* and *myogenin* expression occurred at the same somite stage at 5, 8, and 12°C, and concluded that the effects of temperature on the appearance of organized myofibrils must occur downstream of myosin heavy chain transcription, either at the level of translation or at the assembly stage. However, Xie *et al.* (2001) found that in rainbow trout, *MyoD* and *myogenin* were expressed in a greater number of somites for a given developmental stage at 4°C compared to 12°C. In either case, larvae of both species hatch with well differentiated muscles at all temperatures studied, although the volume density of the fast fibres occupied by myofibrils has been shown to be significantly less in Clyde herring at 5°C (39.2%) than at either 8°C (49.6%) or 12°C (50.2%) (Johnston, 1993). Temperature also affected the timing of myofibril formation and the development of AchE activity in the Blackwater (Spring spawning, Irish sea) and Manx (Autumn spawning, North Sea) populations of herring, although the patterns of response were significantly different to those observed in Clyde herring (Johnston *et al.*, 2001). These population differences may reflect genetic differences or maternal effects (growth factors, mRNAs in the egg yolk) related to different environmental and/or feeding conditions, but are not related to egg volume (Johnston *et al.*, 2001).

Swimming performance of fish larvae is, in part, a function of body length. Foraging capacities generally increase whereas maximum tail-beat frequencies during escape responses decrease with increasing body length. Due to their small size, larvae swim at Reynolds numbers where viscous forces predominate until they reach around 12mm (Batty, 1984). The developmental-stage specific expression of myofibrillar proteins in relation to body length has been shown to vary considerably at different rearing temperatures in Atlantic herring (Crockford and Johnston, 1993; Johnston *et al.*, 1997; Johnston *et al.*, 1998). For example, Johnston *et al.* (1997) showed that the adult pattern of myosin LC2 expression was established and the embryonic isoform down regulated at 11mm TL at 15°C, but not until 15mm TL at 5°C. Similarly the complex expression patterns of embryonic> larval> adult isoforms of TnI and TnT were delayed with respect to body length as the rearing temperature was decreased from 15°C to 5°C. The transition of appearance of each myofibrillar protein occurred over a different body length range and each showed a slightly different response to temperature. Thus the myofibrillar phenotype in fast muscle at any given stage was a function of sea temperature until the larvae reached around 19mm TL and the adult pattern of proteins was established. Similarly, Clyde herring expressed the fast muscle isoform of myosin LC3 until 22mm TL at 12-16°C, but didn't switch it off until 24-28mm TL at 5-8°C. Slow muscle fibres in Atlantic herring are initially innervated at their myoseptal ends, as are the fast muscle fibres at all ontogenetic stages. Johnston *et al.* (1997) showed that the adult multi-terminal pattern of innervation began to appear at 12-14mm TL under a regime starting at 12 and rising to 16°C, but was delayed until 16-19mm TL at lower temperatures (5°C rising to 11.5°C). The development of innervation of the anal fin ray muscles was investigated by staining larvae for AchE activity. For larvae of 17mm TL and 22mm the number of anal fin

ray muscles and the number of endplates with AchE staining increased with rearing temperature. The fin ray muscles serve to alter the stiffness and camber of the unpaired fins contributing to the effectiveness of swimming (Videler, 1985). Temperature effects on the development of myofibrillar proteins and muscle innervation appear to occur over the range of body sizes when swimming changes from an anguilliform to a sub-carangiform style, and might be expected to influence performance and behaviour.

Perhaps surprisingly, a difference in the temperature prior to first feeding has been found to be sufficient to produce similar heterochronic shifts in the development of the dorsal and anal fins and associated musculature later during larval life. Johnston *et al.* (2001) reared larvae at either 5°C or 12°C until first feeding (8.5-9.5mm TL) and then transferred them to a common ambient temperature (9-11°C). The development of dorsal and anal fin ray muscles and AchE staining started later in the 5°C than 12°C groups with respect to body length. The functional consequences of the delay in unpaired fin development were investigated by filming escape responses at 200Hz. Over the length range 11.2 to 18.5mm TL maximum swimming velocity was 24% higher in larvae hatched from 12°C than 5°C eggs. The adaptive significance of this result is unclear. Herring larvae less than 25mm TL probably rely on their transparency to avoid encounters with fast moving fish predators rather than swimming performance (Blaxter and Fuiman, 1990; Fuiman, 1991). Escape swimming, may however; be important for evading slow moving predators such as jellyfish. Moreover, Morely and Batty (1996) reported that initial rearing temperature affected behaviour and velocity in Clyde and Manx herring during feeding strikes. Experimental studies in mesocosms would be required to discover whether

temperature-induced heterochronic shifts in fin muscle development were adaptive and associated with an increase in fitness.

The temperature during early development has long been known to alter aspects of the adult phenotype in some species including pigmentation patterns (Schmidt, 1919), meristic characters (Tåning, 1952) and sex (Lagomarisino and Conover, 1993; Strussman *et al.*, 1996). Recently it has been shown that development temperature affects the number and size of the slow and fast muscle fibres present at hatch and first feeding in a wide range of genera (Table 1). Potentially, temperature can influence the number and behaviour of the myogenic precursors involved in all three phases of myogenesis. Evidence that embryonic temperature can have lasting effects on fibre number is beginning to accumulate. Interestingly, the effects of temperature on fibre number vary during ontogeny (Johnston and McLay, 1997; Nathanailides *et al.*, 1995) and between geographically distinct populations of the same species (Johnston *et al.*, 2000a; Johnston *et al.*, 2000b). Johnston *et al.* (2000b) incubated eggs from Atlantic salmon (*Salmo salar*) trapped on their final spawning migrations to upland and lowland tributaries of a large river system at the temperatures normally experienced in their native streams, differing in average temperature by 2.8°C. The number of undifferentiated myogenic cells at first feeding was identified using the molecular marker, *c*-met. Fish were examined at hatch, first feeding and at various points after transfer to constant environmental conditions (12-14°C; 8h dark: 16h light). The number of myogenic cells and muscle fibres in S1 smolts at the end of the freshwater stage was a function of egg incubation temperature for the upland, but not the lowland population (Johnston *et al.*, 2000b). In another recent study with a population of farmed salmon, fish reared at cooler temperatures during the freshwater stages were found to have around 25% more fibres once all

fibre recruitment had ceased in seawater stages than fish that had been reared in heated water, indicating a permanent effect of temperature on the adult phenotype (Johnston *et al.*, 2002).

The total density of cells with the ultrastructural characteristics of undifferentiated myoblasts was quantified in Clyde herring embryos incubated at 5, 8 and 12°C (Johnston *et al.*, 1998). The density of myogenic precursor cells at hatch was around three-fold higher at 8°C than at 5°C, and intermediate at 12°C, suggesting that the temperature of embryonic development may affect larval growth characteristics. In order to test this hypothesis Clyde herring from the same families were incubated at 5 and 8°C until first feeding and then transferred to a common temperature (Johnston *et al.*, 1998). Fish were sampled at transfer to the common temperature and after approximately 1400 degree-days of total growth per group. The total cross-sectional area of muscle increased by around 60% more over the course of the experiment for the 8°C than 5°C group, due to a greater rate of muscle fibre recruitment (Johnston *et al.*, 1998; Johnston *et al.*, 2001). The total density of myonuclei was similar in both groups at first feeding but was 36% higher by the end of the experiment in the 8 than 5°C group. The influence of early development temperature (10 or 13.5°C) on fibre recruitment was also investigated for the autumn spawning population of Manx herring which experience substantially higher temperatures until first feeding, and then falling temperatures with the onset of winter (Johnston *et al.*, 1998). Manx herring were shorter at first feeding and grew more slowly than the Clyde population. After 86d at a common temperature the 10°C group had 30% more fast muscle fibres than the 13.5°C group, indicating that development temperature also influenced fibre recruitment, but in a different manner to that observed with the spring spawning stock.

An ecological perspective on the phenotypic plasticity of muscle in fish larvae

Herring populations in the Eastern Atlantic, Iceland, North Sea and Baltic spawn in almost every month of the year. Marked morphological differences between stocks are found with respect to maximum body size, number of fin rays, vertebrae and otolith shape. In spite of this, with the exception of certain deep-water resident populations in Norwegian fjords, there appears to be very little genetic structuring between stocks (Jørstad *et al.*, 1994; Turan *et al.*, 1998). Ryman *et al.* (1984) suggested that some of the lack of correspondence between patterns of genetic and morphological variability in this species might be explained on the basis of differences in environmental conditions during early development, particularly temperature and salinity. In this context the finding that muscle fibre number is sensitive to development temperature is of particular interest because fisheries biologists have proposed that fast muscle fibre number can be used as a method of stock identification (Greer-Walker *et al.*, 1972). However, the finding that early myogenesis showed a different response to temperature in several herring populations also suggests that variation in genotype-temperature interactions may also contribute to the observed morphological variability (Johnston *et al.*, 2001).

There have been very few studies of muscle development and growth in natural populations. Temple *et al.* (2000) studied a population of herring that spawn in the Blackwater estuary, Essex, England. This population spawns in waves over about two months in the spring at a time of increasing water temperature. The larvae stay within the estuary for at least 2 months allowing the cohorts hatching at different times to be identified by otolith analysis and sampled at regular intervals. Estimated

average temperatures at hatch for early-, mid-, and late- spawners were 7.5, 9.8 and 14.9°C respectively. The number and size distribution of slow and fast trunk muscles varied between different spawning groups with respect to age, and there was some indication of different patterns of fibre growth with respect to body length for the fast muscle (Temple *et al.*, 2000). In the field, muscle growth is undoubtedly influenced by numerous factors including temperature, feeding and foraging activity. Muscle atrophy and/or the degree of muscle fibre separation have previously been used as an index of severe starvation (e.g. Catalán and Olivar, 2002). Studies of muscle growth could provide a useful adjunct to other information on larval nutrition in wild populations. Feeding has been shown to increase the number of myogenic precursor cells expressing MyoD in the Antarctic fish, *Notothenia coriiceps* (Brodeur *et al.*, 2002). The expression patterns of markers of myogenic cell activation and division would be expected to be sensitive to variations in environmental conditions and feeding over relatively short time scales of a few days. In contrast, the addition and expansion of muscle fibres integrates information about feeding and environmental conditions over several weeks or months. A combination of both sorts of measurement in wild populations, in conjunction with carefully calibrated laboratory experiments, has the potential to provide new insights into the survival and nutritional condition of fish larvae in relation to mesoscale variability in physical oceanographic features.

Chapter 2: Stages of embryonic development in the Atlantic cod *Gadus morhua* L.

Introduction

The Atlantic cod, *Gadus morhua*, is an economically important marine fish of the northern hemisphere. It's natural distribution covers Cape Hatteras to Ugava Bay along the North American Coast, the east and west coasts of Greenland, around Iceland and throughout coastal Europe from the Bay of Biscay to the Barents Sea (Cannon, 1997). Cod are seasonal batch spawners, which undergo migration to gather in spawning aggregations during the winter months. They are highly fecund, with each female producing 17-20 batches of eggs over the course of a season, and each batch containing up to 300,000 eggs (Kjesbu, 1989). The spawning season varies geographically between January and May, and usually lasts for a period of 60-90 days.

There has been a cod fishery in the north Atlantic since the eighth century, but since the early nineteen-eighties, fisheries management issues and the prevailing socio-economic climate have seen retail prices rise high enough to make cod farming an attractive commercial proposition. Interest in the potential of cod farming was fuelled by the successes of the Atlantic salmon industry, although important problems remained; most notably the difficulty of larval feeding (Knutsen and Tilseth, 1985; Øiestad *et al.*, 1985; Kjørsvik *et al.*, 1991; Pedersen and Falk-Petersen, 1992), and cannibalism among the juveniles (Folkvord, 1991). The first economically successful attempts at raising cod were piloted in lagoon systems in the Norwegian fjords, fed by a natural crop of concentrated zooplankton (Øiestad, *et al.*, 1985; Øiestad, *et al.*, 1987;

van der Meeren and Næss, 1993). However, scaling-up this approach has been limited by geographic and environmental constraints. Since 1995 however, significant advances in larval feeding strategies have improved the viability of intensive culture, to the point where it is now a commercial reality. It is estimated that by 2005, approximately 6000 tonnes of cod will be produced in Scotland, and by 2020, analysts predict a world production of 400,000 tonnes p/a, just under half the current wild catch (Solsletten, 2001).

Despite their commercial importance, there is a dearth of literature on the development of cod or any gadoid species. The first annotated figures were produced by Sars (1876), but since this time there have only been a handful of studies of embryonic development (Russel, 1976; Fridgiersson, 1978; Thompson and Riley, 1981), all based on observations of gross morphology with no quantitative or histological information presented. The current commercial interest, and sparsity of developmental data on the species, justifies the production of a comprehensive, up-to-date staging series, to provide a context for future experimental studies and as a reference for the normal development of the species.

Materials and Methods

Six female and six male cod (*Gadus morhua*) broodstock, of approximately six years of age and caught by gillnet from Ardtoe Bay, Scotland, were kept in spawning tank systems of the type described by Huse and Jensen (1983). Three discrete clutches of newly fertilized eggs from different females were collected during March 2000. Clutches taken on consecutive days from the same tank were known to be spawned from different individuals, since the release of eggs is subject to an ovarian cycle lasting 36 hours or more (Kjesbu *et al.*, 1996). A third clutch was taken

from a separate closed system. Within clutches, eggs were almost certainly of multiple paternity due to the spawning behaviour of this species (Hutchings *et al.*, 1999; Bekkevold *et al.*, 2002). Eggs were incubated under constant aeration at 7°C ± 0.2°C (range) in 10 litre containers supplied with 0.1μm filtered, UV sterilized seawater (34‰) with a flow rate of 10mls/minute. Photoperiodicity was 8 hours light (1.4 lux):14 hours total darkness. The live embryos were anaesthetized in 0.1% (m/v) MS-222 before being mounted on glass slides and observed under Nomarski Differential Interference Contrast (DIC) optics at ×400 - ×4000 magnification.

Specimens were processed for histology by fixation in Bouin's fixative overnight before being transferred to 70% ethanol. Embryos were embedded in paraffin and 7μm sagittal and transverse sections cut and stained with haematoxylin and eosin (H&E). Specimens for whole mount acetylcholinesterase (AchE) staining and somite counts were fixed overnight in 4% (m/v) paraformadehyde in phosphate buffered saline (PBS) and stored in 0.1% (m/v) sodium azide in PBS. AchE staining was carried out using a method modified from Karnovsky and Roots (1964) as described in Johnston *et al.* (1995). Embryos were dehydrated through graded ethanol and cleared in 0.1% (m/v) celloidin in methyl-benzoate for five minutes. The most posterior somites containing myotubes, myofibres, and AchE staining were scored.

Photographs were taken on a Ziess Axiocam Color, and illustrations were prepared using Adobe Photoshop software (Adobe Systems Inc., CA, USA)

Results

The results are organized as a staging series. It is appreciated that embryogenesis is a process of gradual change and the moments described are not intended to represent thresholds. For this reason stages have been named rather than

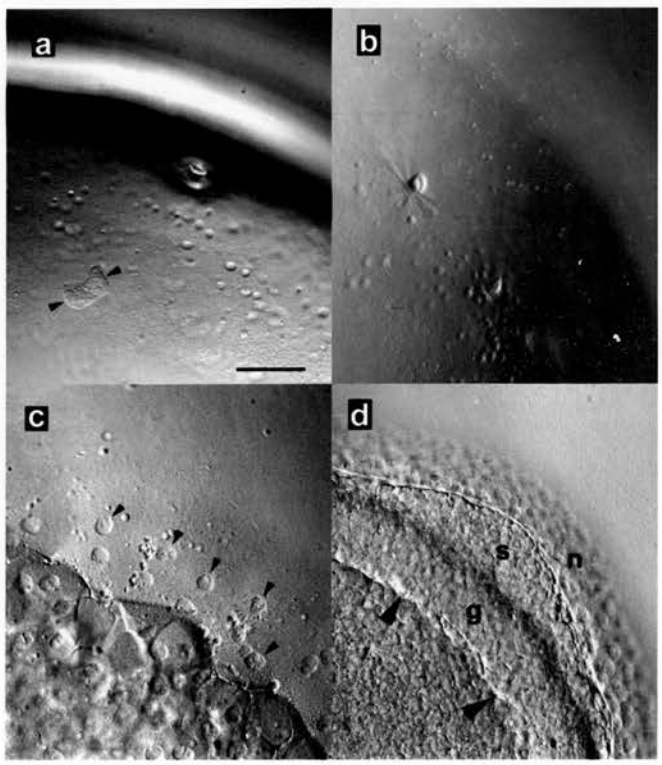


Fig. 1. Early events from fertilization to the onset of epiboly (Nomarski, bar - 50µm).

a. Fusion of egg and sperm pronuclei (arrowheads).
b. Extrusion of the second polar body following fertilization.
c. The yolk syncytial layer (YSL) at the periphery of the blastodisc. Arrowheads show YSL nuclei.
d. Formation of the germ ring (g) and embryonic shield (s) at the onset of involution. Brachet's cleft is indicated by arrowheads, YSL nuclei are still apparent at the periphery (n).

numbered to facilitate future sub-division, and are grouped into larger time blocks called periods. Where possible, nomenclature follows that of the zebrafish *Danio rerio* (Kimmel *et al.*, 1995). Development time is expressed in minutes post-fertilization (mpf) until after the cleavage period, when it is rounded to the nearest hour post-fertilization (hpf). The average egg size in this study was found to be 1.47mm ± 0.04 (mean ± st. dev.), and did not differ significantly between the three clutches (one way ANOVA, $P > 0.05$, n = 50 eggs per clutch). Similarly, the rates of somitogenesis, myotube synthesis and myofibril synthesis were consistent between clutches (ANCOVA, $P > 0.05$, n = 10 individuals per 24h sample).

Fertilization

The egg cell nucleus is located close to the periphery, often beneath the micropyle. Following sperm entry, the sperm nucleus condenses inside the egg and the two nuclei can be seen to fuse (Fig. 1a), forming the zygote embryo. Fertilization initiates the second meiotic division in the egg, and extrusion of the 2nd polar body (Fig. 1b).

Zygote Period (330-405mpf)

When the fertilization reaction is complete, the cytoplasm covering the yolk begins to accumulate at the animal pole collecting at a disc-shaped bulge. The free-floating egg orientates itself in the water column animal pole side-down and the newly formed blastodisc can be viewed through the yolk.

1-cell stage (300 mpf, Figure 2)

The cytoplasm gradually collects in a bulge at the animal pole. 1-cell eggs can be unreliable for staging due to their large time window, and small but significant proportion which are either infertile, or do not successfully complete a first cleavage.

Cleavage Period (335mpf-22hpf)

Cleavages occur very synchronously between embryos, and the cell cycles are easy to follow within clutches. The first cleavage furrow begins to form at 335 mpf, spanning the blastodisc soon after the 1-cell stage. Mitotic spindles can be seen as the disc cleaves to form two distinct cells (Fig. 3). The second cleavage is also horizontal and results in a 2x2 array (Fig. 4). The third cleavage involves two parallel cleavage planes and produces two rows of four daughter cells lying side-by-side (Fig. 5). These rows are again cleaved vertically in parallel planes producing a 4x4 array of 16 cells (Fig. 6). As described for the tilapia (Morrison *et al.*, 2001) the fifth cleavage is uneven, resulting in a partially stratified disc with two cell layers in the centre, and a single layer on the periphery (Fig. 7). The arrangement of cells at the 32-cell stage is variable, and subsequent cleavage events become more and more irregular. Until this stage the cell cycle time between cleavages is 135 minutes. However, the cell cycle is only synchronous enough to follow reliably until the seventh cleavage (producing 128 cells; Fig. 8), after which the embryo enters the blastula period.

2-cell stage (405 mpf, Figure 3)

The first cleavage is vertical and divides the blastodisc into two cells.

4-cell stage (540 mpf, Figure 4)

The second cleavage is also vertical, but occurs perpendicular to the first, resulting in a 2x2 arrangement of cells

Figs. 2-26. (Opposite and overleaf) Stages of embryonic development in the Atlantic cod (bar - 300μm). In Figs. 2-15, **a** shows the dorsal view, whilst **b** shows the lateral view. Figs. 16-24 show lateral views only.

Fig. 2. 1-cell stage.

Fig. 3. 2-cell stage.

Fig. 4. 4-cell stage.

Fig. 5. 8-cell stage.

Fig. 6. 16-cell stage.

Fig. 7. 32-cell stage.

Fig. 8. 64-cell stage.

Fig. 9. 128-cell stage.

Fig. 10. Oblong stage.

Fig. 11. Sphere stage.

Fig. 12. Germ ring stage.

Fig. 13. 10% epiboly.

Fig. 14. 25% epiboly.

Fig. 15. 50% epiboly.

Fig. 16. 75% epiboly.

Fig. 17. 10-somite stage.

Fig. 18. 20-somite stage.

Fig. 19. 30-somite stage.

Fig. 20. 40-somite stage.

Fig. 21. 50-somite stage.

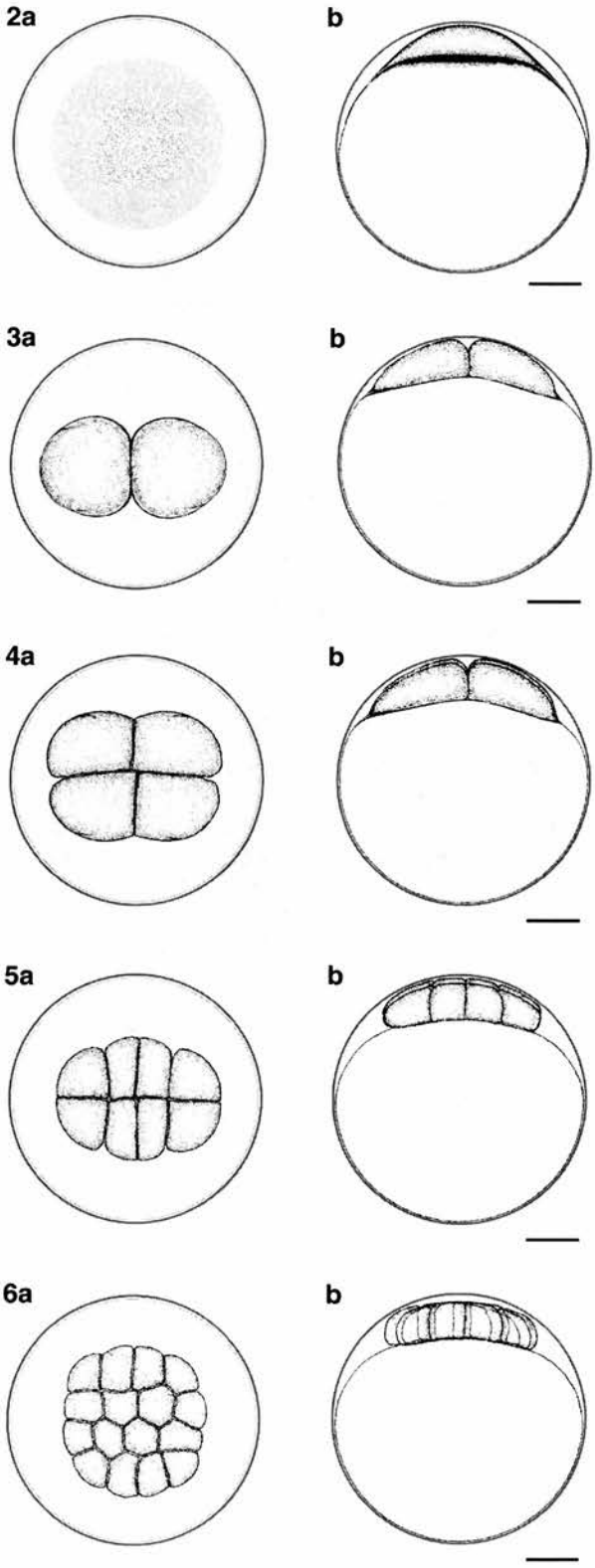
Fig. 22. Goldeneye stage.

Fig. 23. Hatching gland stage.

Fig. 24. Bladder stage.

Fig. 25. Hindgut stage.

Fig. 26. First feeding stage.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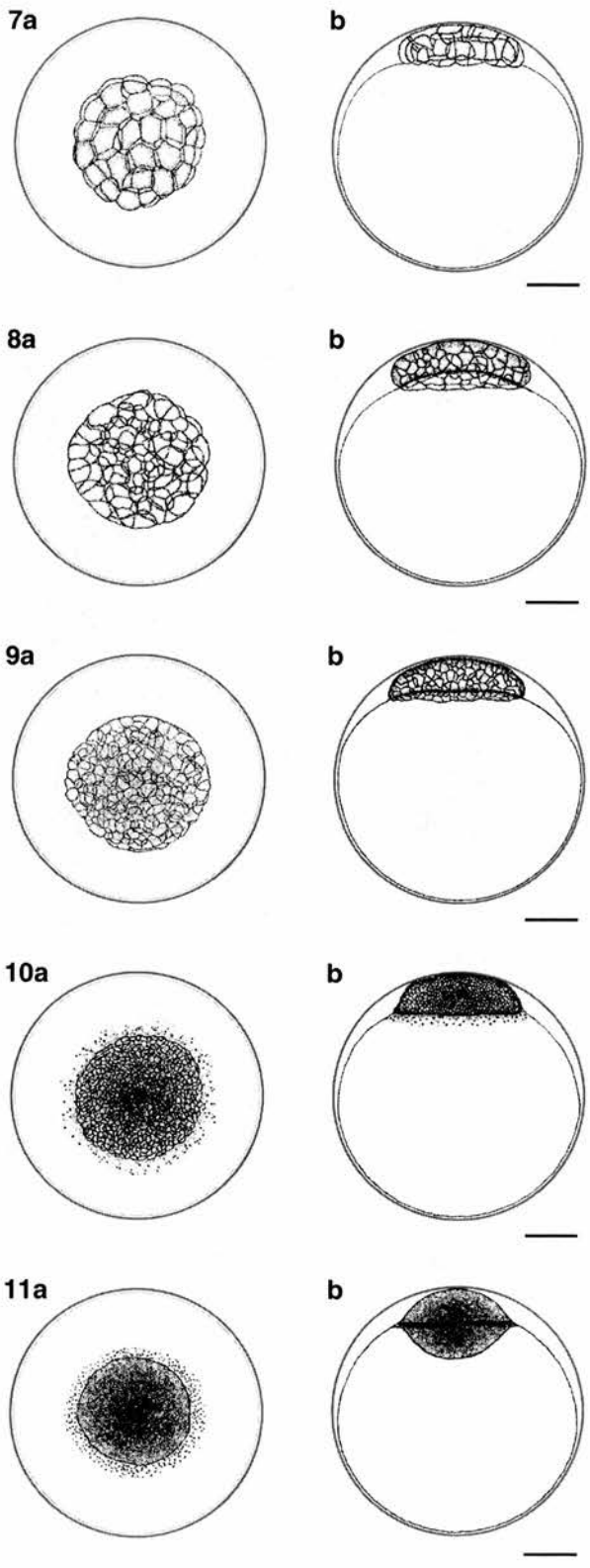
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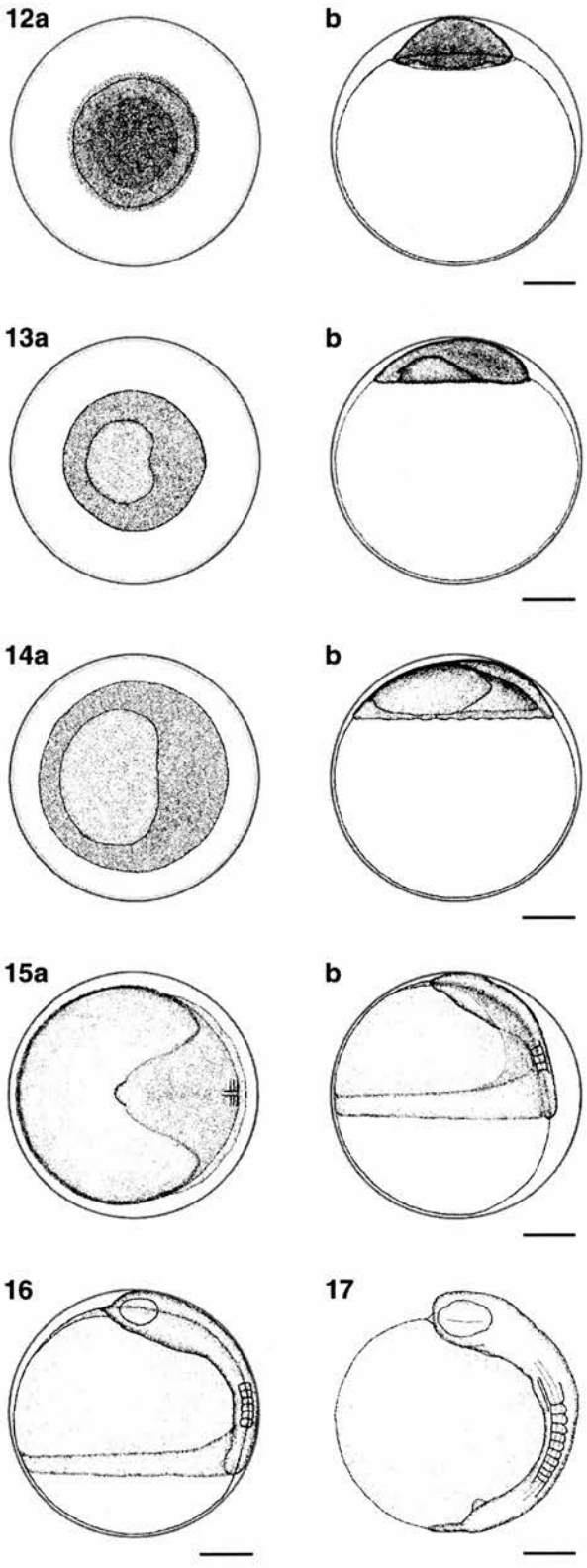
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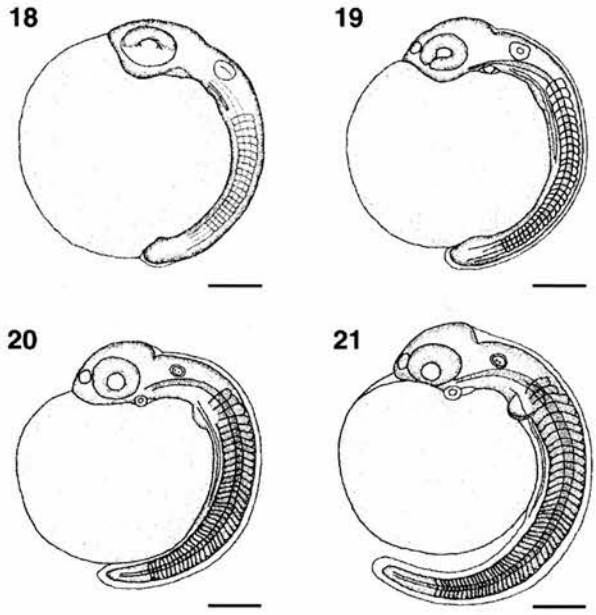
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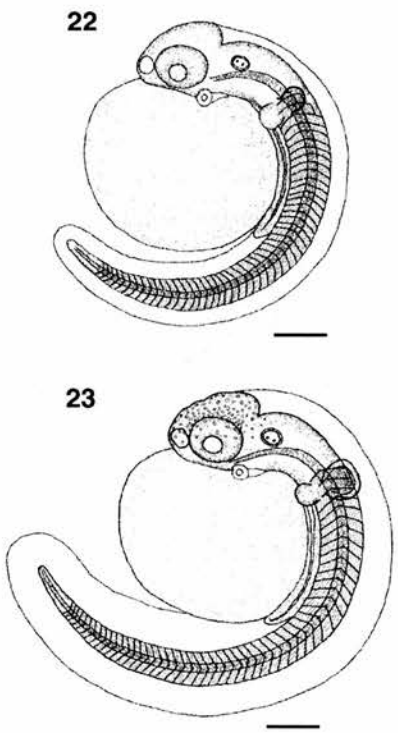
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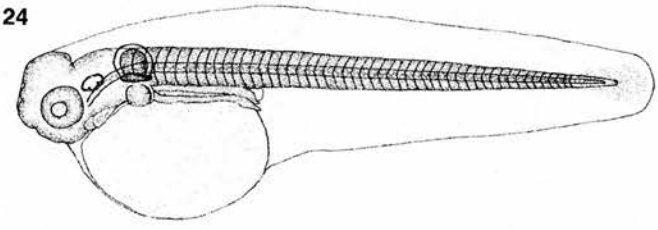

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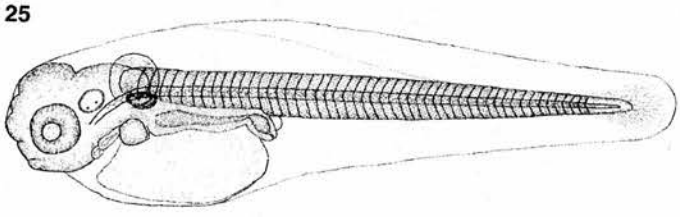

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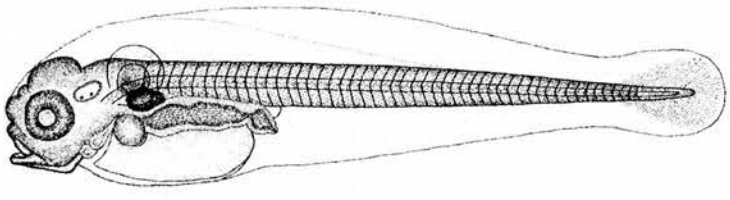


8-cell stage (675 mpf, Figure 5)

The third cleavage is again vertical and proceeds in two planes, parallel to the plane of the first cleavage. The cells of the blastodisc thus display a 2x4 array.

16-cell stage (810 mpf, Figure 6)

The fourth cleavage occurs at right angles to first, again in two vertical planes along the centre of each row of four cells, resulting in a 4x4 array.

32-cell stage (945 mpf, Figure 7)

The fifth cleavage is uneven, resulting in a partially stratified disc of daughter cells, whose arrangement is variable.

64-cell stage (1080 mpf, Figure 8)

The sixth cleavage is the first in a fully horizontal plane, and bisects the blastodisc into two layers, one above the other.

128 cell stage (1215 mpf, Figure 9)

The seventh cleavage is the last that can be discretely followed before cell cycle regularity breaks down. It again occurs in a horizontal plane and results in ball of 128 cells, which divide metasynchronously.

Blastula Period (22-56hpf)

By 36 hpf around nine-ten cleavages have occurred and the blastodisc consists of a solid ball (blastula) of approximately 500 cells (oblong stage; Fig. 10). No synchrony in cell division is observed and it is assumed that the embryo has entered the mid-blastula transition (MBT), where the blastomeres begin to transcribe their own mRNA. The peripheral blastomeres around the rim of the cell ball and adjacent to the yolk, fuse to form the yolk syncytial layer (YSL; Figs. 1c, d). This multinucleate layer is an organ unique to teleosts which no-longer undergoes cytoplasmic cleavage (Kimmel *et al.*, 1995). The nuclei continue to divide mitotically,

and spindles may be seen in the cytoplasm as the YSL spreads beneath the blastodisc, separating the true blastomeres from the yolk. When viewed from below, a ring of protruding YSL can be seen surrounding the blastula (Figs. 10, 11, 12). The cells of the blastodisc continue to divide and begin to exert a pressure on the yolk. Creases can be seen in the yolk membrane during this sphere stage. Gradually however, the pressure subsides and the cell ball sits atop the yolk once more, rather than pressing down into it. A circular furrow is now visible on the underside of the disc, demarking the germ ring (germ ring stage; Fig. 12).

Oblong stage (36 hpf, Figure 10)

The blastodisc sits on top of the yolk amid a ring of peripheral YSL nuclei. As the blastodisc expands and the cell ball presses against the chorion, the vegetal surface is slowly pushed into the yolk.

Sphere stage (49 hpf, Figure 11)

The blastodisc has become ovoid and pushes down strongly into the yolk giving creases in the membrane at the peripheries. A narrower ring of smaller, more prolific YSL nuclei surrounds the blastodisc.

Gastrula Period (56-113hpf)

The appearance of the germ ring marks the beginning of involution and the onset of gastrulation. Cells at the periphery of the disc involute, forming a second layer, the hypoblast, between the YSL and the remainder of the disc, the epiblast (Figs. 1d, 12, 13). Brachet's cleft, the fissure separating the cells of the hypoblast from those of the epiblast, is visible from the side. The cell ball spreads laterally, and the cells of the blastodisc, lead by the peripheral YSL begin a migration over and around the yolk towards the vegetal pole. The embryo now attains dorso-ventral and

anterio-posterior axes. The thickened portion of blastoderm adjacent to the developing cavity becomes the embryonic shield, which will develop into the trunk of the body (Figs. 14, 15, 16). Stages are defined by percentage epiboly (coverage of the yolk as viewed two-dimensionally from the side). In theory this allows description of an infinite number of stages, although it is convenient to describe only those which accompany other important developmental events. Involution continues until the closure of the blastopore at 100% epiboly (Fig. 17). Following completion of epiboly, cells in the epiblast give rise to the ectoderm, which eventually becomes the epidermis and neural tissue. The hypoblast gives rise to the mesoderm, which forms such tissues as muscle and bone, and the endoderm, which forms such tissues as the liver and gut. During gastrulation, the free floating embryo orientates shield side-up until closure of the blastopore, when the weight of the embryonic body pulls it beneath the yolk. Following completion of epiboly, a transient structure known as Kupffer's vesicle can often be seen within the cells of the tail bud (Fig. 17, 27a). Its function is not well understood (Kanki and Ho, 1997) and its size is extremely variable between embryos of the same clutch. Occasionally it is only visible in sectioned material. Following epiboly the vesicle shrinks and eventually disappears.

Germ ring stage (56 hpf, Figure 12)

Pressure on the yolk has subsided and the blastodisc again sits atop the yolk surround by a narrow ring of YSL. Cell size has decreased dramatically from the sphere stage, and a circular infolding on the vegetal side of the disc demarks the germ ring.

10% epiboly stage (73 hpf, Figure 13)

An asymmetric yolk filled cavity is present on the underside of the blastodisc. The cell mass is thicker on one side, indicting the position of embryonic shield

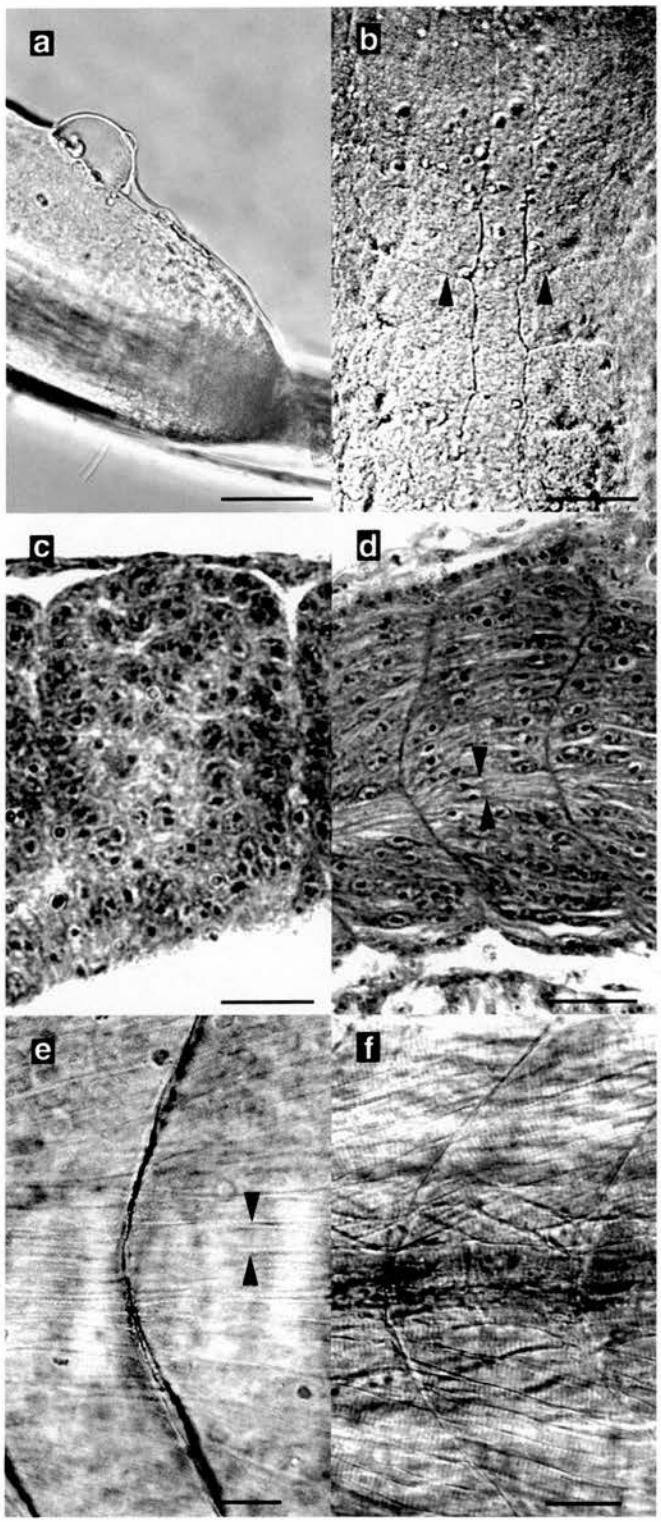


Fig. 27. Kupffer's vesicle (a) and somitogenesis (b-f).

a. Kupffer's vesicle at the 10-somite stage (Nomarski, bar - 50μm).
b. Division of the presomitic mesoderm into somites. Arrowheads show the first somite furrow (Nomarski, bar - 50μm).
c. The first somite, shortly after its formation. Note the irregular array of indifferentiated myoblasts (sagittal section, H&E, bar - 100μm,).
d. The first mononucleate myotubes (arrowheads) at the horizontal myoseptum at the 20-somite stage (sagittal section, H&E, bar - 100μm).
e. Myotubes (arrowheads) and acetylcholinesterase staining at the myosepta (Nomarski, bar – 100μm).
f. Striated myotubes showing the characteristic A-I banding pattern (Nomarski, bar - 100μm).

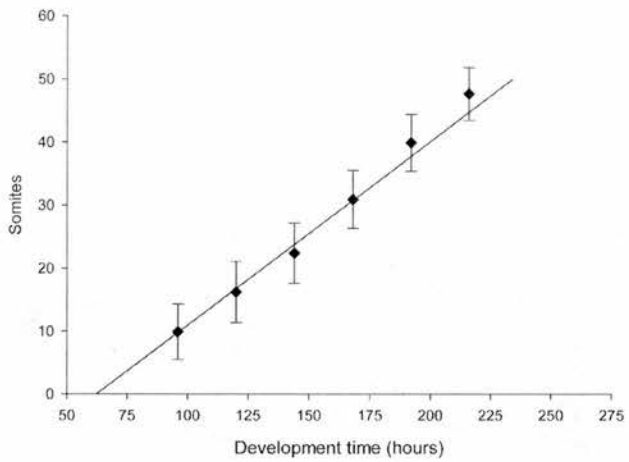


Fig. 28. Development of somites (s) over time (t). The regression equation is s = 0.29t – 18.14, r^2 (adjusted) 0.89, residual df 208. Error bars show standard deviations from the mean.

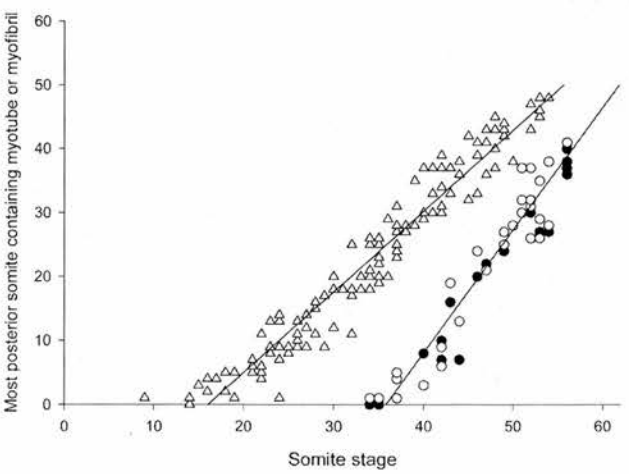


Fig. 29. Development of myotubes (triangles), myofibrils (filled circles), and acetylcholinesterase staining (open circles). The regression equation for myotube (mt) development in relation to somite stage (s) is mt = 1.26s – 20.27, r^2 (adjusted) 0.94, residual df 123. There was no significant difference between development of myofibrils (mf) and acetylcholinesterase staining (ANCOVA $P > 0.05$), such that the common regression equation for both is given by mf/AchE = 1.92s – 68.66, r^2 (adjusted) 0.93, residual df 178.

10-somite/100% epiboly stage (113 hpf, Figure 17)

A thin invagination of the optic primordia can be seen in lateral view. The heart field is visible posterior to the diencephalon. Kupffer's vesicle may be present ventral to the developing tail bud.

20-somite stage (142 hpf, Figure 18)

The solid otic placode is midway between the eye and the first somite. A small invagination may be visible in the centre under DIC illumination. The heart has taken on the appearance of a ring. The early gut possesses a lumen, and the neural retina and lens are distinct. The rudiment of the embryonic fin fold can be seen at the tail bud.

30-somite stage (167 hpf, Figure 19)

The otic vesicle has a lumen, although no otoliths are yet present. The heart now takes on the appearance of a posteriorly skewed tube and the cells of the neural retina have become squamous. There are nasal placodes in front of the eye and the notochord possesses small vacuoles at the anterior end. A bulge in the anterior intestine indicates the future position of the liver.

40-somite stage (191 hpf, Figure 20)

Two otoliths are visible inside each otic vesicle. The heart tube beats sporadically and is beginning to loop to the right. The notochord is vacuolated to approximately halfway down the body, and the liver has become bi-lobular.

50-somite stage (217 hpf, Figure 21)

The heart possesses an atrium and ventricle and beats sporadically. The notochord is vacuolated the length of the body, and sporadic muscle contractions occur.

Golden eye stage (225 hpf, Figure 22)

The eye has undergone pigmentation and has a golden, reflective appearance.

Somite formation is complete and a mesodermal pectoral fin rudiment is present laterally and distally to the liver. The heart beat has become regular and has frequency of approximately 126 beats/minute.

Hatching gland stage (250 hpf, Figure 23)

The embryo is curled tightly inside the chorion with the tail adjacent to the head. Hatching glands cover the surface of the head and the chorion is soft to the touch. The pectoral fin rudiment is bordered by an apical fin fold.

Organogenesis During Segmentation

Somites and Notochord

The first structure to become visible with the light microscope within the shield of the whole mount embryo is the notochord. The parallel boundaries in the hypoblast between the axial chorda mesoderm (which will form the notochord) and the paraxial segmental plate mesoderm (which will form the somites) develop as longitudinal furrows. As segmentation continues, the central cells of the notochord become vacuolated. Vacuolization is associated with the development of a surrounding thickened sheath of tissue which imparts rigidity (Adams *et al.*, 1990). Somites are formed anterior-posteriorly along the body axis from presomitic mesoderm adjacent to the notochord. The first somite furrow appears at 82 hours, only a matter of minutes after the appearance of the notochord rudiment at 45% epiboly. The anterior somite boundary quickly becomes visible delineating the first pair, shortly before the second, posterior somite furrow (Fig. 27b). Further somites are formed linearly with respect to time, at intervals of 162 minutes. The final number of somites varies between 48 and 56 with a mean of 52 (range: ± 4; n = 16 individuals per clutch). All somites are formed by 220 hpf, approximately 36 hours prior to

hatching.

The ventral portion of the somite comprises the sclerotome, which will form the axial skeleton and cartilage, whereas the dorsal portion comprises the myotome, giving rise to the trunk muscle. Myotubes are formed from adaxial "muscle pioneer" cells, present on the medial surface of the somite, which elongate to span the somite longitudinally (Figs. 27c, d). This first wave of differentiation is followed by a second, more extensive wave, where myoblasts line up within the somite, before fusing to become multinucleate, syncytial myotubes. The appearance of myotubes begins at the 16-somite stage, and progresses posteriorly at a slightly faster rate than that of somite development, at intervals of 119 minutes per somite (Fig. 29). As the first myotubes are produced, the somites begin to acquire the characteristic chevron shape, indicating the central position of the horizontal myoseptum and future division into the dorsal (epaxial) and ventral (hypaxial) muscle masses. Later, at the 35-somite stage, the myofibrils develop in the anterior-most somites, distinguishable by the characteristic A-I banding pattern (Huxley, 1969) of the contractile apparatus (Fig. 27e). Innervation occurs at the same time as organized myofibrils are seen, as shown by the appearance of AchE staining at the myosepta (Fig. 27f).

Neurulation

The neural plate is apparent by 50% epiboly as a thickening of tissue along the medial axis of the embryonic shield. Soon after the beginning of somite formation the anterior neural plate swells and distends into the primordium of the brain. As epiboly proceeds, neural plate cells extend ventrally forming a deepened keel (Figs. 30a-d) above the mesodermal cells of the presumptive notochord. The keel is quickly overridden by adjacent epidermal cells, and forms a solid neural rod. A central lumen,

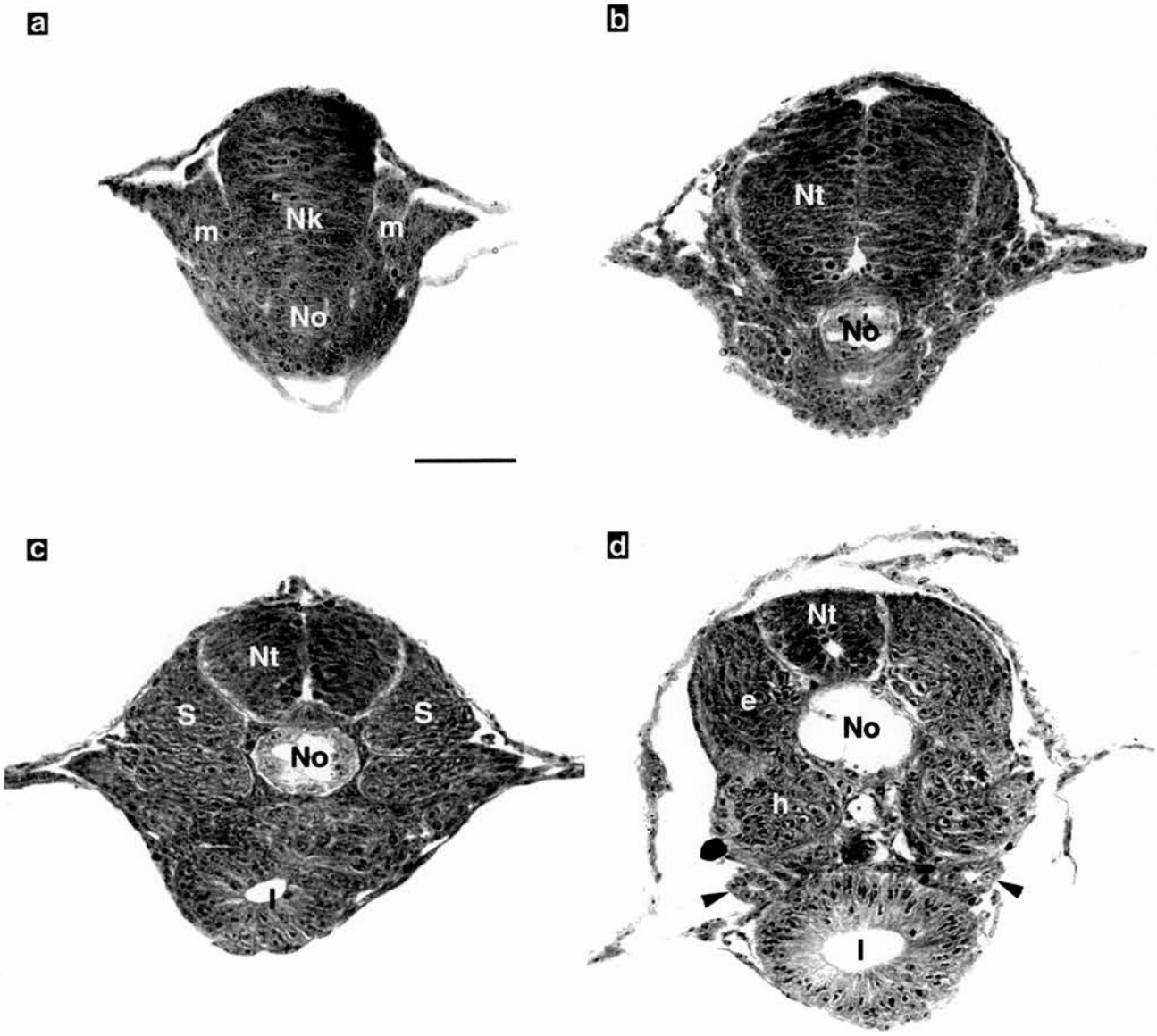


Fig. 30. Development of axial structures. Transverse sections taken immediately posterior to the position of the liver (H&E, bar - 100μm).

a. 75% epiboly. Nk - neural keel, m – mesoderm, No – notochord.
b. 10-somite stage. Nt – Neural tube, Fp – Floor plate, No – Notochord.
c. 30-somite stage. Nt – Neural tube, S – somites, No – Notochord, I – intestine.
d. Goldeneye stage. Nt – Neural tube, No – Notochord, e – epaxial muscle mass, h – hypaxial muscle mass, I – intestine, arrowheads – pronephric ducts.

the neurocoel, originates by cavitation rather than the infolding reported in avian and mammalian systems (Papan and Campos-Ortega, 1994). By the 10-somite stage the floorplate may be discerned in transverse (TS) section above the vacuolating notochord.

Brain

As the anterior neural tube distends, the first three neuromeres are distinguishable as swellings in the head, adjacent to the optic primordia at 70% epiboly (5-somite stage). They remain relatively indistinct however, until the end of epiboly (10-somite stage). These most rostral neuromeres form the midbrain (mesencephalon) and the two subdivisions of the forebrain, the diencephalon and the telencephalon. After completion of epiboly the neuromeres distend further, and by the 20-somite stage, strongly affect the shape of the head (Fig. 31a). Olfactory primordia are now present on either side of the telencephalon. At 25 somites, a further seven neuromeres, the rhombomeres, are barely visible as constrictions, dividing the neural rod into the segments of the hindbrain. An epiphysis can be seen in the roof of the diencephalon (Fig. 31b), and the midbrain/hindbrain boundary is convoluted with a pronounced cleft. By the 30-somite stage the patterning of brain areas is virtually complete (Figs. 31c, 32a). The diencephalon, mesencephalon and telencephalon are clearly delineated although they no longer have such a pronounced effect on the shape of the head, and the cerebellum displays a contorted appearance. The differentiation of neurons and glia is apparent, most obviously in the olfactory primordia where receptor and their supporting cells can be seen with their axons running towards the brain (Fig. 33f).

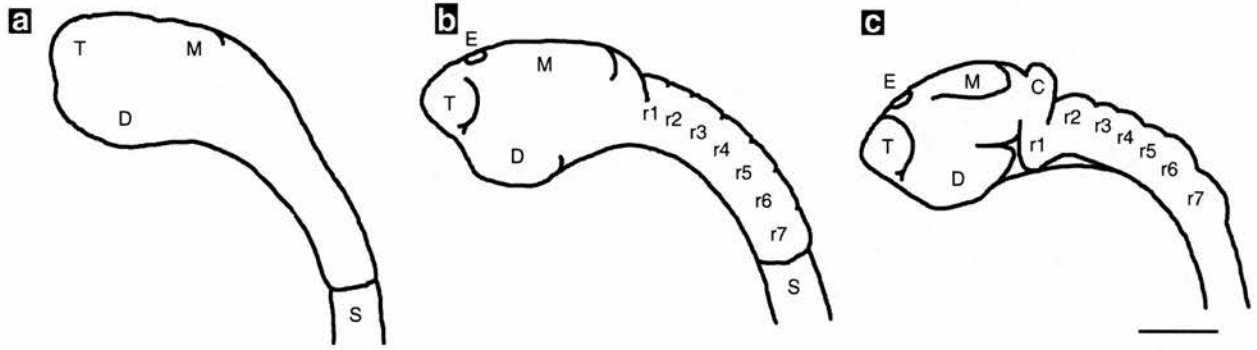


Fig. 31. Development of the brain (bar - 200μm). T – telencephalon, M – mesencephalon, D – diencephalon, C – cerebellum, E – epiphysis, r – rhombomeres, S – somites.

a. 20-somite stage.
b. 25-somite stage.
c. 30-somite stage.

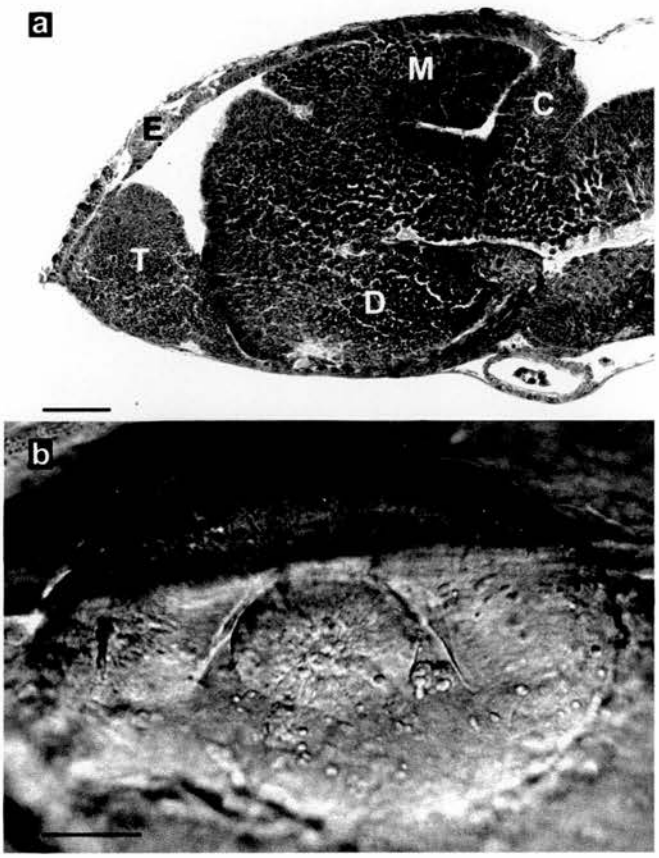


Fig. 32a. Patterning of brain areas at the 30-somite stage (sagittal section, H&E, bar – 50μm). T – telencephalon, M – mesencephalon, D – diencephalon, C – cerebellum, E – epiphysis.

Fig. 32b. The eye at the 20-somite stage. Lateral view (Nomarski, bar - 50μm).

Eyes

The optic placodes start to differentiate as solid masses of cells and appear as two elliptical bulges on either side of the developing neural plate between 50% and 75% epiboly (Figs. 15, 16). By 75% epiboly the placodes have fully differentiated from the shield as solid masses of large, loosely packed cells and are attached to the tube only by the anterior optic stalks. At completion of epiboly (10-somite stage; Fig. 17) a faint infolding becomes visible rostro-caudally in the centre of the eye. Transverse sections reveal the formation of an optic lumen, and a thickening of the overlying ectodermal tissue, the lens placode (Fig. 33a). Invagination of the lateral surface extends until the vesicle is cup shaped at the 17-somite stage. The process continues in a dorsal-ventral wave over the course of the next four hours, whilst a further ten somites are formed (Fig. 32b). As the lens continues to differentiate the cells of the optic cup lose their regular arrangement and columnar form, and take on an irregular squamous appearance, as they form the neural retina (Fig. 33b). Pigment epithelial cells, although for the moment lacking in pigment granules can be seen at the periphery. By the 40-somite stage the first indications of retinal lamination are discernible. Adjacent to the lens is the ganglion cell layer surrounded by the inner plexiform layer (Fig. 33c). Two nuclear layers make up the outer retina, separated by another thin plexiform layer. Deposition of pigment granules in the epithelium does not occur until after the completion of somitogenesis (Fig. 33d). When it does so however, the eye takes on a bright, reflective golden appearance, which is particularly striking under strong illumination. The onset of pigmentation appears to be highly synchronous and is a useful staging tool (golden eye stage, Fig. 22). Retinal lamination and pigment deposition continue rapidly until the eye becomes functional (Fig. 33e), between the bladder and hind-gut stages.

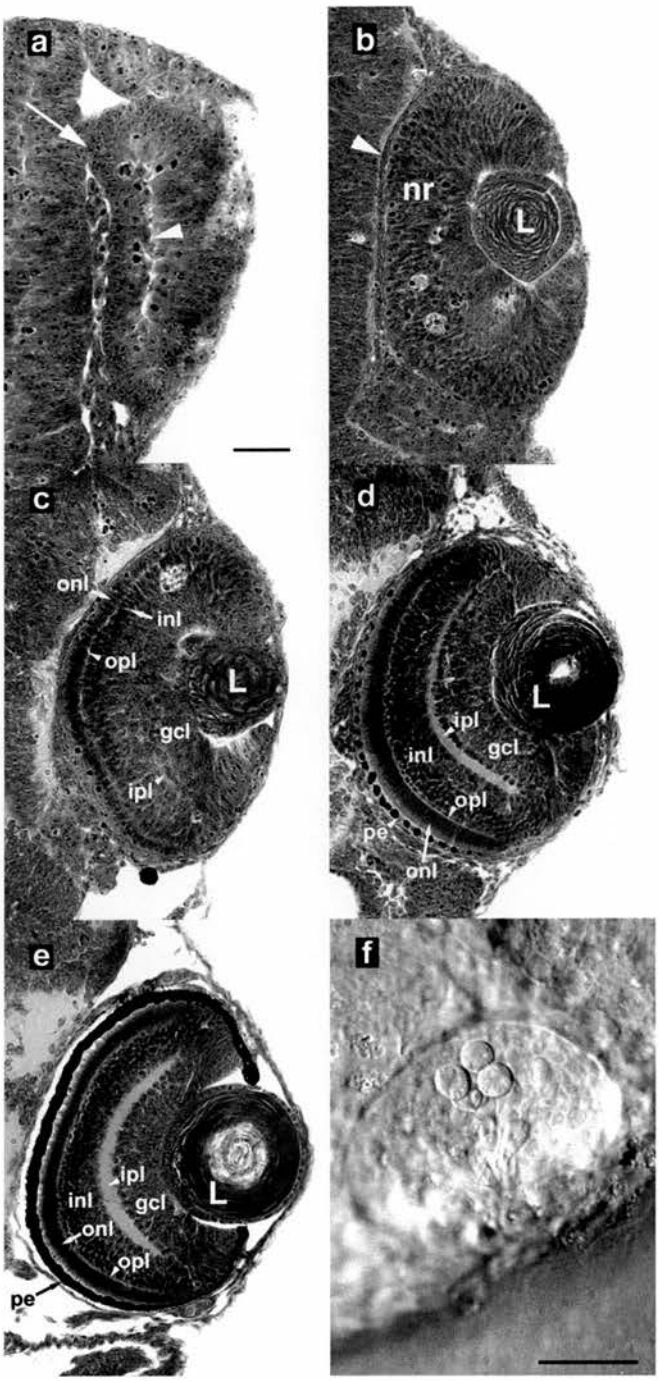


Fig. 33. Development of the eye (a-e; sagittal sections, H&E, bar – 50μm) and olfactory placode (f; Nomarski, bar - 50μm).

a. 10-somite stage. Arrow shows the optic stalk, connecting the optic vesicle to the neural tube. Arrowhead shows the developing optic lumen.
b. 25-somite stage. L – lens placode, nr – neural retina, arrowhead – pigment epithelial cells.
c. 40-somite stage. L – lens, gcl – ganglion cell layer, ipl – inner plexiform layer, inl- inner nuclear layer, opl – outer plexiform layer, onl – outer nuclear layer.
d. Goldeneye stage.
e. hindgut stage. Fig. 33f – Neurons in the olfactory vesicle at the 30 – somite stage.

Ears

Like the optic primordia, the otic placodes originate as ectodermal outfoldings of the neural plate. They are just visible as solid masses of ectoderm either side of the hindbrain at the 17-somite stage, lateral to rhombomeres 3-5, positioned approximately halfway between the eye and the first somite (Fig. 34a). The first indication of the development of the lumen comes at the 20-somite stage, when a tiny invagination can be seen in the centre of each placode. This soon enlarges and becomes more distinct, as the placode cavitates to become a fluid filled epithelial ovoid; the otic sac or vesicle (Fig. 19). Two tiny otoliths can be seen at opposite ends of each vesicle as calcium carbonate is deposited inside the lumen (Fig. 34b). The otic lumen expands rapidly, and the otoliths continue to enlarge (Fig. 34c). Transverse sections reveal a thickened macula containing hair cells in the ventral lining of the vesicle by the golden eye stage (not shown), although further development of the inner ear occurs during the larval period.

Gut and Liver

The intestinal lumen can be seen as early as the 10-somite stage (Fig. 17). Endodermal cells migrate and differentiate, and the blind-ending tube extends posteriorly, until by the 30-somite stage (Fig. 19, 34d) transverse striations of smooth muscle are apparent in the anterior gut wall. By the 50-somite stage (Fig. 21) the internal epithelium of the gut is highly villiarized, but a cloaca does not develop until the early larval period (Fig. 24). Later still (first-feeding stage, Fig. 26), the epithelium of the mid-gut is thrown into deep folds, greatly increasing the surface area for absorption. The liver originates as an endodermal bud from the anterior of the mid-gut. From the 20-somite stage a thickening in the ventral wall marks its future

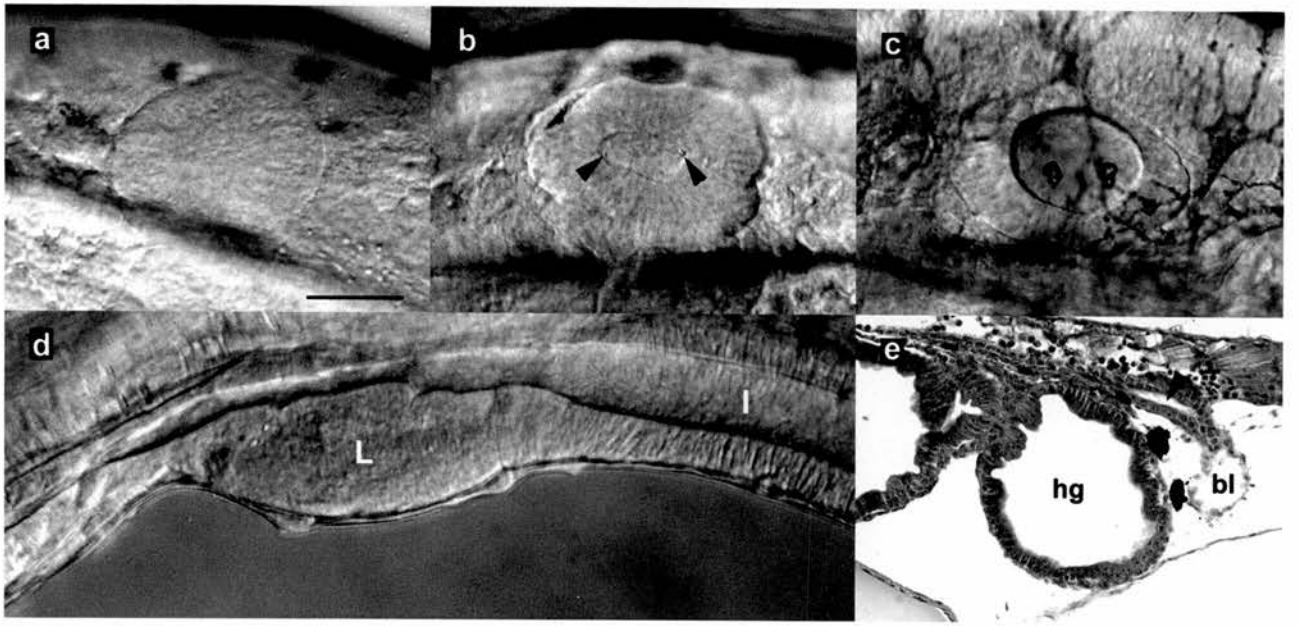


Fig. 34. Development of the otic vesicle (a-c), liver (d; Nomarski, bar - 50μm) and bladder (e; sagittal section, H&E, bar - 50μm)

a. The otic placode, immediately after formation at the 17-somite stage.
b. The fluid filled otic vesicle at the 25-somite stage. Two tiny otoliths are just visible (arrowheads).
c. Otoliths at the goldeneye stage
d. The intestine (I) and presumptive liver (L) at the 30-somite stage. Transverse striations of smooth muscle can be seen in the intestinal wall.
e. The intestine and nephros at the hindgut (hg) stage. Pronephric ducts (arrowhead) end in a tubal, urinary bladder (bl).

position (Fig. 34d). The gall bladder, situated between the liver and pancreas is visible in histological section from the goldeneye stage (Fig. 35).

Kidney

The first evidence of the development of a kidney is in the pronephric ducts, which run dorsal to the mid-gut on either side of the body. Although very difficult to see in living specimens, their primordia are apparent (as yet without lumina) in transverse section at the 30-somite stage (Fig. 30c, d). The glomerulus is visible in longitudinal section, and in post-hatch fish the pronephric ducts adjoin the a urinary bladder (Fig. 24, 34e).

Heart

The heart field, although not yet a true pericardial cavity, is visible by the 20-somite stage as a seemingly empty space posterior to the developing diencephalon (20-somite stage; Fig. 18). By the 25-somite stage, two mesodermal tubular primordia are seen on either side of the ventral midline, which have fused by the 30-somite stage into a cone. The base of the heart cone rests on the yolk, where it is visible as a ring (Figs. 19, 36a, b). Over the course of the next 24 hours, and the addition of a further 10 somites, the cone skews posteriorly (Fig. 36c). The venous end that was sitting on the yolk faces anteriorly, and the apex of the cone becomes continuous with the dorsal aorta. The now bell-shaped heart contains two epithelial layers, an outer myocardial layer which will form the heart muscles, and a very thin, endocardial layer forming the inner lining. Sporadic heart beats occur after the 40-somite stage, with an estimated average frequency of $30s^{-1}$. Between 45 and 50-somites, the heart begins to loop to the embryos' right (Fig. 36d), and the beats become more frequent and

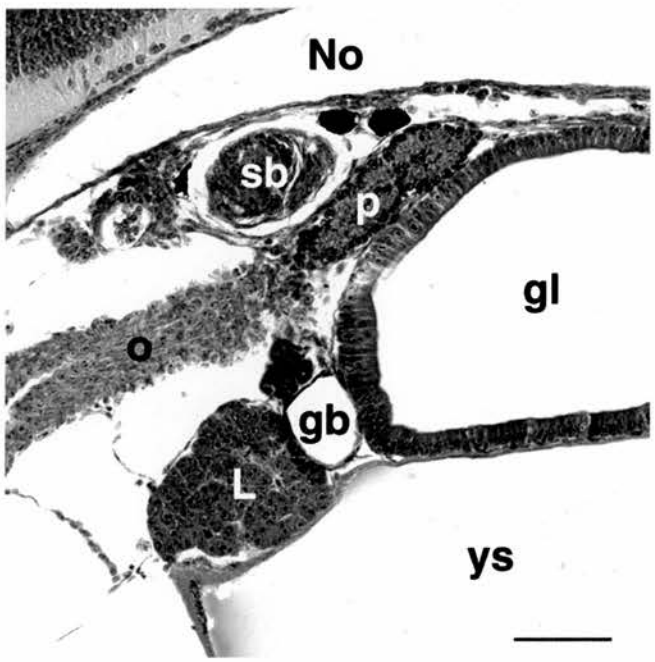


Fig. 35. Sagittal section showing the region immediately posterior to the head at the first feeding stage (H&E, bar - 25μm). No – notochord, sb – swimbladder, p – pancreas, o – oesophagus, gl – gut lumen, gb – gall bladder, L – liver, ys – yolk sac.

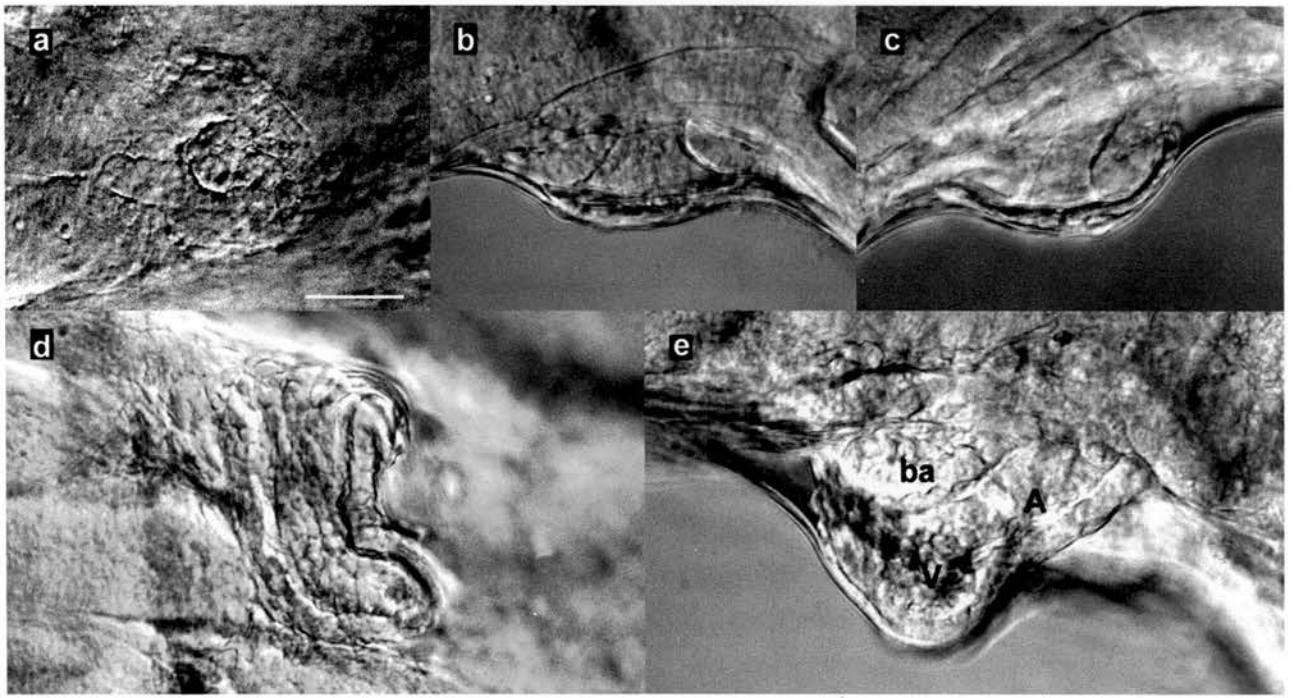


Fig. 36. Development of the heart (Nomarski, bar - 50μm).

a. 30-somite stage, the heart primordium is visible as ring when viewed ventrally.
b. 30-somite stage, heart primordium, (lateral view).
c. 35-somite stage, the heart is visible as a posteriorly-skewed cone, (lateral view).
d. 45-somite stage, the heart begins to loop to the embryo's right (ventral view).
e. 50-somite stage. Demarcations of the heart chambers are visible (lateral view). ba – bulbous arteriosus, V – ventricle, A – atrium.

gradually more regular. The demarcations of the atrium (on the left) and the ventricle (on the right) become apparent as development proceeds in an arterial to venous direction. After the 50-somite stage the two minor chambers, the sinous venosus and the bulbous arteriosus delineate at the extremities. Beyond this stage (Fig. 36e), it becomes difficult to view the heart in its entirety from any particular angle, since the chambers are in different focal planes. The heart now acquires a "double" beat, wherein to prevent backflow the atrium does not relax until the ventricle has contracted. The frequency of beating is approximately 126 bpm, and transparent haemolymph can be seen flowing through the chambers.

Fins

The first protrusion of the unpaired embryonic fin fold is just visible at the 17-somite stage (Fig. 37a). By the 25-somite stage it has expanded to surround the entire body, with the exception of the head. However, it remains obscure in the live embryo, due to the twisting of the embryonic tail so that the transparent fin fold lies against the chorion. Unjointed collagenous fibres of the early fin rays or "actinotrichia" are visible in the caudal portion of the fin fold upon hatching (Figs. 24, 37b). Development of the pectoral fins is comparatively late. The first visible sign of these structures comes at the 50-somite stage when mesenchymal fin buds protrude distally from the ventro-lateral region adjacent to the liver (Fig. 37c). Their expansion is rapid and by the hatching gland stage an apical fin fold surrounds the periphery of the proximal mesenchyme (Fig. 37d). These early appendages are characterized by their vertical orientation with reference to the anterior-posterior body axis. By first-feeding however, they have undergone rotation to a near horizontal position (Fig. 26).

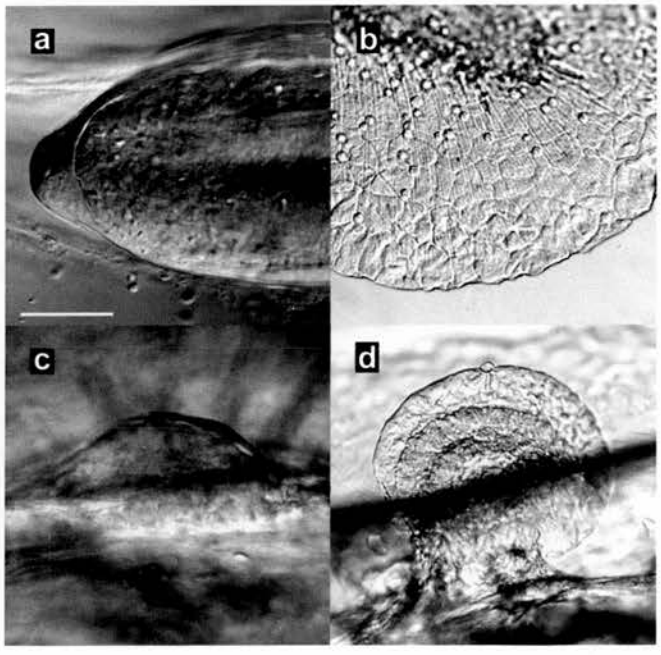


Fig. 37. Development of the paired and unpaired fins (Nomarski, bar - 50µm).

a. The first protrusion of the unpaired medial fin fold from the tail bud (17-somite stage).
b. Unjointed collagenous actinotrichia present in the caudal portion of the fin fold immediately after hatching.
c. First protrusion of the mesenchymal fin bud which will become the pectoral fin at the 50-somite stage.
d. Early pectoral fin at the hatching gland stage.

Hatching and Early Larval Period (>256hpf)

After completion of the last somites, and pigmentation of the eye, the embryo is tightly curled inside the chorion. Most of the body organs are developed, with the notable exceptions of the jaw and the ear, but the yolk sac is still relatively large, indicating no immediate need for external nutrition. The chorion is noticeably softer to the touch after being weakened by a hatching enzyme secreted from the hatching glands. The frequency of sporadic muscular contractions is increased until the embryo can completely re-orientate itself inside the chorion. It is possible that such movements improve the perivitelline circulation and gas exchange (Peterson *et al.*, 1983), and it is certain that they finally cause the chorion to tear. The event of hatching occurs fairly synchronously, at around 256 hpf, although it is not generally considered a reliable marker of development, as embryos within the same clutch hatch within too broad a developmental window.

The newly hatched, free-floating larva remains upside-down and motionless at the waters' surface, initiating sharp movement only in response to external stimuli. As the size of the yolk sac diminishes, the embryos re-orientate to the adult position, and undergo negative phototaxis away from the water's surface. Seven pairs of free neuromasts are barely visible on the epidermis, arranged symmetrically on either side of the body. Two pairs are usually located on the head, one on the upper jaw and the other above the eye. The remaining five pairs are distributed along the primordium of the lateral line. The cupulae extend to approximately 40μm by the first feeding stage, when five-six more neuromast pairs can be counted, mainly surrounding the jaw, although there is variation between larvae in both number and position.

Bladder stage (262 hpf, Figure 24)

The hatched embryo floats upside-down at the surface of the water. A urinary

bladder is present at the posterior of the gut adjoining the pronephric tubules. The swim bladder is seen for the first time in live embryos dorsal to the liver. Actinotrichia are present in the caudal embryonic fin fold.

Hind-gut stage (304 hpf, Figure 25)

It is possible to recognise three areas of the gut, the fore-gut, mid-gut and hind-gut. The hind-gut is separated from the mid-gut by a muscular constriction. The lower jaw has extended, although the mouth still does not open.

First-feeding stage (346 hpf, Figure 26)

The mouth is able to open and is used to gulp prey items. The mid-gut is convoluted and thrown into folds. Sporadic swimming movements occur without external stimuli.

Discussion

All teleosts show a discoidal meroblastic cleavage pattern, where the large yolk volume restricts cell division to a small area at the animal pole. The first divisions are vertical, and there is no cytoplasmic growth between early divisions, resulting in a decrease in blastomere size with each successive division. In most species the blastomeres are regular in size and shape, and blastomere morphology has been used as a predictive indicator of egg viability within a clutch (Shields *et al.*, 1997). However, inter and intra-specific variation exists on the general pattern. In cod, horizontal stratification of the cell mass usually occurs at the sixth cleavage, between the 32 and 64 cell stage, but is frequently observed at the previous or following cleavage. In the medaka *Oryzias latipes*, the first horizontal cleavage is the fifth, occurring between the 16 and 32 cell stages (Iwamatsu, 1994), and in the ice goby (*Leucopsarion petersii*) it occurs even earlier, between the four and eight cell stages

(Nakatsuji *et al.*, 1997). Unusually within the teleosts, eggs of the wolfish *Anarhichas lupus* have unequal blastomere sizes during early divisions (Pavlov *et al.*, 1992).

Timing of the cell cycle during the first cleavage events is highly synchronous, with each cycle having approximately the same length (Marrable, 1965). The cell cycle time in the cod at 7°C was 135 minutes. Fridgeirsson (1978), presents comparable data on five species of gadoid including cod, but indicates a positive correlation between egg diameter and cell cycle time. Similarly, Ignatieva (1976) compared cleavage at optimal temperatures for seven different teleosts, and found that the duration of the cleavage period was closely correlated with yolk volume. The mid-blastula transition (MBT) in cod starts soon after the seventh cleavage (128 cell stage). Beyond the seventh cleavage embryos begin to show metasynchrony, and within a further two or three, differential cell cycle lengthening has ensured that individual cleavage events can no longer be distinguished. The timing of the MBT in cod mirrors that seen in the zebrafish, which also shows metasynchronous cleavages beyond cycle seven, and in which the MBT is defined as beginning at cycle 10. Kane and Kimmel (1993), made a detailed analysis of the zebrafish MBT. Time lapse imaging and radiolabelled uracil injections revealed that it was characterized not only by a lengthening of the cell cycle and asynchronous division, but by the activation of transcription and the onset of cell motility. Interestingly, the onset of cell cycle lengthening was found to be correlated with nucleocytoplasmic ratio, rather than cleavage cycle number.

In the cod, the beginning of gastrulation, as defined by the onset of involution, starts early with respect to some other teleosts, and coincides with the thinning of the blastodisc at the start of epiboly. The site of the embryonic shield is visible immediately as a peripheral thickening on one side. The common carp, *Cyprinus*

carpio and the medaka follow a similar pattern (Verma, 1971; Iwamatsu, 1994). In the mummichog *Fundulus heteroclitus* however, involution begins at approximately 25% epiboly, and in the zebrafish it does not begin until 50% epiboly (Warga and Kimmel, 1990; Kimmel *et al.*, 1995). This variation appears not to be correlated with future developmental processes, in particular somite development, which provides the most useful method of staging during the segmentation period. Unusually, the first somites of the cod are formed well before closure of the blastopore at 45% epiboly, but in the carp, mummichog, medaka and zebrafish somitogenesis is not initiated until completion of epiboly (Armstrong and Child, 1965; Verma, 1971; Iwamatsu, 1994; Kimmel *et al.*, 1995).

The process of somite development in fish is similar to that in birds, amphibians and mammals (Stickney *et al.*, 2000). The linear development pattern and reiterative nature of somite formation has lead to models of a molecular clock, which would allow translation of a smooth positional gradient into uniform blocks of cells (Schnell and Maini, 2000). Somites give rise to the axial skeleton via the ventrally situated sclerotome, and the axial muscle via the differentiation of the dorsal myotome. Fish, in possessing a swim bladder and being neutrally buoyant within an aqueous medium, are less reliant on a robust skeleton than terrestrial vertebrates, and consequently the sclerotome occupies a much smaller portion of the somite than in other vertebrates (Morin-Kensicki and Eisen, 1997). It is likely that the fish somite also contains dermatome, which in the chick is the precursor to the dermis of the skin (Brand-Saberi and Christ, 2000). As yet, a fish dermatome has not been characterized (Stickney *et al.*, 2000) although it has been observed that some zebrafish somite cells contribute to blood vessel formation (Morin-Kensicki and Eisen, 1997).

There are obvious inter-specific differences in final somite number within teleosts, cod having 50 somites, herring 62 (Hill and Johnston, 1997b), salmon 60 (Gorodilov, 1996), trout 60 (Killeen, 1999), and zebrafish 32 (Kimmel *et al.*, 1995). Final somite number is variable even within a clutch, and is influenced by environmental variables such as temperature. Brooks and Johnston (1994) found that plaice reared at 5°C had 9% more somites on hatching than plaice reared at 12°C. Similarly, Hempel and Blaxter (1961) found that an increase in somite number could be induced by lowering the rearing temperature, or increasing salinity. The final number of somites is usually correlated with vertebral number (Blaxter, 1969).

Myogenesis in cod is essentially similar to the process described in zebrafish (Kimmel *et al.*, 1995), herring (Vieira and Johnston, 1992) and trout (Killeen *et al.*, 1999). Myoblast cells within the myotome produce myotubes, within which the contractile apparatus is produced as they differentiate into muscle fibres. The first myotubes are mononuclear "muscle pioneer cells", with later myotubes arising by the fusion of adjacent myoblasts to form multinucleate syncytial cells. Early somite development is profoundly influenced by and indeed appears to be "under the control of" the notochord, which also serves as the major supportive structural element of the embryo until ossification of the skeleton. Development of the notochord is highly conserved in the Chordata, and in cod parallels that of other species such as the trout (Killeen *et al.*, 1999) and zebrafish (Kimmel *et al.*, 1995). It is the source of many of the early inductive signals for neural differentiation, body axis formation and somatic cell specification and patterning (Currie and Ingham, 1996; Odenthal *et al.*, 1996; Stemple *et al.*, 1996; Appel, 2000). Removal of the notochord during early development prevents formation of the entire sclerotome and results in fusion of the somites beneath the neural tube (Münsterberg and Lassar, 1995). In the cod,

vacuolization of the notochord coincides with the appearance of striations in the muscle fibres suggesting that the change in structure may be correlated with alterations in its regulatory role.

Development of the heart and vascular system varies substantially among teleosts and again reflects egg and larval size. The first heart beat in cod is seen shortly before the 40-somite stage, which makes it the first organ to form and function, as in other vertebrate embryos (Yelon, 2001). The yolk sac never vascularizes as it does in salmonids (Gorodilov, 1996; Killeen *et al.*, 1999), and the blood develops haemoglobin only at metamorphosis, well beyond first feeding. Zebrafish embryos, which are of a similar size, show the same characteristics (Isogai *et al.*, 2001), possibly influenced by the lack of diffusional constraints for respiration over such small distances.

All vertebrate embryos, including teleosts, use a series of transient kidney structures to regulate fluid balance and metabolic waste during development. The embryonic kidney is the pronephros, which arises from cells in the intermediate mesoderm (Serluca and Fishman, 2001) and is first apparent in the cod at the 30-somite stage. It consists of two fused glomeruli, sprouting a pair of laterally projecting pronephric tubules, each adjoining a pronephric duct running posteriorly down the lateral trunk of the body. The glomeruli are situated ventral to the notochord and dorsal aorta at the level of the first somites and are the site of blood filtration. The adjoining tubules are the site of selective secretion and reabsorption, and the pronephric ducts carry the modified urine to the exterior (Tytler, 1988). Upon hatching a tubal urinary bladder arises from a terminal expansion of the two pronephric ducts. A urinary bladder is also seen upon hatching in the herring (Hill and Johnston, 1997a; Tytler and Ireland, 2000) and is an important site of reabsorption of

water and ions in marine species, reducing the amount that has to be drunk (Morrison, 1993). Later in development, during the juvenile to adult transition, a more complex, mesonephric kidney arises along the course of the pronephric duct (Drummond *et al.*, 1998).

Overall, early eye development in the cod is similar to that in other vertebrates, despite the fact that the optic vesicle originates as a solid outgrowth of the neural tube. The process of retinal lamination and neurogenesis is clearly highly conserved (Schmitt and Dowling, 1994). Eye pigmentation was seen to occur very suddenly in the cod and provides a useful staging tool. The timing of pigmentation is possibly adaptive, and may be due to the predation cost of possessing a highly reflective retina versus the necessity of a functional visual system before the stage of first feeding. Until the golden-eye stage the embryos are virtually transparent, and eye pigmentation and swim bladder inflation are two factors known to increase vulnerability to visual predators (Blaxter, 1988). Upon hatching, the larvae hang upside-down at the water's surface and show no response even to high levels of light. After 12-24 hours however, they begin to undergo negative phototaxis (Ellertsen *et al.*, 1980). By the first feeding stage at approximately 3 days post-hatch, the eyes have gained sufficient functionality to allow selective feeding. Cod larvae are visual feeders preferring low light levels with an optimum of 1.4 lux (Ellertsen *et al.*, 1980). In conditions of total darkness, they are unable to feed, but light levels rising above the optimum give an increasing mortality, presumably as a result of negative phototactic behaviour at the expense of feeding activity. This assumption is strengthened by the observation that larvae reared in darkly coloured tanks (which do not reflect light) show a lower mortality (P Smith, personal communication).

Almost all teleosts hatch with a quantity of yolk remaining, and the first exogenous feeding supplements the endogenous nutrient supply throughout the transition period (Meijide and Guerrero, 2000). Cod larvae have a large jaw gape, and are able to take relatively large prey items in comparison to their body size. However, initially they show a limited capacity to digest the larger nauplii, and attempts to immediately wean onto *Artemia* have been met with little success (Fridgeirsson, 1978; Tilseth *et al.*, 1992). In mesocosm studies it has been shown that first feeding larvae ingest small algae such as diatoms and dinoflagellates by filter feeding, inter-dispersed with the capture of larger prey items such as rotifers and ciliates. Copepod nauplii aren't seen until around two weeks post-hatch (van der Meeren, 1991; van der Meeren and Næss, 1993), which coincides with a change in gut morphology; a looping and extensive folding of the gut wall, and a marked increase in the digestive and absorptive capacities for lipid and protein (Kjørsvik *et al.*, 1991).

Embryonic development between teleost species is highly variable, and analysis to date has demonstrated a striking range of heterochronic shifts, between the developmental timing of processes as diverse as fin bud development (Richardson *et al.*, 1997), ossification (Mabee *et al.*, 2000), innervation (Vieira and Johnston, 1999) and final somite number (Richardson *et al.*, 1998). Future studies of comparative development, coupled with genomic approaches such as those underway for the pufferfish *Fugu rubripes*, zebrafish and medaka (Cyranoski and Smaglik, 2000), will not only provide information for the further expansion of aquaculture, but will be central to studies of speciation and morphological evolution.

Chapter 3: Temperature, developmental heterochrony and plasticity of embryonic myogenesis in the Atlantic cod *Gadus morhua* L.

Introduction

Changes in the rate or timing of developmental processes, a phenomenon known as heterochrony, is fundamental to morphological evolution (Gould, 1977; Hall, 1992; Mabee *et al.*, 2000). Teleosts in particular have been singled out as a group which show a striking range of heterochronic shifts, both between species (Richardson *et al.*, 1997), and within species from differing environments (Pavlov, 1984; Johnston, 1993; Gorodilov, 1996). Typically the timing of developmental events is reported under a single set of precisely controlled conditions (Armstrong and Child, 1965; Iwamatsu, 1994; Kimmel *et al.*, 1995; Gorodilov, 1996; Hill and Johnston, 1997a; Meijide and Guerrero, 2000; Morrison *et al.*, 2001; chapter 1, this thesis), as a "normal" series for the species. However, the ambient conditions experienced by different individuals can uncouple developmental events, resulting in the expression of a multitude of phenotypes. Phenotypic plasticity in teleosts has most commonly been reported in counts of meristic characters such as fin rays (Ali and Lindsey, 1974), vertebrae (Brander, 1979) and somites (Hempel and Blaxter, 1961). Somite and subsequent muscle development is a particularly interesting model, both in terms of the causes and consequences of plasticity, and its commercial implications (Johnston, 1999; Johnston *et al.*, 2000c; Johnston *et al.*, 2000d; Johnston, 2001).

Development of muscle in the teleost embryo begins with the induction of a sub-population of mesodermal cells by secretion of hedgehog glycoproteins from the

notochord (Munsterberg and Lassar, 1995; Munsterberg *et al.*, 1995; Currie and Ingham, 1996). A subset of these adaxial cells, the "muscle pioneers" are the first to elongate and span the somite, forming mononuclear myotubes (van Raamsdonk *et al.*, 1978; Vieira and Johnston, 1992). The majority of myotubes however, arise from the fusion of several myoblasts which align horizontally (Waterman, 1969; Nag and Nursall, 1972). Recently, Devoto *et al.* (1996) showed that the adaxial myotubes migrate laterally from their innermost position adjacent to the notochord, through the somite, to an outermost position beneath the epidermis. It is this migrating population which forms the superficial red layer at the periphery of the developing myotome. Presomitic cells within the remainder of the somite remain in approximately the same position and become the deep white fibres which form the bulk of the myotome (Blagden *et al.*, 1997; Stoiber *et al.*, 1998)

The influence of early thermal experience on muscle development in fish has received previous attention. In Atlantic herring (*Clupea harengus*), rostral to caudal progression of myofibril synthesis begins at earlier somite stages as temperature is increased (Johnston *et al.*, 1995). The development of the unpaired fins and associated musculature was also seen to occur at shorter body lengths at higher temperatures, and slow muscle innervation began earlier (Johnston *et al.*, 1997). In a separate study, Johnston (1993) found that development of the spinal cord, pronephros, pectoral fin buds and axial muscle fibres was relatively retarded at 5°C relative to 10°C or 15°C whereas the gut, notochord, eyes and haemocoel all appeared at the same somite stage.

Muscle fibre cellularity can be influenced by early rearing temperature, with long-term consequences for post-embryonic growth, which has incited considerable interest in the aquaculture industry (Johnston, 1999). Stickland *et al.* (1988) incubated

Atlantic salmon (*Salmo salar*) alevins at either ambient temperature fluctuating at around 1.6°C, or in heated water at a constant 10°C. Alevins grown under heated conditions had significantly fewer muscle fibres, with a greater diameter than those of the alevins reared at ambient temperature. Similar results have since been found by Usher *et al.* (1994), Nathanailides *et al.* (1995), and in the brown trout (*Salmo trutta*) by Killeen (1999). In contrast, herring show a greater fibre number with elevated temperature. Vieira and Johnston (1992) reared Clyde herring at 5, 10 and 15°C. The number of muscle fibres increased from 187 to 257 to 311, but the average diameter of the fibres was inversely proportional to temperature, so that the total cross sectional area of the fish remained the same.

Galloway *et al.* (1998) studied the effect of low temperature on cod development and found that eggs reared at 1°C had fewer muscle fibres upon hatching than eggs reared at 5°C, but the results were confused by asynchronous hatching and a small sample size (3 individuals from each temperature group). It was concluded that 1°C is close to, or below the lower thermal tolerance limit for normal functional development.

This study on Atlantic cod (*Gadus morhua* L.), examines the effect of environmental temperature on the timing of appearance of developmental landmarks, muscle cellularity characteristics, and the rates of development of somites and the contractile apparatus, within the normal thermal range for development of the population studied.

Materials and Methods

Six female and six male cod (*Gadus morhua*) broodstock, of approximately six years of age and caught by gillnet from Ardtoe Bay, Scotland were kept in spawning tank

systems of the type described by Huse and Jensen (1983). Discrete clutches of newly fertilized eggs were collected during March 2001. Each was divided and incubated under constant aeration at 4°C, 7°C and 10°C ± 0.2°C (range) in 10 litre containers supplied with 0.1μm filtered, UV sterilized seawater (34‰) with a flow rate of 10mls/minute. Photoperiodicity was 8h light (1.4 lux):14h total darkness.

Embryos were taken at twelve hour intervals and fixed for 24h in 4% paraformaldehyde (m/v) in phosphate buffered saline (PBS), before being transferred to 0.1% sodium azide in PBS. After dechorionation, graded ethanol dehydration and clearing in 1% celloidin in methyl-benzoate, stage of somite development, and the posterior extent of myotube and myofibrils were scored under Nomarski differential interference contrast (DIC) optics at ×10,000. Acetylcholinesterase (AchE) staining was carried out as described in Chapter 2.

For muscle cellularity measurements, larvae were anaesthetized in 0.1% (m/v) tricaine (MS-222) and set in 3% gelatin before being snap-frozen in isopentane-cooled liquid nitrogen. Histological sections (7μm) were made at the level of the bladder (Fig. 1) on a cryostat before being stained with haematoxylin and eosin (H&E). Cellularity and total cross sectional area were measured on a Videoplan image analysis system (Kontron Elektronic GmbH, Basel, Switzerland). Measurements of embryo length were made using the UTHSCSA Image Tool software package (University of Texas Health Science Centre, San Antonio, USA).

Observations of discrete events in development, such as the appearance of the otic vesicle were made on live specimens under DIC, and were recorded as the moment when 50% of embryos within a clutch expressed a particular characteristic. Three replicate batches were followed at each temperature. Percentage development time was expressed as the percentage of time between fertilization and first feeding,

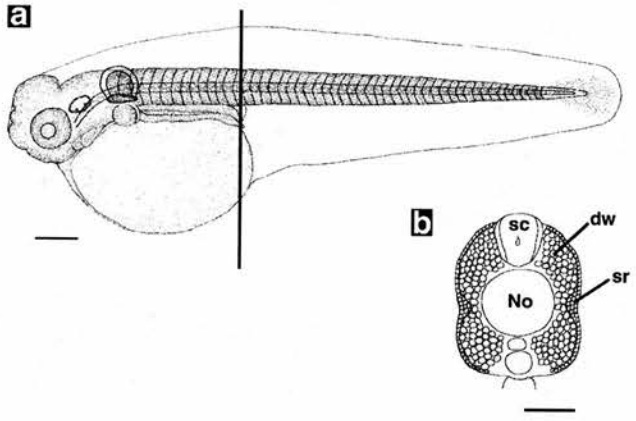


Fig. 1a. Larval cod at the bladder stage. bar - 300μm

Fig. 1b. Myotomal cross section. No – notochord, sc – spinal chord, dw – deep white fibres, sr – superficial red fibres, bar - 100μm.

since previous studies of teleosts have shown that hatching is often a poor marker of developmental stage (Gorodilov, 1996; Galloway *et al.*, 1998).

Comparisons of standard length (SL), cross-sectional area and fibre number were, in each case, made between 16 individuals from each temperature group using simple one-way analysis of variance (ANOVA). Fibre diameters were also compared between 16 individuals from each temperature group, using a two-level nested general linear model (GLM) design. The development of myotubes, myofibrils and AchE staining were compared using analysis of co-variance (ANCOVA) between 10 individuals from each temperature group at intervals of 12h. All statistical analysis was carried out using Minitab (data analysis software, Minitab, Inc., PY, USA), with the exception of ANCOVA which was performed according to Zar (1999).

Results

Development Time

Absolute development time was inversely correlated with temperature, with embryos raised at higher temperatures hatching and reaching the first feeding stage in a shorter time. Embryos reared at 4, 7 and 10°C reached first feeding (100% development time) after development and opening of the jaw, at 23, 14 and 11 days respectively. Hatching occurred at approximately 75% development time in all groups (Fig. 2). Newly hatched and first feeding larvae from each temperature group were indistinguishable in terms of their gross morphology, indicating that the hatching and first feeding stages are suitable developmental landmarks on which to base comparisons, at least over the temperature range studied. Standard length (SL) at hatching was 4.26mm ± 0.17 (mean ± st. dev.), and by first feeding the larvae had reached 4.83mm ± 0.20. No significant differences in length were apparent between

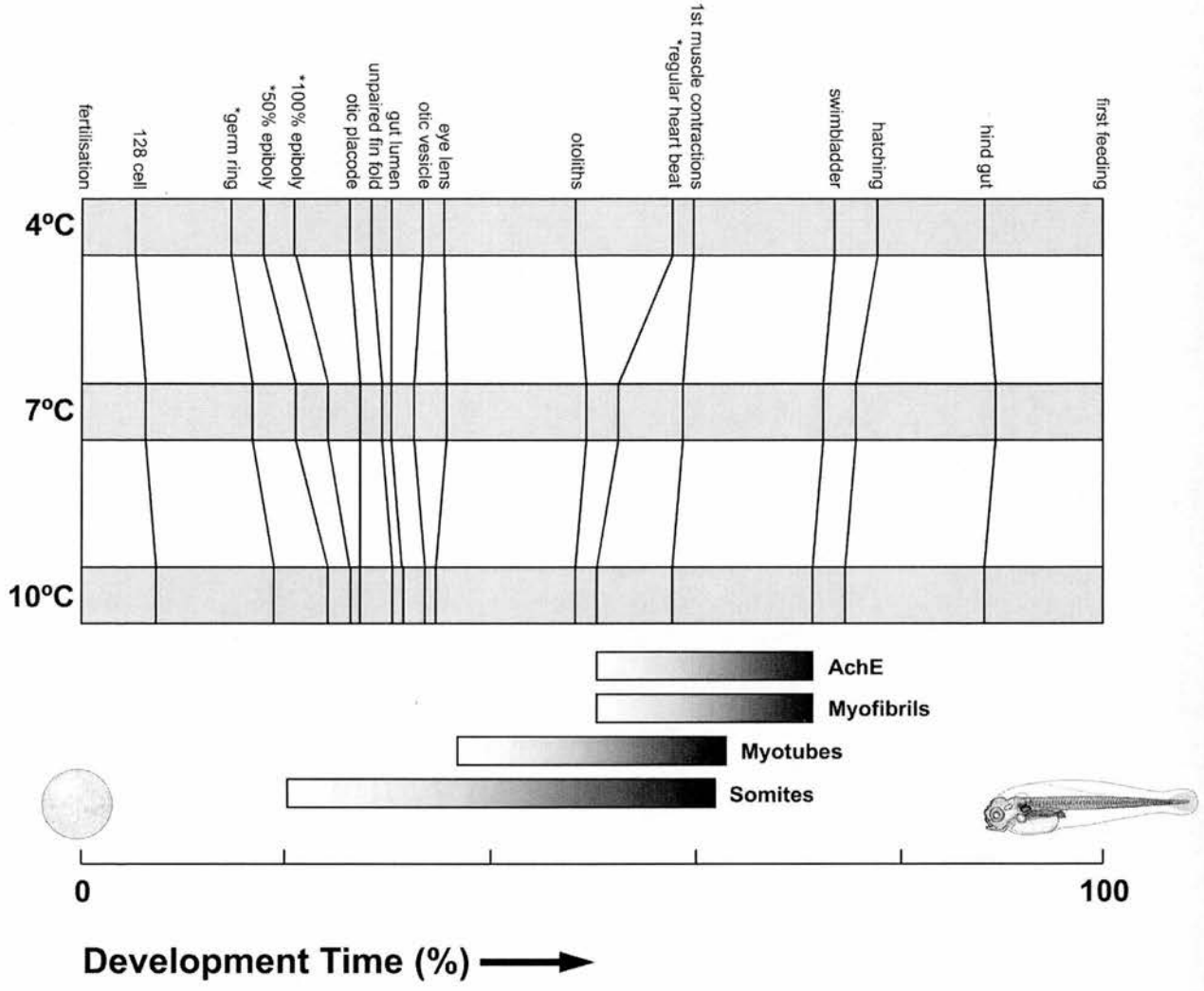


Fig. 2. Relative timing of developmental events during embryogenesis at different temperatures. Differences of greater than 5% development time are indicated by an asterisk.

temperature groups at the hatching or first feeding stages (one-way ANOVA, $P > 0.05$, n = 16 individuals per temperature group). Only minimal differences were seen between the 7°C group and those raised in chapter 2.

Muscle Development

Somite development proceeded in an anterior-posterior wave beginning at 122h in the 4°C group, 75h in the 7°C group and 53h in the 10°C group. Further somites were added linearly with respect to time at intervals of 4.5h, 3.0h and 2.3h respectively (Fig. 3), up to a maximum of 52 somites ± 4 (range). There was no significant difference in final somite number between temperature groups (one-way ANOVA, $P > 0.1$). As somitogenesis progressed, the appearance of myotubes, and subsequently myofibrils, was evident, beginning in the anterior-most somites. In both cases development proceeded linearly with respect to time (Figs. 4, 5). The first myotubes appeared at 209h (4°C), 130h (7°C) and 89h (10°C), progressing posteriorly at intervals of 3.2, 2.1 and 1.7h per somite respectively. Myofibrils were first visible at 268h (4°C), 180h (7°C) and 125h (10°C), progressing posteriorly at intervals of 2.8, 1.6 and 1.5h per somite respectively. Regression equations for somite, myotube and myofibril development are given in Table 1.

Endplate formation.

Cod embryo myotomes are focally innervated at the myosepta, giving rise to dark brown lines of acetylcholinesterase staining between the somites. AchE activity first appeared at the anterior-most myoseptum at 269h (4°C), 179h (7°C), and 123h (10°C). The development of AchE activity and the development of myofibrils were closely correlated such that often, the most posterior somite containing myofibrils was also

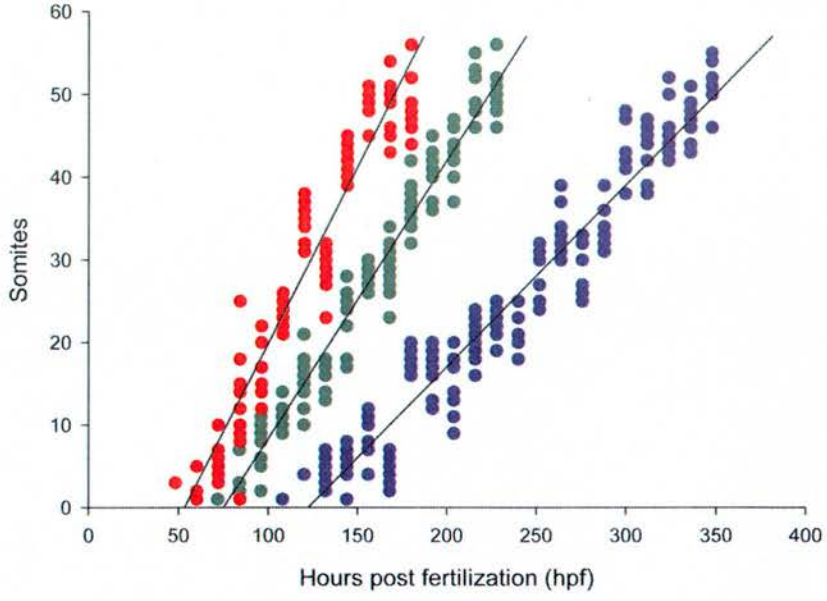


Fig 3. Somite development over time in embryos reared at 4°C (blue), 7°C (green) and 10°C (red).

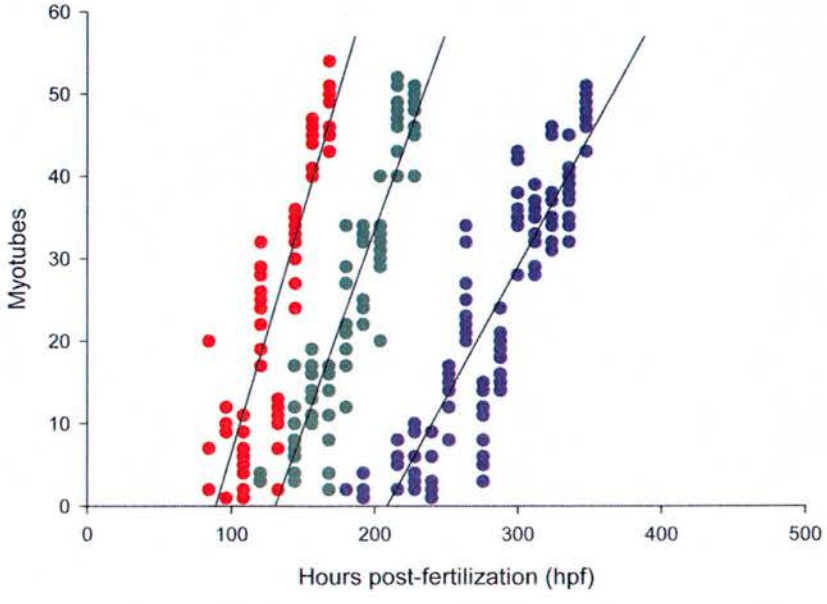


Fig 4. Myotube development over time in embryos reared at 4°C (blue), 7°C (green) and 10°C (red).

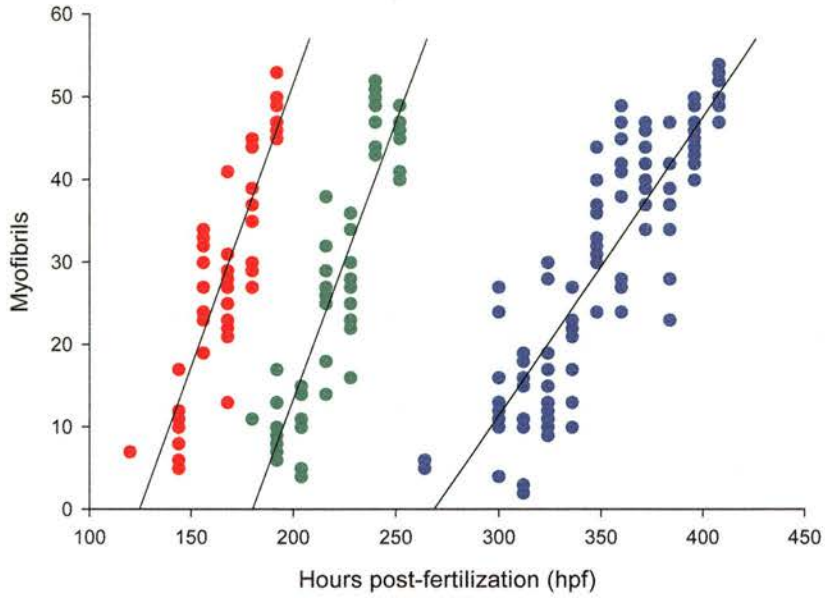


Fig. 5. Myofibril development over time in embryos reared at 4°C (blue), 7°C (green) and 10°C (red).

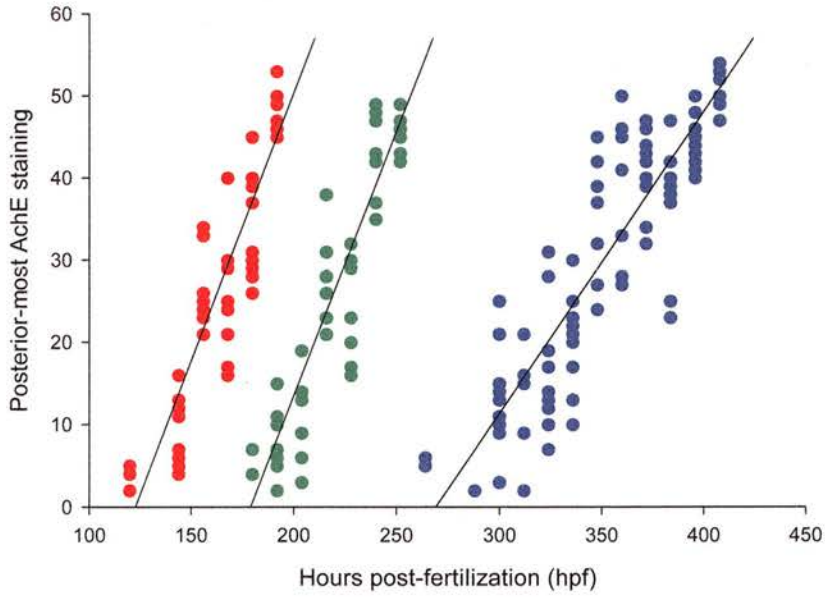


Fig. 6. Development of acetylcholinesterase activity at the myosepta in embryos reared at 4°C (blue), 7°C (green) and 10°C (red).

Table 1. Regression equations for the various aspects of myogenesis examined. $P < 0.001$ for all regressions. Residual DF = residual degrees of freedom, equal to n - 2.

Development of		Relative to	Slope	Intercept	r^2 (adjusted)	Residual DF
Somites	4°C	Hours-post fertilisation	0.220	-26.89	93.6	187
	7°C	Hours-post fertilisation	0.337	-25.28	95.7	127
	10°C	Hours-post fertilisation	0.427	-22.80	91.7	103
Myotubes	4°C	Hours-post fertilisation	0.317	-66.12	79.6	112
	7°C	Hours-post fertilisation	0.481	-62.69	85.0	80
	10°C	Hours-post fertilisation	0.586	-52.24	74.0	64
Myofibrils	4°C	Hours-post fertilisation	0.362	-97.08	77.4	99
	7°C	Hours-post fertilisation	0.671	-120.88	80.5	55
	10°C	Hours-post fertilisation	0.685	-85.46	77.2	48
AchE Activity	4°C	Hours-post fertilisation	0.367	-98.61	77.6	99
	7°C	Hours-post fertilisation	0.642	-114.91	83.9	57
	10°C	Hours-post fertilisation	0.651	-79.93	78.2	51
Somites		% Development time	1.184	-25.73	94.0	540
Myotubes		Somite stage	1.430	-26.59	94.0	270
Myofibrils		Somite stage	2.166	-80.47	88.0	118
AchE Activity		Somite stage	2.326	-88.14	89.6	123

the most posterior somite expressing AchE activity. The rate of posterior progression of AchE activity at the myosepts was 2.7h per somite at 4°C, 1.6h per somite at 7°C, and 1.5h per somite at 10°C (Fig. 6, Table 1).

Relative timing of organogenesis

Rate of somitogenesis was compared against percentage development time (Fig. 7). No significant difference was seen over the temperature range studied (ANCOVA, $P > 0.05$). Development of the contractile apparatus was compared using both somite stage (Fig. 8), and development time as independent variables. No significant difference was seen between timing of appearance and rate of posterior progression in the development of myotubes, myofibrils or AchE activity in each case (ANCOVA, $P > 0.05$).

Developmental heterochrony between the other ontogenetic landmarks measured was low (Fig. 2), and there was no change in their order of appearance. Time of hatching and timing of appearance of the otic placode, unpaired median fin fold, gut lumen, otic vesicle, lens of the eye, otoliths, first muscular contractions, swimbladder, and hindgut were all unaffected by developmental temperature, occurring within the same 5% of development time between temperature groups and between replicates at the same temperature (data not shown). Epiboly however, was relatively delayed at higher temperatures such that at closure of the blastopore a 4°C embryo possessed three somites, a 7°C embryo ten somites and a 10°C degree embryo twelve somites. Conversely, the first regular heart beat occurred earlier at higher temperatures, 50.5% development time at 10°C, 52% at 7°C and 57% at 4°C.

Table 2. Number of days until hatching, larval length at hatching, mean area of transverse sections, number and diameter of deep and superficial fibres, final somite number and percentage survival at 4, 7 and 10°C. Number of days until hatch was invariant between clutches, all other value represent the mean ± standard deviation (n = 16 individuals per temperature, except survival where n = 3 per temperature). Different superscripted letters represent significant differences between values.

Temp (°C)	Days until hatch	Larval length (mm)	TS area (mm^2)	Deep fibres		Superficial fibres		Final somite number	Survival (%)
				No.	Diam. (μm)	No.	Diam. (μm)		
4	18	4.32 ± 0.19	0.0134 ± 0.0011	192 ± 11[a]	6.8 ± 1.2[a]	97 ± 10	4.0 ± 0.5	52 ± 3	73 ± 7
7	11	4.19 ± 0.14	0.0141 ± 0.0021	188 ± 17[a]	6.2 ± 1.0[a]	89 ± 13	4.2 ± 0.7	52 ± 3	78 ± 10
10	8	4.29 ± 0.17	0.0139 ± 0.0023	217 ± 12[b]	5.1 ± 0.7[b]	96 ± 13	4.8 ± 0.5	53 ± 3	71 ± 9

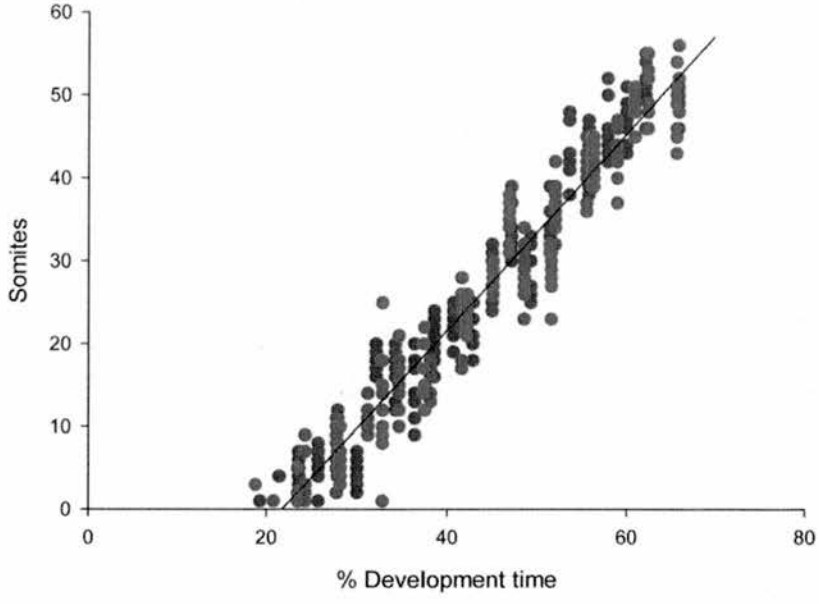


Fig. 7. Somite development in relation to percentage development time in embryos reared at 4°C (blue), 7°C (green) and 10°C (red). No significant difference was seen between temperature groups (ANCOVA, $P > 0.05$).

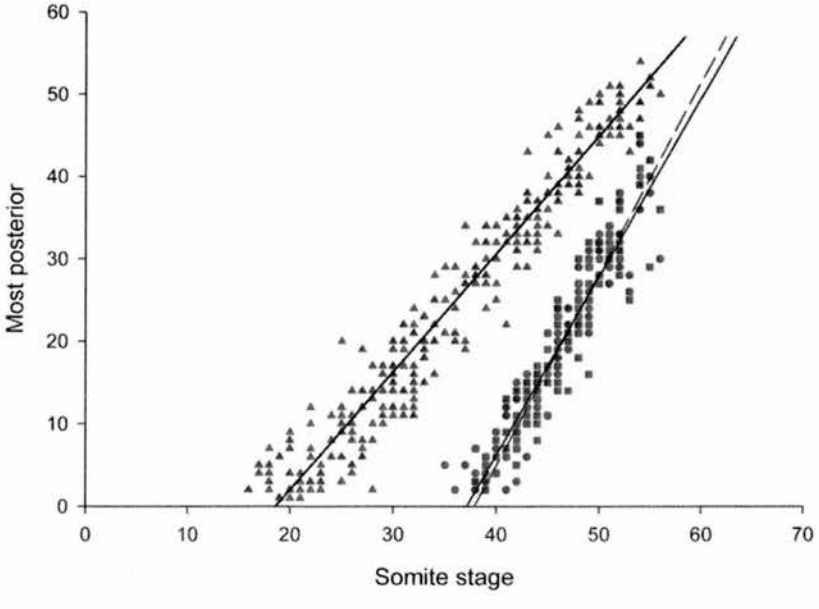


Fig 8. Development of myotubes (triangles), myofibrils (squares) and acetylcholinesterase activity (circles, dashed regression line) at 4°C (blue), 7°C (green) and 10°C (red), in relation to somite stage. No significant differences were seen between temperature groups (ANCOVA, $P > 0.05$).

Muscle cellularity at hatching

Upon hatching, the total area of transverse (TS) sections taken at the level of the bladder was 0.0138mm² ± 0.0019 (mean ± st. dev.), and was not significantly different between temperature groups (one-way ANOVA, $P > 0.05$). Two separate layers of muscle fibres could be distinguished under the light microscope. Immediately beneath the epidermis was a monolayer of "superficial" fibres which continued around the entire circumference of the trunk (Fig. 1). At the horizontal myoseptum the monolayer extended inwards to approximately three fibres deep. The majority of the muscle mass was occupied by "deep" fibres which had a larger diameter (Table 2) than the superficial fibres. Deep fibre number was not significantly different between the 4 and 7°C degree groups (one-way ANOVA followed by Tukey test, $P > 0.05$), but was 14% higher in the 10°C group relative to the other temperature groups (Tukey test, $P < 0.001$). Deep fibre diameter was also less in the 10°C group (two-level nested GLM followed by Tukey test, $P < 0.001$; Table 2). In contrast to the deep fibres, the number and diameter of superficial fibres did not differ between temperature groups (Table 2, two-level nested GLM, $P > 0.05$).

Discussion

Development time in the cod is inversely proportional to temperature, as is invariably the case in eggs of poikilothermic species (Laurence and Rogers, 1976; Bermudes and Ritar, 1999; Polo *et al.*, 1991). The relationship is linear, except at extremes of the range (Guma'a, 1978) and presumably reflects the effect of temperature on metabolic rate. Embryos have a window of temperature tolerance, beyond the limits of which, survival drops off sharply (Kinne and Kinne, 1962). In the cod, the environmental

limits of temperature tolerance are between approximately 1°C (Galloway *et al.*, 1998) and 12°C (Iversen and Danielssen, 1984).

At hatching and first feeding there was a lack of any gross morphological difference between temperature groups. In a variety of teleosts however, the extent of differentiation at hatching has often been observed to vary between clutches reared at different temperatures. For instance Atlantic salmon hatch at an earlier developmental stage at lower temperature (Pavlov, 1984), as do plaice (*Pleuronectes platessa*; Ryland, 1975), turbot (*Scophthalmus maximus*; Gibson and Johnston, 1995), and tambaqui (*Colossoma macropomum*; Vieira and Johnston, 1999). It has been suggested that earlier hatching might be due to oxygen constraints of the chorion, since oxygen solubility decreases with increasing temperature (Vieira and Johnston, 1999). However, Herzig and Winkler (1986) found that hatching in three cyprinid species occurred relatively earlier at higher temperatures. In a separate study Guma'a (1978) found that perch raised at temperatures between 6 and 20°C hatched earlier at both the lower and the higher extreme. It is possible that early hatching is a general response to sub-lethal conditions, since freedom from the constraints of the chorion allows locomotion, and some degree of active movement away from a particular locality.

Final number of somites is highly variable within teleosts ranging from 16 in the ocean sunfish *Mola mola* (Brainerd *et al.*, 2001), to over 200 in some eels (Richardson, 1998). The average final somite number in the cod was 52, and did not vary with incubation temperature. However, Brander (1979) compared cod fished from different areas of the North Atlantic and found an inverse relationship between temperature and mean vertebral number (of which somite number is predictive). Recent knowledge of the genetic diversity of North Atlantic stocks (Nielson *et al.*,

2001), and the results obtained here, suggest that number of somites in cod might have a stronger heritable than temperature induced basis. Temperature has been shown to influence final somite number in other fish. For instance Pavlov (1984) showed that salmon reared at 2°C had an average of 58.3 somites, whereas those reared at 9°C had 56 somites.

The rate of somite development in cod is linear, as it is in herring (Johnston *et al.*, 1995), trout (Killeen, 1999) and salmon (Gorodilov, 1996). In other species such as turbot (Gibson and Johnston, 1995) and plaice (Brooks and Johnston, 1994) an initial linear rate of somite development slows around the time of the first muscular contractions. In both these species somite formation also continues after hatching and into the larval period.

The timing of development of the myotubes, myofibrils and AchE activity is clearly highly canalized in cod unlike in the herring, where the timing of muscle fibre formation can vary by 10 or more somite intervals over a range of 7°C (Johnston *et al.*, 1997). A similar lack of variability was reported in brown trout by Killeen (1999), who attributed it to characteristics of the early life history of the salmonids. It was argued that the more variable thermal regimes experienced by salmonid embryos in upland river tributaries, would necessitate the evolution of regulatory mechanisms, to ensure coordinated development over a broad temperature range. Whether or not this is the case, this argument cannot be applied to the cod, which is a marine species unlikely to experience the same thermal fluctuation in early life.

Muscle cellularity characteristics are often highly labile and different species can show a variety of responses to environmental temperature. Over the temperature range studied, cod showed a similar response to that seen in herring (Vieira and Johnston, 1992), with a greater number of smaller muscle fibres being layed down at

the higher temperature, such that total cross sectional area remained the same. Measurements of total cross sectional area and fibre diameter were both approximately 10% smaller in the current study than found by Galloway *et al.* (1998), but this could be due to differences in embryo sectioning, since these authors give no indication of the position of their transverse sections.

The timing of the developmental characteristics measured was also highly canalized and invariant compared to recent studies of other species (Fukuhara, 1990; Johnston, 1993). As in this study, a delay in the timing of the first heart beat at low temperatures was also seen by Pavlov (1984) in salmon, who attributed it to the higher oxygen solubility in colder water. Recently, studies in *Xenopus laevis* (Territo and Burggren, 1998) and the zebrafish *Danio rerio* (Pelster and Burggren, 1996), have shown that the heart begins to beat well before the metabolic needs of the embryo outstrip material transport by diffusion. Rather, early beating facilitates structural development, in particular angiogenesis (Burggren *et al.*, 2000). This does not necessarily discredit Pavlov's hypothesis however, since an early heartbeat might still confer an advantage in conditions of environmental hypoxia (Pelster, 1997).

The finding that the extent of epiboly is uncoupled with somite formation is potentially extremely significant. Epiboly and closure of the blastopore are relatively delayed at higher temperatures, which has implications for the extent of proliferation of the mesodermal cells prior to somite formation, and for the timing of the inductive signals they are exposed to. Such "process" heterochronies can be important for the generation of "pattern" heterochronies later in development (Richardson, 1999). It would be fascinating to follow the expression patterns of mesodermal and myogenic inductive factors under different temperature conditions. However, this would first require the cloning and isolation of the cDNAs of interest.

Despite the prevalence of temperature induced heterochrony and phenotypic plasticity in teleosts, the majority of authors stop short of discussion of adaptive significance. Indeed, the relative lack of heterochrony and phenotypic plasticity found in this study when compared to others (Fukuhara, 1990; Johnston, 1993) might lead to the conclusion that many incidences of heterochrony or plasticity are in fact detrimental sub-lethal rather than adaptive responses. This possibility is emphasized in view of the fact that the current study was specifically confined to a "normal" temperature range.

Chapter 4: Temperature and the expression of seven muscle-specific protein genes during embryogenesis in the Atlantic cod *Gadus morhua* L.

Introduction

Muscle is an unusual and highly specialized tissue in that it consists predominantly of post-mitotic syncytial cells. Many proteins are expressed uniquely in muscle cells (termed muscle-specific proteins or MSPs; Xu *et al.*, 2000). These include structural and contractile proteins (e.g. α-actins, myosins, troponins etc.) as well as soluble muscle proteins and enzymes (e.g. muscle creatine kinase and parvalbumin). During differentiation, non-muscle isoforms of many proteins are down-regulated, while muscle specific isoforms begin to be expressed (Rubenstein and Spudich, 1977; Goncharova *et al.*, 1992; Phillips *et al.*, 1995). Much is known about the biochemistry of muscle contraction, but comparatively less is known about the process of myofibrillogenesis, and there are relatively few models. Most studies have relied on cell culture systems (Lin *et al.*, 1994; Yoshimi *et al.*, 1995; van der Ven and Furst, 1998), which may not reflect the *in-vivo* situation (Costa *et al.*, 2002). At the current time, whole embryo studies are limited to the mouse *Mus musculus* (Furst *et al.*, 1989), zebrafish *Danio rerio* (Xu *et al.*, 2000) and *Xenopus laevis* (Martin and Harland, 2001), and the relative timing of the onset of MSP expression appears to differ considerably between these species (see Costa *et al.*, 2002). Teleost muscle is unique in many aspects of its structure compared to other vertebrates (Luther *et al.*, 1995), and the extent to which myofibril assembly shows phylogenetic variation is unknown.

The first aim of the present study was therefore to characterize and investigate the expression of MSP genes required for myofibril assembly in the Atlantic cod (*Gadus morhua* L.), including a full-length cDNA of *MyoD*. Myogenic regulatory factors (MRFs) of the *MyoD* gene family play a key role in lineage determination (*MyoD*, *Myf-5*) and in initiating and stabilizing the differentiation programme (*myogenin*, *MRF4*) in co-operation with other basic helix-loop-helix and MADS box transcription factors (MEF-2 proteins) (reviewed in Sabourin and Rudnicki, 2000; Pownall *et al.*, 2002). The promoter regions of most muscle-specific genes, including *MyHC*, contain *MyoD* and *MEF-2* recognition sites (Giger *et al.*, 2000; Wheeler *et al.*, 1999). It has been shown that *MyoD* mRNA expression precedes the *de novo* expression of *MyHC IIB* mRNA in rat fast muscle following to hind limb suspension (Wheeler *et al.*, 1999). Other MSP cDNA clones characterized included: *α-actin*, which forms the backbone of the thin filament in the myofibril (Gordon *et al.*, 2000); *myosin heavy chain* (*MyHC*), which is the major component of the thick filament and the most abundant protein of the sarcomere (Lu *et al.*, 1999); *muscle creatine-kinase* (*CK-M*), which plays a central role in the catalysis of ADP to form high energy ATP (Walliman *et al.*, 1992), and the three troponin (Tn) subunit genes *troponin C* (*TnC*), *troponin I*, (*TnI*) and *troponin T* (*TnT*), which are involved in calcium binding and signal transduction (Filatov *et al.*, 1999).

Temperature is known to have major effects on early muscle development in teleosts altering the timing of myofibril assembly with respect to somite stage (Atlantic herring; Johnston *et al.*, 1995; chapter 3, this thesis), as well as the number and size of embryonic muscle fibres in numerous species (Stickland *et al.*, 1988; Vieira and Johnston, 1992; Brooks and Johnston, 1993; Gibson and Johnston, 1995; Hanel *et al.*, 1996; Matschak *et al.*, 1998); including Atlantic cod, *Gadus morhua*

(Galloway *et al.*, 1998; chapter 3, this thesis). During ontogeny, embryonic isoforms of the myofibrillar proteins are gradually replaced by larval and adult isoforms reflecting increases in body sizes and associated changes in swimming behaviour (Martinez *et al.*, 1991; Chanoine *et al.*, 1992; Mascarello *et al.*, 1995). The relative timing of expression of developmental-stage specific isoforms varies for different myofibrillar components and is altered by rearing temperature (Johnston *et al.*, 1997; Johnston *et al.*, 1998). For example, in Atlantic herring (*Clupea harengus*) the appearance of adult isoforms of myosin light chain 2, troponin T, troponin I occurred at longer body lengths in larvae reared at 5°C compared to 12°C (Johnston *et al.*, 1997).

In a recent study in rainbow trout (*Oncorhynchus mykiss*) it was shown that MRF expression was delayed and prolonged at low compared to high egg incubation temperatures, and it was suggested that this resulted in a higher number of muscle fibres in hatched embryos due to a longer period for proliferation of the myogenic precursor cells prior to terminal differentiation (Xie *et al.*, 2001). In contrast, the onset of *MyoD* and *myogenin* expression in relation to developmental stage was found to be similar at a range of temperatures in the Atlantic herring (Temple *et al.*, 2001).

The second aim of the present study was therefore to test the hypothesis that differences in muscle cellularity with temperature in cod embryos (chapter 3) were correlated with changes in the relative expression of MSP genes required for myofibril assembly. Somite stage was used as a normalized index of development at the different temperatures studied.

Materials and Methods

Larval rearing was carried out as in chapter 3.

Isolation of cDNA clones

Total RNA was extracted from embryos of mixed developmental stages using Tri-reagent (Sigma). mRNA was purified from the total RNA using a poly-T^+ spin column (Amersham Pharmacia Biotech). A first-strand reaction was carried out with 1μg poly A^+ RNA using Superscript II reverse-transcriptase (Gibco BRL) and either an oligo-DT, 3' RACE cDNA synthesis primer (Gibco BRL) or an oligo-DT, 5' RACE cDNA synthesis primer (Clontech) in conjuction with a SMART II oligonucleotide (Chenchik *et al.*, 1998). Primers were designed to conserved regions of multiple nucleotide sequence alignments of genes from related species (Tables 1 and 2); prepared using the Clustal algorithm in Lasergene (DNAstar, Inc). PCR conditions were complex and often involved multiple rounds and touchdown cycles (details are given in Appendix II). Final PCR products were purified by agarose gel electrophoresis followed by gel extraction on a spin column (Qiagen). cDNAs were ligated into the PCR-4-TOPO vector (Invitrogen) and transformed into TOP-10-F competent cells (Invitrogen) according to manufacturers instructions. All clones were sequenced twice in either direction, and the nucleotide and deduced amino acid sequences submitted to the GenBank database (NCBI, Bethesda, USA; Table 1).

In addition to the MSP genes, the 60S ribosomal subunit gene L15 was cloned for use as an internal standard during RT-PCR analysis. None of the clones have been previously isolated or sequenced elsewhere, all are novel sequences reported for the first time. Sequence manipulation was carried out using DNAman (Lynnon Biosoft) and Lasergene (DNAstar Inc.). The phylogenetic tree was constructed from a Clustal alignment followed by neighbour joining in PHYLIP (Felsenstein, 1995). Initial homology searches were carried out on the DNA data bank of Japan (DDBJ) protein-blast engine.

Table 1. Details of MSP clones and the most homologous cDNAs, determined by the DDJP blast engine.

Gene name	Abbreviation	Most homologous cDNA (species, Genbank accession no.)	AA sequence identity	Whole insert length (nt)	Coding region (nt) (F, full length)	Genbank accession no.
Myogenic Determination Factor	*MyoD*	zebrafish, Z36945	64%	1568	804 F	AF329903
Skeletal α-Actin	*α-actin*	Alaskan pollack AB073380	100%	1598	1131 F	AF500273
Myosin Heavy Chain	*MyHC*	chum salmon AB076182	88%	897	782	AY093703
Troponin T	*TnT*	Atlantic salmon AF072687	86%	767	690 F	AF500272
Troponin I	*TnI*	Atlantic salmon U84394	62%	817	516 F	AF498091
Muscle Creatine Kinase	*CK-M*	Mozambique tilapia AY034098	89%	1187	777	AF329904
Troponin C	*TnC*	African clawed frog AB003080	81%	589	367	AF500274

Table 2. Primer sequences used for first-strand cDNA synthesis, and 3' and 5' RACE. Final product sizes calculated include the primer sequences. Underlined bases represent RNA rather than DNA residues. V= A, G or C, N= A, G, C or T. AUAP - Abridged universal adapter primer, UP - Universal primer. *Clones used for *in-situ* hybridization.

cDNA	3' RACE Forward Primer	Product Size (bp)	5' RACE Reverse Primer	Product Size (bp)
MyoD	AGATGCACGTCCACCAACCCGAACC	1143*	AAGGATCCCCACTTTGGGCAGCCTCTGG	512
α-actin	ACCTTCCAGCAGATGTGGATCAGCA	494*	AAGCACTTCCTGTGGACGATGGAGG	1194
MyHC	CAAGGAGCAGGCTGCTATGGTTGAGC	934*		
TnT			CCAAGCAGCAGAAGGGCCCGTT	447*
TnI	GATGAGGAGCGGTACGATGCTGCGG	516*	GCAGACATGCGCACCTTCTTCAGGG	447
CK-M	GGTGGCGATGACCTGGACCCC	1205*		
TnC	TGCATCAGTACCAAGGA	606*		

RT-PCR Analysis Primers

cDNA	Forward	Reverse	Product Size (bp)
MyoD	GAGCCTTGGTCGAACATCACCGAC	GGAACTTTGATGAGCTTGCTTCTTATCAG	1262
L15	GCTCCCAGACCCACCAGACCC	ATGGCCTTGTGGAAGGTGTCG	311

First-Strand cDNA synthesis	
3' RACE oligo-DT cDNA synthesis (Gibco)	GGCCACGCGTGCACTAGTACTTTTTTTTTTTTTTTT
5' RACE oligo-DT cDNA synthesis (Clontech)	TTTTTTTTTTTTTTTTTTTTTTTTVN
5' RACE Smart II oligo (Clontech)	AAGCAGTGGTATCAACGCAGAGTACGC<u>GGG</u>

RACE adapter primers	
3' end (AUAP, Gibco)	GGCCACGCGTCGACTAGTAC
5' end (Short UP, Clontech)	CTAATACGACTCACTATAGGGC

Preparation of DIG-labelled cRNA probes

Aliquots of plasmid were linearized using SpeI or NotI restriction endonucleases and purified on an enzymatic cleanup spin column (Qiagen). 50pg/µl of linear plasmid was used to transcribe the probes in a reaction containing $1/10^{th}$ volume DIG-RNA labeling mix (Roche), transcription buffer and 2 units/µl of the appropriate RNA polymerase (T3 for NotI digests, T7 for SpeI digests). Following incubation at 37°C for 2h the labeled probe was purified by lithium chloride/ethanol precipitation and dissolved in diethyl-pyrocarbonate (DEPC) treated water, before storage at -80°C. All probes were made using the longest plasmid insert possible, with the exception of α-actin, the 3' UTR of which was used to avoid cross-hybridization with β-actin which is ubiquitously expressed (Xu *et al.*, 2000).

***In-situ* hybridization**

Twenty embryos of mixed somite stages from each temperature group were used for *in-situ* hybridization per cRNA probe. Sense probes were also used in each case as negative controls. *In-situ* hybridization was carried out using a procedure incorporating aspects of those described by Wilkinson *et al.* (1992) and Ennion *et al.* (1999) which permitted a high through-put with small embryos and gave an excellent signal. The tri-ethanolamine wash, antibody pre-absorption step and RNAse digestion step were found to be unnecessary, and use of CHAPS rather than SDS allowed briefer stringency washes carried out at a single temperature. Importantly, the length of hybridization was lengthened considerably and appeared to result in a stronger final signal.

Details of the *in-situ* hybridization are as follows. Embryos were dechorionated on ice under a dissecting microscope using no. 10 watchmaker's forceps, then rehydrated at room temperature through 75% methanol (MeOH):25% 0.1% Tween-20 in phosphate buffered saline (PBST), 50:50 MeOH:PBST, 25:75 MeOH:PBST, followed by two washes in 100% PBST. Permeablization was achieved by digestion in 20µg/ml proteinase K in PBST for 10min, followed by 2×5min washes in PBST. Embryos were refixed in 4% (m/v) paraformaldeyde, 0.1% gluteraldehyde in PBST, followed by 3×5min washes in PBST. A pre-hybridization step was carried out in hybridization buffer (50% formamide, 2% [m/v] blocking reagent [Roche], 0.1% triton-X100, 0.1% [m/v] CHAPS, 20µg/ml yeast tRNA, 50µg/ml heparin in 5mM EDTA) for twenty minutes at 70°C before addition of 0.5µg/ml DIG-labelled cRNA probe. The hybridization step lasted for three days at 70°C. Subsequent to hybridization, the embryos were washed with decreasing stringency to remove unbound probe. The post-hybridization washes consisted of 2×10min in 2×SSC, 3×20min washes in 2×SSC, 0.1% (m/v) CHAPS, and a further 3×20min washes in 0.2×SSC, 0.1% (m/v) CHAPS. Embryos were rinsed 2×10min in "Heaven Seven" (HS) solution (150mM NaCl, 1% Tween-20 in 100mM Tris pH 7.5) followed by a 20 minute blocking step in 20% sheep serum in HS. Bound probe was conjugated to an alkaline-phosphatase labeled anti-digoxygenin antibody (Roche), which was used at a dilution of 1:4000, overnight at 4°C. Free antibody was removed by 4×1h washes in 1mM levamisole in HS. The final colour reaction was carried out in "Divine Nine" (DN) solution (100mM NaCl, 1% Tween-20, 1mM levamisole, 100mM Tris pH 9.5) containing 1mg/ml of nitroblue tetrazolium (NBT) and 0.5mg/ml of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). After 24h of development in the dark at 4°C, the reaction was stopped with 4% paraformaldeyhde

in PBS. Photographs were taken on a Lieca MZ7.5 binocular microscope using darkfield illumination and Ziess Axiocam imaging system.

Analysis of expression patterns.

Expression patterns were determined for the structural/contractile mRNAs by scoring the most posterior somite expressing a particular mRNA against somite stage. For *MyoD* however, this was not considered appropriate since expression preceded somite formation and was confined transiently to a small number of somites. Instead, expression was compared visually and RT-PCR analysis was carried out on RNA→cDNA extracted from different stages of development, as in Wysykowski *et al.* (2002) and Lin-Jones and Hauschka (1997). Primers are shown in Table 2. Statistics, one-way analysis of variance (ANOVA) and multiple analysis of covariance (MANCOVA) were performed according to Zar (1999).

Results

The sequence of development of somites, myotubes and myofibril assembly (Fig. 2) was identical to that described in chapter 3.

Sequence Analysis

Of the amplified cDNAs, deduced amino acid sequence conservation was generally high. *α-actin* was the most highly conserved, with an identical deduced amino acid sequence to those of Alaskan pollack *Theragra chalcogramma* and rat-tail fish *Coryphaenoides cinereus* (Fig. 3).

The MyHC cDNA was a partial sequence (the full coding cDNA is >5000bp in carp; Hirayama and Watabe, 1997). The isolated clone came from the 3' end and

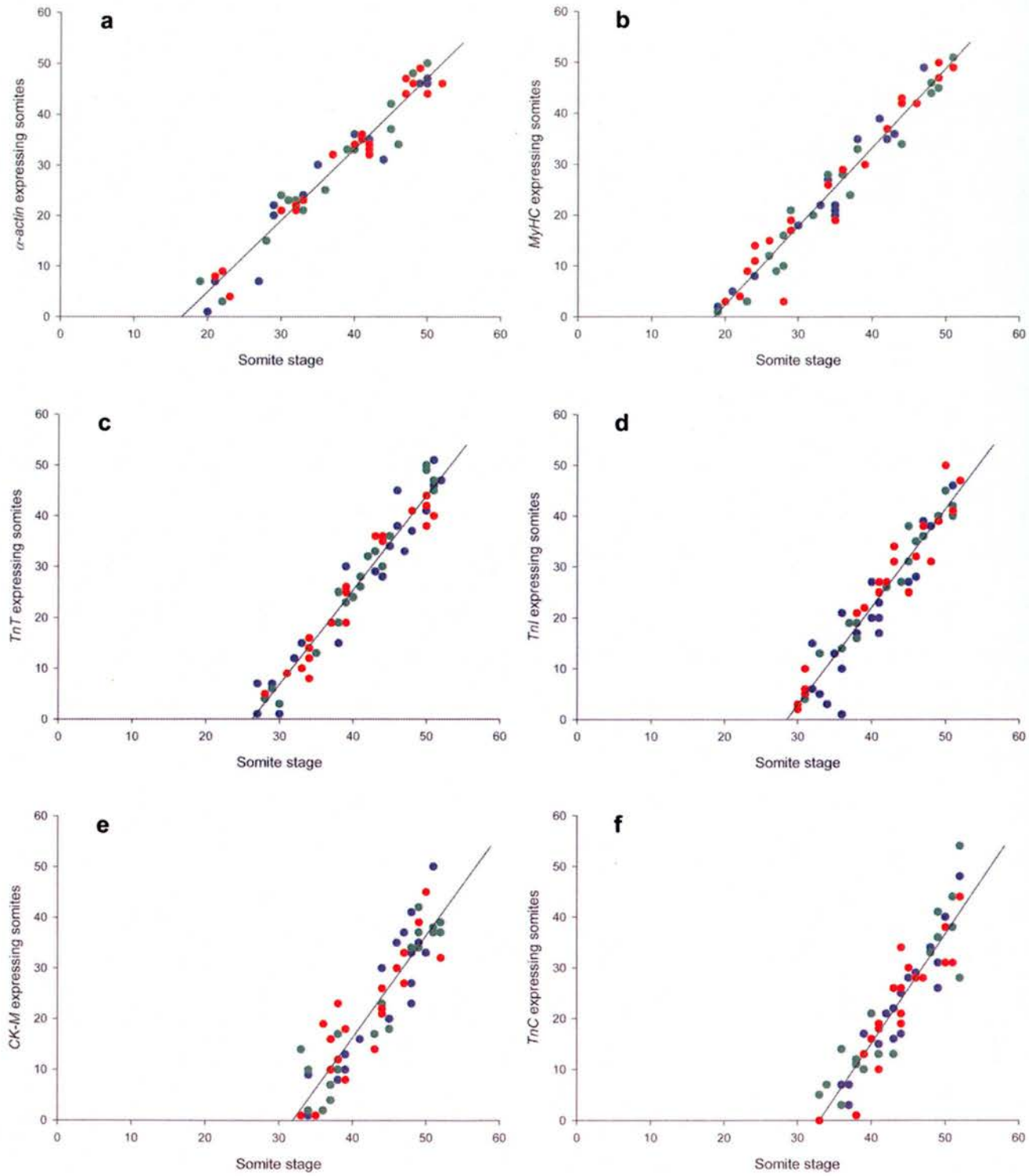


Fig. 1. Expression of myofibrillar MSP mRNAs at different temperatures according to somite stage. A - *α-actin*, B - *myosin heavy chain* (*MyHC*), C - *troponin T* (*TnT*), D - *troponin I* (*TnI*), E - *muscle creatine kinase* (*CK-M*) F - *troponin C* (*TnC*).

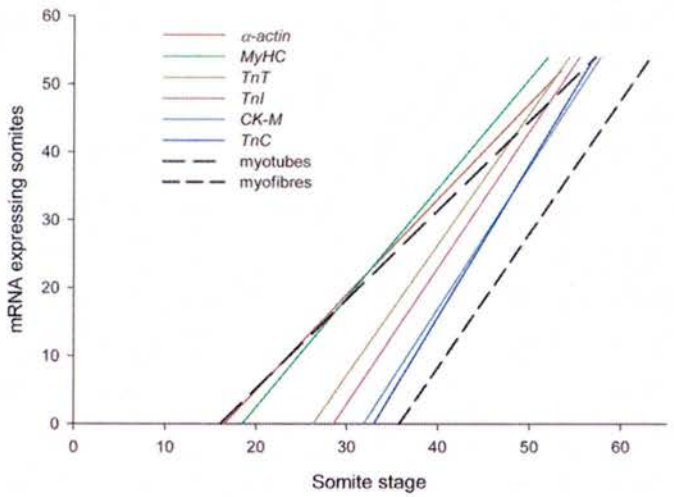


Fig. 2. Comparison of the regression lines of MSP expression with somite stage, with the regression lines of myotube and myofibril synthesis against somite stage, from chapter 3.

Fig. 3. Multiple sequence alignment of the deduced amino acid sequence of Atlantic cod *Gadus morhua* α-actin with those of Alaskan pollack *Theragra chalcogramma* and rat-tail fish *Coryphaenoides cinereus*. Sequence identity was 100%.

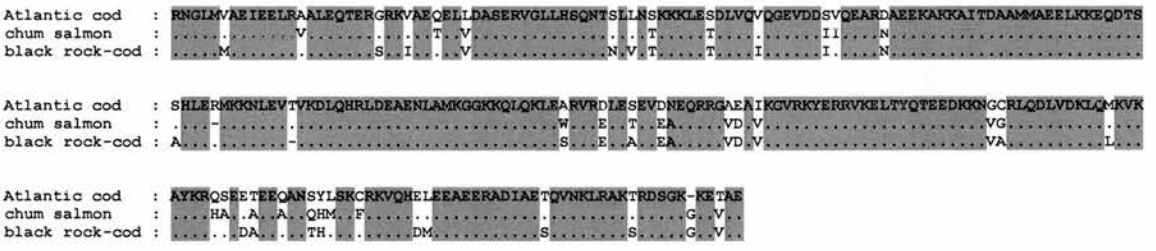


Fig. 4. Multiple sequence alignment of the deduced amino acid sequence of the C-terminus region of Atlantic cod *Gadus morhua* myosin heavy chain (MyHC), with those of chum salmon *Onchorynchus keta* (sequence identity 88%) and black rock-cod *Notothenia coriiceps* (sequence identity 86%).

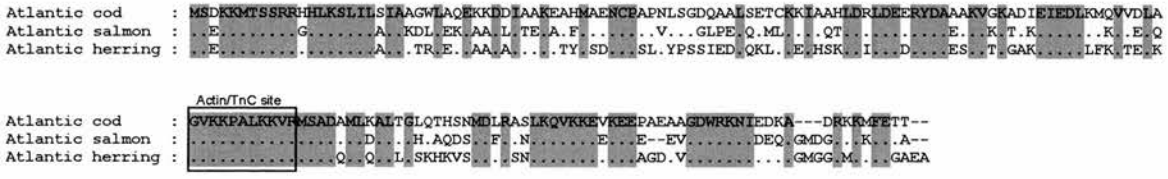


Fig. 5. Multiple sequence alignment of the deduced amino acid sequence of Atlantic cod *Gadus morhua* troponin I (TnI), with those of Atlantic salmon *Salmo salar* (sequence identity 62%) and Atlantic herring *Clupea harengus* (sequence identity 55%). The actin\Troponin C (TnC) binding site is boxed.

Fig. 6. Multiple sequence alignment of the deduced amino acid sequence of Atlantic cod *Gadus morhua* troponin T (TnT), with those of Atlantic salmon *Salmo salar* (sequence identity 86%) and zebrafish *Danio rerio* (sequence identity 81%). A region of low homology near to the N-terminus is boxed.

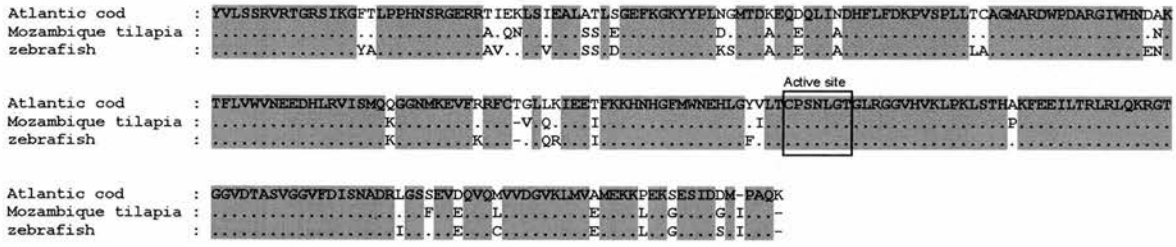


Fig. 7. Multiple sequence alignment of the deduced amino acid sequence of Atlantic cod *Gadus morhua* creatine kinase (CK-M), with those of Mozambique tilapia *Oreochromis mossabicus* (sequence identity 89%) and zebrafish *Danio rerio* (sequence identity 87%). The active site motif is boxed.

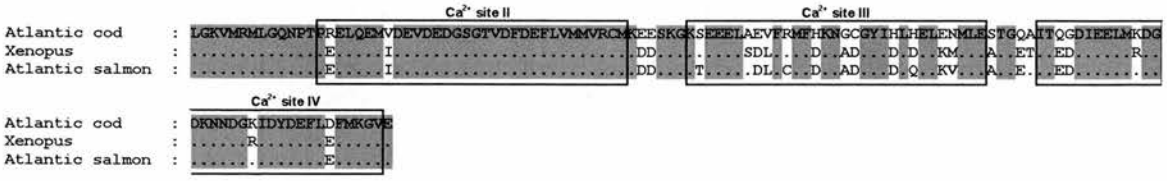


Fig 8. Multiple sequence alignment of the deduced amino acid sequence of Atlantic cod *Gadus morhua* troponin C (TnC), with those of *Xenopus laevis* (sequence identity 81%) and zebrafish *Danio rerio* (sequence identity 62%). Ca^{2+} binding sites II, III and IV are boxed.

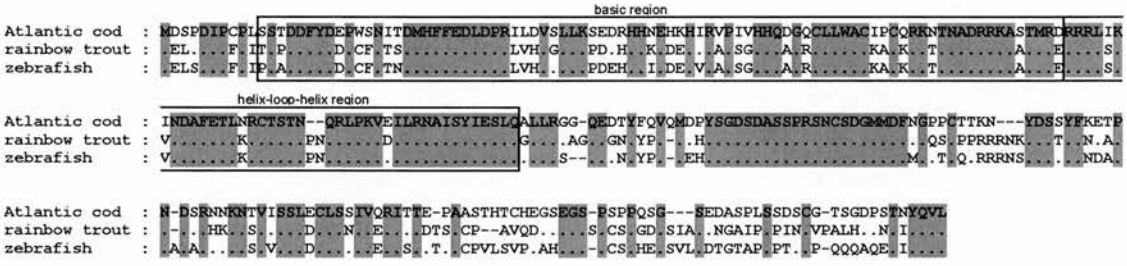


Fig 9. Multiple sequence alignment of the deduced amino acid sequence of Atlantic cod *Gadus morhua* MyoD, with those of zebrafish *Danio rerio* and rainbow trout *Oncorhynchus mykiss* (sequence identity 64% in both cases). Basic and helix-loop-helix domians are boxed.

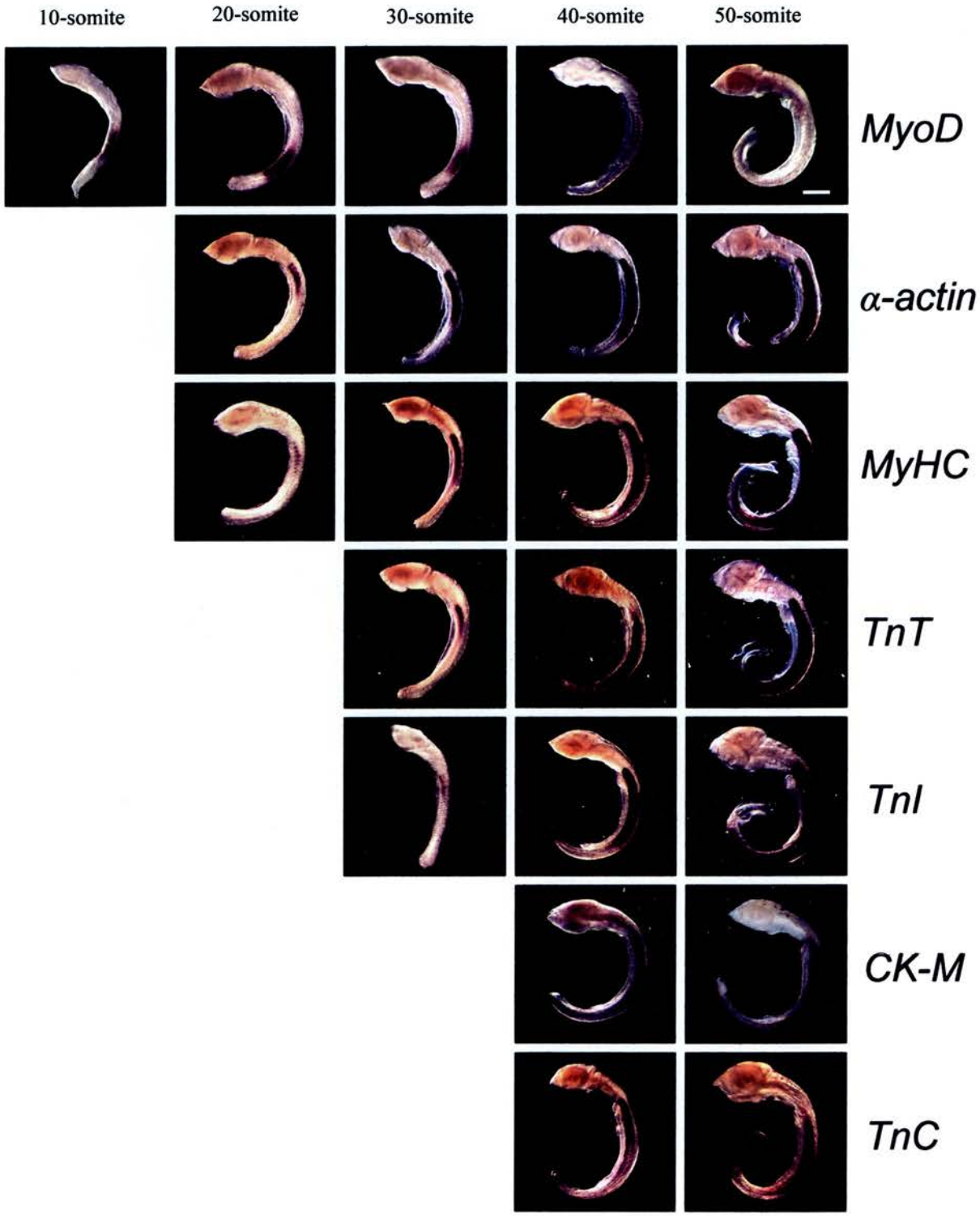


Fig. 10. Whole mount *in-situ* hybridization of MSP mRNAs in developing cod embryos at sequential somite stages at 4°C. Patterns at 7 and 10°C were identical (Fig. 1). Gene abbreviations are *MyHC - myosin heavy chain*, *TnT - troponin T*, *TnI - troponin I*, *CK-M - muscle creatine kinase*, *TnC - troponin C*. Scale bar - 200μm.

spanned 779bp of coding region and 89bp of UTR. The deduced amino acid sequence most closely matched that of chum salmon *Oncorhynchus keta* adult fast skeletal muscle MyHC exhibiting 88% sequence identity (Table 1). No specific functional domains were defined in this region (Fig. 4).

Troponin I exhibited the lowest identity with published sequences, 62% when compared with Atlantic salmon *Salmo salar*, and just 55% when compared to Atlantic herring (Fig. 5). The most highly conserved region within the TnI molecule was the actin/troponin C binding site located towards the center of the sequence. This binding site exhibited the motif characteristic of invertebrates (*K*PXL*K*) rather than that usually seen in vertebrates (*R*PXL*R*).

Troponin T showed 86% sequence identity with the Atlantic salmon isoform 3 (Table 1). A short region of low conservancy at the N-terminus (Fig. 6) was present in a position that spans several known splice sites in avian and mammalian TnTs (Smillie *et al.*, 1988).

Surprisingly, troponin C most closely matched the trout cardiac/slow isoform (Table 1) despite being expressed exclusively in the skeletal muscle and not in the heart (Fig. 10). Sequence identity was 83% with the trout cardiac/slow sequence and 81% with the *Xenopus* cardiac/slow sequence. The portion of the TnC sequence isolated incorporated the second, third and fourth Ca^{2+} binding sites (Fig. 8). Sites II and IV were highly conserved, exhibiting only 7 differences in 65 residues (89% identity) with the trout sequence. Site III showed less conservation, sharing 64% sequence identity with that of the trout.

The fast muscle creatine kinase clone was most closely related to that from Mozambique tilapia (*Oreochromis mossambicus*) showing 89% identity in deduced amino acid sequence (Table 1), and 87% with that of the zebrafish. It was possible to

recognize the active site motif CPSNLGT (Fig. 7), which is absolutely conserved between all known CK isoforms (Taylor *et al.*, 1990; Fritz-Wolf *et al.*, 1996).

When blast searched on the DDBJ protein-blast engine, the full-length cod MyoD amino acid sequence showed greatest identity with zebrafish MyoD (64%). The most conserved regions were the basic and the helix-loop-helix domains, with the extent of sequence identity declining towards the C-terminus (Fig. 9).

***In-situ* hybridization (ISH)**

mRNA signals for all of the MSP genes began in the most differentiated somites at the anterior of the embryo and progressed rostro-caudally, mirroring the pattern of somite formation (Fig. 10). The onset of expression of different genes was sequential, with *MyoD* being the first to be expressed in a single band in the presomitic mesoderm. Upon formation of somites, expression was seen in the posterior-most 7, plus a single band equivalent to the field of the next somite to be formed (Fig. 11). After approximately the 35-somite stage, expression faded, and somites continued to be added caudal to the anal pore, unstained for *MyoD* mRNA. However, RT-PCR analysis showed that transcripts were still present at the forty and fifty somite stages despite the lack of ISH staining (Fig. 12). No expression was seen in the adaxial cells adjacent to the notochord at any stage (Fig. 11).

Subsequent expression of the contractile/structural MSPs was correlated with myotube and myofibril synthesis (Figs. 1 and 2). Expression occurred throughout the fibres/myotubes and no localization of message was seen either within cells or within the myotome. Unfortunately it was not possible to deduce whether expression occurred differentially in the slow and fast muscle. Whilst slow muscle fibres were undoubtedly stained, their presence as a superficial monolayer meant that unspecific

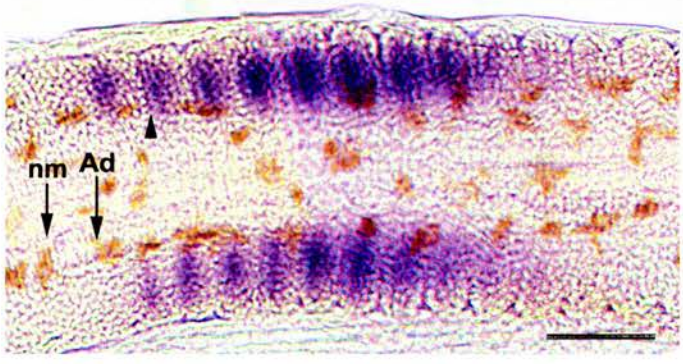


Fig. 11. *MyoD* expression in the developing somites of a 15-somite embryo. Expression can be seen the anterior-most 7 somites, and in a single band in the presomitic mesoderm at the site of imminent somite formation. The posterior-most somites are unstained for *MyoD*. Arrowhead, first somite furrow. nm, notochord/mesoderm boundary. Ad, unstained adaxial cells. Scale bar - 100μm.

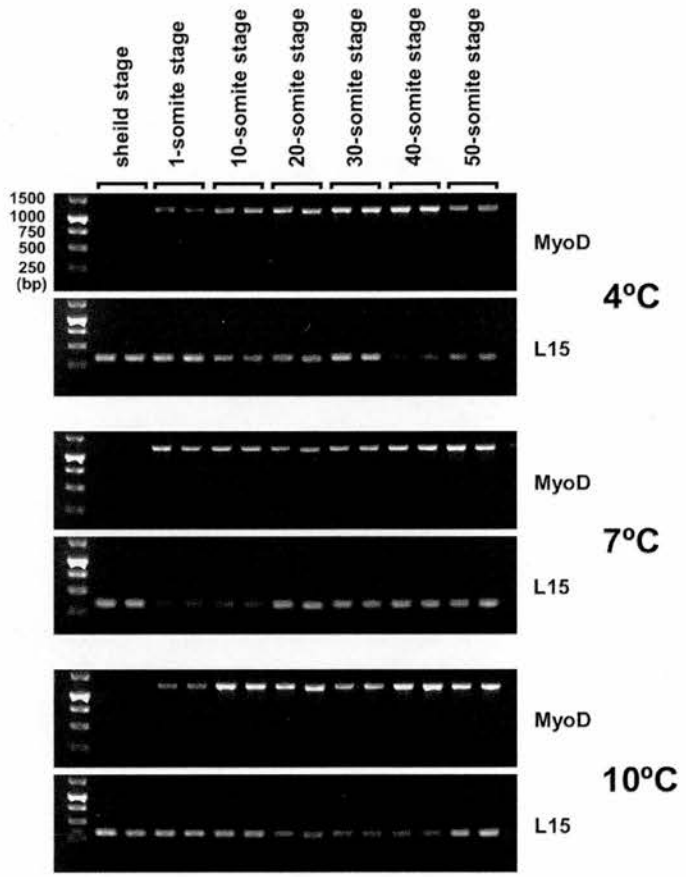


Fig. 12. RT-PCR analysis of *MyoD* expression in different stage embryos between temperature groups. At all temperatures, *MyoD* transcripts are switched on as the somites began to form. Note that despite the lack of in-situ staining for MyoD beyond approximately the 35-somite stage (Fig. 10), transcripts are still present. L15 is a 60S ribosomal subunit "housekeeping" gene, expressed at all temperatures and in all stages, for use as an internal standard.

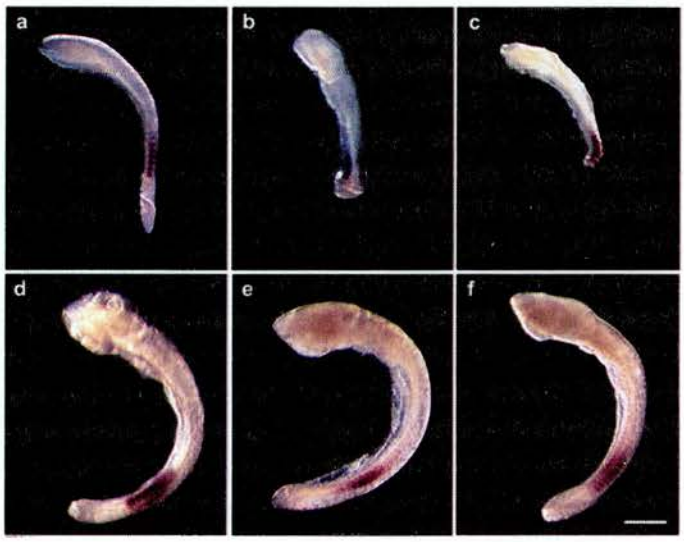


Fig. 13. *MyoD* expression in 5-somite (a-c) and 35-somite (d-f) embryos at 4, 7 and 10°C. Note that the 7 and 10°C embryos are shorter than the 4°C embryos, reflecting differences in the extent of epiboly between temperature groups (see chapter 3). However, the expression of *MyoD* in relation to somite development remains constant. Scale bar - 300µm.

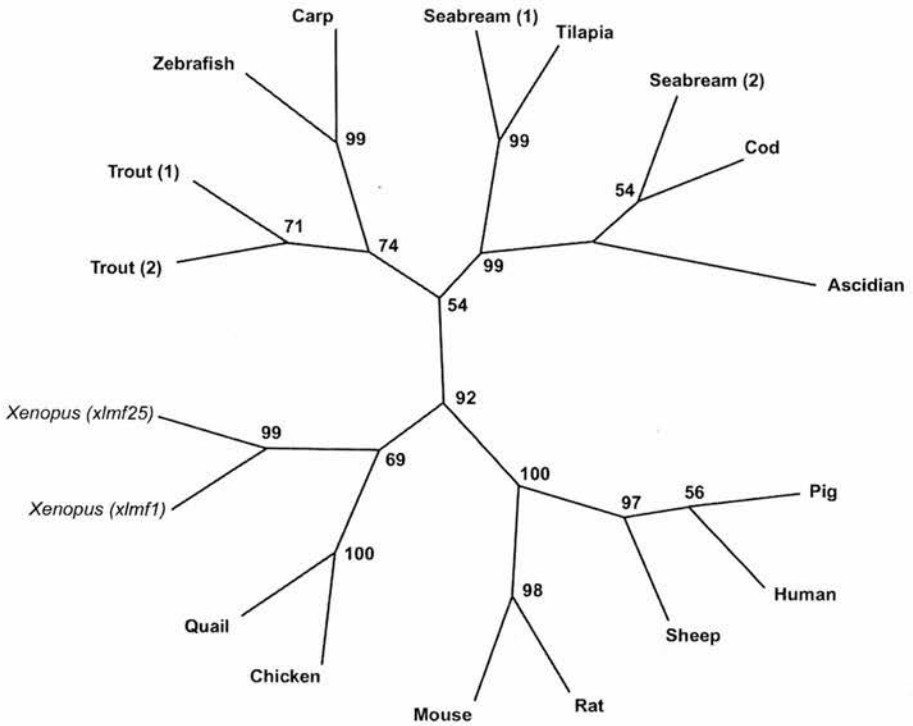


Fig. 14. Phylogenetic relationships of the vertebrate *MyoD* sequences published to date. Importantly, the cod sequence clusters with the seabream *MyoD2* sequence. Similarites are also evident in the in-situ expression patterns. The tree was constructed in PHYLIP (Felsenstein, 1995) by the neighbour joining method. Node numbers refer to the percentage of bootstrap trials supporting a clade. Bootstrap confidence is based on 1000 pseudoreplications. The amino acid sequence for the single myogenic factor (AMD1) of the ascidian *Halocynthia roretzi* (accession no. D13507) was used as the outgroup. Other accession numbers are seabream *Sparus aurata MyoD1* AF478568, *MyoD2* AF478569, blue tilapia *Oreochromis aureus* AF270790, Atlantic cod *Gadus morhua* AF329903, Pig *Sus scrofa* U12574, human *Homo sapiens* NM_002478, sheep *Ovis aries* X62102, rat *Rattus norvegicus* M84176, mouse *Mus musculus* XM_124916, chicken *Gallus gallus* L34006, Quail *Cotornix cotornix* L16686, *Xenopus laevis xlmf1* M31116, *xlmf25* M31118, rainbow trout *Oncorhynchus mykiss MyoD1* X75798, *MyoD2* Z46924, zebrafish *Danio rerio* NM_131262, common carp *Cyprinus carpio* AB012882.

Table 3. Regression data for the expression of MSP genes in relation to somite stage ($P < 0.001$ for all regressions). F values represent the results of analysis of co-variance (ANCOVA) between 4, 7 and 10°C groups. No significant interaction was found between temperature and expression in all cases ($P > 0.05$).

Expression of	Slope	Intercept	r^2 (adjusted)	F (slopes)	F (intercepts)	Residual DF
α-actin	1.42	-23.55	94.6	0.031	0.326	58
MyHC	1.55	-28.62	94.9	0.079	0.235	58
TnT	1.85	-48.64	94.4	1.064	0.358	58
TnI	1.93	-55.09	90.9	0.051	0.152	58
CK-M	2.01	-63.96	83.1	0.708	0.140	58
TnC	2.16	-71.15	83.8	0.204	0.724	58

Table 4. MANCOVA comparison of the slopes of the regression lines of expression between different MSP genes (all temperatures)

	MyHC	*TnT*	*TnI*	*CK-M*	*TnC*
α-act	NS	p<0.001	p<0.001	p<0.001	p<0.001
MyHC		p<0.05	p<0.01	p<0.001	p<0.001
TnT			NS	NS	NS
TnI				NS	NS
CK-M					NS

Table 5. MANCOVA comparison of the intercepts of the regression lines of expression between different MSP genes (all temperatures)

	MyHC	*TnT*	*TnI*	*CK-M*	*TnC*
α-act	NS	p<0.001	p<0.001	p<0.001	p<0.001
MyHC		p<0.001	p<0.001	p<0.001	p<0.001
TnT			p<0.01	p<0.001	p<0.001
TnI				p<0.001	p<0.001
CK-M					NS

bleed-through of products from the alkaline-phosphatase colour reaction was indistinguishable from any specific staining. Expression of all MSPs preceded development of organized myofibrils (Fig. 2).

No significant differences in expression of any genes were seen between embryos raised at different temperatures (Table 3, Fig. 1). Expression of *MyoD* was not scored against somite stage, due to it being expressed before the first somite formed, but the expression pattern remained the same at different temperatures nonetheless. In all cases the characteristic pattern of expression of a single stained band in the presomitic mesoderm followed by seven stained somites was observed. However, at the initiation of myogenesis the body axis of embryos incubated at 10°C was frequently slightly shorter than those incubated at lower temperatures, as a result of asynchrony between the extent of epiboly and segmentation in these fish (Fig. 13; chapter 3). RT-PCR analysis showed that no transcript was detectable at the embryonic shield stage, shortly prior to the initiation of myogenesis, but as segmentation began, expression of *MyoD* was switched on (Fig. 12). Furthermore, low-level expression persisted even after completion of formation of somites at the ~50-somite stage, when transcripts are no longer visible by *in-situ* hybridization (Fig. 10)

When the expression patterns of different MSP transcripts were compared with each other by MANCOVA, all were highly significantly different in terms of slope or intercept, with the exception of *MyHC*/*α-actin* and *TnC*/*CK-M* (Tables 4 and 5, Fig. 2)

Discussion

Despite the differences in fibre number shown previously (chapter 3), there was no difference in the relative timing of expression of the 7 MSPs studied between temperature groups. Although there are many additional MSPs involved in myofibrillar assembly, these data do not support the hypothesis that differences in fibre number are related to differences in the timing of expression of myofibrillar genes. However, the discrete, sequential patterns seen between most of the mRNAs reinforces the notion of muscle differentiation as a set of centrally regulated, concomitant but autonomous steps (Costa *et al.*, 2002).

A common feature of many muscle proteins is that they have multiple isoforms, which are generated either from separate genes or by alternate mRNA splicing from the same gene. This situation is complicated in fish by an ancestral whole-genome duplication event (Meyer and Schartl, 1999; Taylor *et al.*, 2001a), which is thought to have occurred after the radiation of the sarcopterygian lineage, which includes all terrestrial vertebrates. A second tetraploidization of the salmonid genome is also thought to have taken place more recently (Allendorf, 1984; Rescan and Gauvry, 1996). Evidence for genome duplication events is far from conclusive (see Taylor *et al.*, 2001a; Taylor *et al.*, 2001b; Robinson-Rechavi *et al.*, 2001a; Robinson-Rechavi *et al.*, 2001b), but whatever the reason, teleost genomes are characterized by expanded gene families (Robinson-Rechavi *et al.*, 2001c).

There are at least six different actin genes present in mammals the products of which are expressed in various and overlapping cell types. β- and γ- actins are constituents of the cytoskeleton and are ubiquitously expressed. A further four actins are specific to muscle cells. These include two striated muscle (α- skeletal and α-cardiac) and two smooth muscle (α-aortic and γ-enteric) actins (Vandekerckhove and

Weber, 1978; Vandekerckhove and Weber, 1979). Polymerized actin chains form the backbone of the thin filament, consisting predominantly of mixed α and β chains (Gordon *et al.*, 2000). Single cDNAs encoding skeletal muscle α-actin have been isolated from channel catfish *Ictalurus punctatus* (Kim *et al.*, 2000), common carp and goldfish (Watabe *et al.*, 1995a). However, in a recent study of the Japanese Pufferfish *Fugu rubripes*, Venkatesh *et al.* (1996) isolated nine distinct genomic actin clones. These were classified as two skeletal α-actins, three α-cardiac actins, one testis-type α-actin, two β-actins and one vascular β-cytoplasmic actin. The two skeletal muscle types had identical genomic organization and differed in only five amino-acid residues. Such high sequence identity is a common feature of actins, even between distantly related species. This was consistent with the finding that cod skeletal *α-actin* exhibited 100% deduced amino acid sequence identity with that of Alaskan pollack and rat-tail fish (Fig. 3). The expression pattern was also comparable with that of zebrafish skeletal *α-actin* (Xu *et al.*, 2000), being switched on shortly after the onset of somitogenesis and before the expression of *MyHC*.

The MyHCs of vertebrates are encoded by a multi-gene families and are expressed in a tissue-specific manner (Konig *et al.*, 2002). It has been reported that there are at least eight skeletal and two cardiac MyHCs in humans (Soussi-Yanicostas *et al.*, 1993). However, in the common carp *Cyprinus carpio* over 29 different genomic sequences have been identified (Kikuchi *et al.*, 1999). Different *MyHC* mRNA isoforms in teleosts have been shown to be expressed under different conditions of temperature (Imai *et al.*, 1997), at different stages of development (Ennion *et al.*, 1999) and in different fibre types (Rescan *et al.*, 2001). In the present study, no difference in *MyHC* expression was seen with different rearing temperature. It is possible that the probe used in this study bound heterologously to multiple *MyHC*

mRNA isoforms, since the proportion of 3' UTR:cds was much greater in this case than with the other clones used (89bp:779bp respectively). It is equally possible that the probe hybridized to a specific isoform, which showed a broad pattern of expression.

Troponin (Tn), is an actin associated protein complex, consisting of three interacting subunits, each receiving its identifying letter from the first identified property: troponin C (TnC) binds Ca^{2+}, troponin I (TnI) binds to actin and inhibits the actomyosin ATPase, and troponin T (TnT) links the Tn complex to tropomyosin (Gordon *et al.*, 2000). Three *TnT* genes exist in mammals, encoding fast, slow and cardiac forms (Huang *et al.*, 1999). In the mouse, these are alternatively spliced into at least 13 fast isoforms (Wang and Jin, 1997) and 3 slow isoforms (Jin *et al.*, 1998). Splicing usually occurs in proximity to the N-terminus and different splice variants are thought to be involved in ontogenetic changes in phenotype and in different fibre-typing in the adult (Briggs and Schachat, 1996). Five TnT protein isoforms have been isolated from Atlantic salmon, two from slow muscle and three from fast muscle (Waddleton *et al.*, 1999). A single fast muscle cDNA has been isolated from the zebrafish (Xu *et al.*, 2000). The *TnT* isoform isolated from cod showed greatest identity with an Atlantic salmon fast muscle isoform. The most degenerate area of sequence was at the N-terminus, in a region spanning several known splice sites in mammals (Smillie *et al.*, 1988).

As with *TnT*, the *TnI* isoform found in this study showed greatest identity with an Atlantic salmon fast muscle isoform. Three genes code for slow, fast and cardiac isoforms of TnI in birds and mammals (Guenet *et al.*, 1996; Mullen and Barton, 2000). Much less is known about TnI in fish. Three cDNAs have been isolated from salmon (Jackman *et al.*, 1998) and one from herring (Hodgeson *et al.*, 1996).

Surprisingly, however, in each case the amino acid sequence identity was considered too low to assign orthology to either the fast, slow or cardiac varieties seen in other vertebrates. In addition, the actin/TnC binding site contained a motif peculiar to invertebrates (*K*PXL*K*) rather than that seen in avian and mammalian species (*R*PXL*R*). This same, invertebrate-type motif was also seen in the cod sequence found in this study (Fig. 5). The significance of this finding is unknown, but it appears that TnI is more heterologous in fishes than in other vertebrates. At the protein level too, Crockford *et al.* (1991) resolved two different isoforms of TnI being co-expressed in the fast fibres of *Oreochromis niloticus* and *O. andersoni*, using 2d gels and affinity chromatography. No genomic, cDNA or proteomic information on TnI has yet been presented for the zebrafish.

Troponin C exists as two distinct tissue-specific types rather than the three exhibited by the other Tn sub-units. In birds, mammals, and amphibians, a fast-fibre type, and a type specific to both slow and cardiac fibres have been identified (Reinach and Karlsson, 1988; Gahlmann and Kedes, 1990; Parmacek *et al.*, 1990; Jin *et al.*, 1995; Tiso *et al.*, 1997; Warkman and Atkinson, 2002). Numerous studies have investigated aspects of TnC function in teleosts at the protein level (Demaille *et al.*, 1974; McCubbin *et al.*, 1982; Gerday *et al.*, 1984; Feller *et al.*, 1989; Francois *et al.*, 1997), but until now the only published nucleotide sequences were those from zebrafish (showing highest identity to *Xenopus* fast-type; Xu *et al.*, 2000) and trout (showing highest identity to *Xenopus* slow/cardiac-type; Moyes *et al.*, 1996). Because protein sequences with homology to both fast-skeletal and slow/cardiac forms have been isolated from fish, it has been supposed that their nature is equivalent to those in birds and mammals (Yuasa *et al.*, 1998). Importantly however, both types have never been isolated from a single species. In this study, the cod *TnC* sequence showed

highest identity with the trout cardiac form, but was only expressed in the myotomal muscle, and not in the heart. Tissue specific expression has not been investigated in the trout, but the timing of expression shown in this study was quite different to that of the zebrafish fast-type *TnC* shown by Xu *et al.* (2000). In zebrafish, fast-type *TnC* was one of the first MSPs to be switched on, whereas in the cod, expression occurred last out of the seven MSPs, towards the end of somitogenesis. It may be therefore, that TnC expression in teleosts is more complex than previously thought, as has been demonstrated in the case of TnI.

Fast skeletal muscle TnC contains four Ca^{2+} binding sites, which facilitate conformational changes in the protein according to calcium concentration. Sites I and II, in close proximity to the N-terminus, have a lower Ca^{2+} affinity than sites III and IV, which are located towards the C-terminus. In the cardiac/slow form of TnC, site I is non-functional. The nucleotide sequence isolated from cod did not cover site I, so comparison of this region could not be made with fast and slow isoforms from other species. However, binding sites II and IV were highly conserved, sharing 89% identity with the trout sequence. Site III was less highly conserved, sharing 64% sequence identity with the trout sequence.

In cells and tissues with intermittently high and fluctuating energy requirements, including skeletal muscle, creatine kinase plays a central role in the catalysis of the reversible transfer of a phosphate ion from phosphorylcreatine to ADP to form high energy ATP (Walliman *et al.*, 1992). Creatine kinase enzymes constitute a family of different isoforms with tissue-specific expression and iso-enzyme specific subcellular localization (Stolz and Walliman, 1998). Four isoform types are present in all vertebrates, a cytosolic brain-type (CK-B), a cytosolic muscle-type (CK-M) and two mitochondrial types, CK-MiA and CK-MiB (Benfield *et al.*, 1984; Ordahl *et al.*,

1984; Sun *et al.*, 1998). The genetic organization of the creatine-kinase enzymes has been little studied in any species, although it is known that three sub-isoforms of CK-M are encoded by three different genes in the common carp (Sun *et al.*, 1998) and that at least two *CK-M* genes exist in the zebrafish (Harder and McGowan, 2001) and in the channel catfish (Liu *et al.*, 2001). Only one *CK-M* gene has been identified in any mammalian species and it has been argued that the multiple copies found in teleosts play a role in overcoming the rate-depressing effect of seasonal cooling, and therefore help to retain muscle function over a broad temperature range (Sun *et al.*, 1998). The number of *CK-M* encoding genes in the cod is not known, but the *CK-M* mRNA isoform examined in this study did not show any change in its timing of expression with respect to somite stage, between different rearing temperatures.

There is an apparent paradox regarding the myofibrillar proteins, in that despite the large number of splice variants found, and the enormous number theoretically possible (Miyadzaki *et al.*, 1999), only a relatively small number of protein isoforms have been isolated (Yao *et al.*, 1992). This could be for several reasons; it might be that the differences in protein structure are so subtle as to not be resolved by currently available techniques, or that many proteins are inefficiently transcribed or not stably incorporated into the myofibrils. Alternatively, the explanation could simply be that isoform diversity is not so important at the level of the protein. Duplication of genes at the genomic level might allow them to be placed under different conditions of transcriptional control, and divergence in mRNA primary structure might be a prerequisite for differential timing and maintenance of translation.

Expression of *MyoD* in the cod was very unusual in that the expression pattern appeared to be more limited than that shown previously in the zebrafish (Weinberg *et*

al., 1996) and in the herring (Temple *et al.*, 2001). *MyoD* was not expressed in the adaxial cells adjacent to the notochord, and was undetectable with ISH after approximately the 35-somite stage (Fig. 10). It is well known that two paralogues of *MyoD* exist in the salmonids, apparently produced from a recent tetraploidization of the salmonid genome (Rescan and Gauvry, 1996), and that these genes have diverged in function (Delalande and Rescan, 1999). However, two non-allelic *MyoD* genes have recently been cloned from the (non-salmonid) gilthead seabream *Sparus aurata* (Tan and Du, 2002). In this case *MyoD2* transcripts are much more restricted in their expression pattern than *MyoD1*. To test the hypothesis that the cod *MyoD* clone was an orthologue of seabream *MyoD2*, a neighbour joining phylogenetic tree was constructed in PHYLIP (Felsenstein, 1995), using all available full-length vertebrate *MyoD* sequences. Whilst the two trout paralogues clustered together, the seabream paralogues were more highly divergent. Importantly, seabream *MyoD2* and the cod *MyoD* sequence were more similar to each other than to any other sequence (Fig. 14).

In summary, muscle development in the Atlantic cod is canalized over the range of temperature studied (4-10°). Although the number of fibres has been shown to differ between temperature groups (chapter 3), the relative timing of muscle development and expression patterns of the myofibrillar mRNAs is independent of temperature. Myofibrillar genes are activated asynchronously and follow a distinct temporal order during myogenesis and a potentially exciting prospect is the application of these and related cDNAs to the characterization of teleost embryos from pelagic sampling studies. Surveys of fish egg abundance have been used to estimate spawning biomass in stock assessments, and require the determination of the age distributions and mortality rates of eggs (Armstrong *et al.*, 2001). It is suggested

that the timing of MSP gene expression using ISH could be used as the basis of a convenient species-specific method of identification and staging.

Chapter 5: Temperature and the expression of myogenic regulatory factors (MRFs) and myosin heavy chain isoforms during embryogenesis in the common carp *Cyprinus carpio* L.

Introduction

The myogenic regulatory factors, (MRFs) are a family of basic helix-loop-helix (bHLH) transcription factors essential to the specification and determination of the muscle cell lineage. The four members, *MyoD*, *Myf-5*, *myogenin* and *MRF4* are characterized by their ability to induce myogenic conversion in a variety of cell types including fibroblasts, neurons, adipocytes, chondrocytes and melanocytes (reviewed by Edmondson and Olsen, 1993; Arnold and Braun, 2000). The bHLH domain is central to the role of transcriptional activation and is highly conserved, with the four proteins sharing approximately 80% amino acid sequence identity in this region within species (Edmondson and Olsen, 1993). The HLH region is characterized by two amphiphatic α-helices, separated by an unstructured intervening loop. HLH regions are mutually attractive, and facilitate the formation of functionally active protein dimers (Maleki *et al.*, 2002). The basic region forms an extension of one of the α-helices of the HLH region, and facilitates DNA binding. bHLH dimers specifically bind E-box elements (CANNTG) found in the promoters and enhancers of most, if not all muscle specific genes (Apone and Hauschka, 1995; Spinner *et al.*, 2002) although it is likely that nucleotide variation in the flanking regions and within the motif imparts some specificity (Ludolph and Konieczny, 1995).

During myogenesis, *Myf-5* and *MyoD* are required for the initial determination of the myogenic lineage. Gene knockout studies in mice show that lack of *MyoD* and *Myf-5* results in failure of myoblast formation, and a consequent lack of all head and trunk skeletal muscle (Rudnicki *et al.*, 1993). *Myogenin* and *MRF4* are activated during myoblast differentiation (Edmonson and Olson, 1989; Rhodes and Konieczny, 1989; Wright *et al.*, 1989; Miner and Wold, 1990; Pownall *et al.*, 2002), and likely have cooperative functions with *MyoD* and *Myf-5* as transcription factor regulators for the activation of muscle contractile protein genes (Lassar, 1991). When *myogenin* is "knocked out" in mice, myoblasts form in the correct place but do not fuse into muscle fibres (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993; Venuti *et al.*, 1995). The function of *MRF4* is less clear because in all three mutants constructed to inactivate it, *Myf-5* expression is also affected (Olson *et al.*, 1996; Summerbell *et al.*, 2000; Summerbell *et al.*, 2002).

In the zebrafish *Danio rerio*, *Myf-5* and *MyoD* are initially expressed at approximately 7.5 hours at 28.5°C (80% epiboly) in bilateral bands of cells flanking the presumptive notochord (Weinberg *et al.*, 1996; Coutelle *et al.*, 2001; Chen *et al.*, 2001). Their expression patterns overlap considerably, incorporating the adaxial cells as they form. Expression of *Myf-5* extends further into the presomitic mesoderm than that of *MyoD*, but strikingly, as the adaxial cells become incorporated into the somites, *Myf-5* expression dramatically declines. Expression of *MyoD* persists in the differentiated somites until much later, after they become chevron-shaped, when it is down-regulated. Expression of *myogenin* begins at 10.5h (at 28.5°C) in a subset of the *MyoD*/*Myf-5* expressing cells (Weinberg *et al.*, 1996; Chen *et al.*, 2000). *Myogenin* transcripts first appear in bands of cells extending laterally away from the adaxial cells. However, this lateral extension of expression is narrower than in the case of

MyoD, and due to its later onset, first expression is within the somites rather than the presomitic mesoderm. *Myogenin* expression is also transient, and persists until shortly after the disappearance of *MyoD* expression. Furthermore, there are some differences in MRF expression between fish species. In the rainbow trout *Oncorhynchus mykiss* for instance, *MyoD* expression, rather than spreading laterally, remains confined to the medial domain of the somite for a prolonged period (Delalande and Rescan, 1999). In the herring *Clupea harengus*, *myogenin* expression shows a more transient expression pattern than that seen in zebrafish (Weinberg *et al.*, 1996) and trout (Delalande and Rescan, 1999), disappearing from the somites before the down-regulation of *MyoD* (Temple *et al.*, 2001). A number of species including the trout (Rescan and Gauvry, 1996), gilthead seabream *Sparus aurata* (Tan and Du, 2002) and *Xenopus laevis* (Scales *et al.*, 1990; Scales *et al.*, 1991; Charbonnier *et al.*, 2002) also possess multiple copies of one or more MRF gene.

Temperature has been shown to influence many aspects of development in teleosts including muscle cellurity (Stickland *et al.*, 1988; Nathanailides *et al.*, 1995; Johnston and McLay, 1997; Matschak *et al.*, 1998; Vieira and Johnston, 1992; Galloway *et al.*, 1998; Galloway *et al.*, 1999b; chapter 3, this thesis) and the relative timing of myofibrillogenesis (Johnson *et al.*, 1995; Johnston *et al.*, 1996; Johnston *et al.*, 1997). There is also a small body of evidence to suggest the timing and extent of MRF expression varies with temperature. Xie *et al.* (2001) found that *MyoD* and *myogenin* were expressed in a greater number of somites in trout embryos of the same developmental stage, reared at 12°C compared to 4°C. This change in expression was apparently concomitant with a "relatively advanced" state of muscle development at 12°C compared to 4°C. Similarly, Wilkes *et al.* (2001) used quantitative northern blots to show that *MyoD* and *myogenin* mRNA levels in trout and sea bass *Dicentrarchus*

labrax were highest at temperatures close to those of the usual environmental spawning temperatures for the species. In contrast, Temple *et al.* (2001) found no difference in the timing of *MyoD* or *myogenin* expression in herring embryos reared at 5, 8 and 12°C. In chapter 4, there was also no difference in the timing of *MyoD* expression between Atlantic cod *Gadus morhua* embryos reared at 4, 7 and 10°C, although the timing of blastopore closure relative to somite stage was relatively delayed at 7 and 10°C when compared to 4°C, and the number of deep fibres at hatching in the 10°C group was significantly higher than in the lower temperature groups.

Fishes from cold environments express *MyHC* types with a higher specific myofibrillar ATPase activity and a lower thermal stability than those from warmer environments (Johnston *et al.*, 1973; Johnston *et al.*, 1975a; Johnston *et al.*, 1975b), and there is an apparent trade off between these traits. Species with a broad temperature tolerance, such as the goldfish *Carassius auratus* and the common carp *Cyprinus carpio* can alter their Mg^{2+} Ca^{2+} ATPase activity depending on the ambient temperature, by differential expression of multiple *MyHC* genes (Goldspink *et al.*, 1992, Watabe *et al.*, 1995b; Imai *et al.*, 1997; Cole and Johnston, 2001). The control of such acclimation responses is unknown, and to date has not been demonstrated in embryos, which express many of their own developmental-stage specific isoforms of muscle proteins (Scapolo *et al.*, 1988; Crockford and Johnston, 1993; Johnston *et al.*, 1997). In mammals, there is evidence for involvement of the MRFs in the determination of contractile protein isoform expression and fibre typing (Voytik *et al.*, 1993; Hughes *et al.*, 1999) alongside other influences such as hormones and innervation (Hughes *et al.*, 1993; Lefeuvre *et al.*, 1996).

The common carp is a eurythermal species commonly inhabiting waters which fluctuate between near-freezing and 30°C seasonally (Micheals, 1988). Spawning occurs in the summer months at a minimum temperature of ~18°C, and the eggs and larvae develop normally between temperatures of 18 and 25°C (Penáz *et al.*, 1983). In the current study, the spatial and temporal expression patterns of *MyoD*, *myogenin*, and *Myf-5* were characterized, and the hypothesis that temperature influences expression of the MRFs within the normal limits of thermal tolerance was investigated by comparing embryos and larvae reared at 18 and 25°C. The expression pattern of *Myf-5* was of particular interest since within the Teleostei, to date it has only been described in the zebrafish, and has never been investigated in relation to temperature. In addition, the expression of five different *MyHC* transcripts (2 embryonic types, [Ennion *et al.*, 1999], and 3 temperature-specific types, [Imai *et al.*, 1997]) were characterized and compared between temperature groups. The aims of this study were firstly to investigate whether embryonic isoforms are differentially expressed in response to rearing temperature, secondly, to characterize the timing of expression switching from embryonic to adult isoforms, and thirdly to investigate the initial expression of temperature-specific *MyHC* isoforms in larvae. Since many MRF cDNAs from teleosts have been cloned in recent years, and parologous genes have been identified, a comprehensive phylogeny of vertebrate MRFs was also undertaken. Neighbour-joining and parsimony analyses were used to generate phylogenies, in order to elucidate evolutionary relationships between the genes and the relative timing of gen(om)e duplication events.

Materials and Methods

Spawning and Larval Rearing

Carp spawning and egg incubation were carried out according to Michaels (1988). Briefly, over-wintering adult carp were brought into the laboratory in early January 2000. The water temperature was raised by 3°C per day from 4°C to 25°C, where it was held for a further 6 weeks. Female fish were given 0.6mg kg^{-1} of carp pituitary acetone powder (Sigma) by intramuscular injection, followed by 3mg kg^{-1} twelve hours later. Males were given a single injection of 1.5mg kg^{-1}. After a further 12 hours, eggs and milt were stripped into separate dry containers. They were mixed in the ratio 1:100 (v/v) milt:eggs, and activated with an equal volume of 0.3% urea, 0.3% NaCl. The fertilization reaction was allowed to proceed for 1h, after which the eggs were washed 3 times in 0.5% (v/v) tannic acid to prevent aggregation. Fertilized eggs were transferred to Zuger jars and incubated under constant aeration at 18°C and 25°C ± 1°C (range). Embryos were sampled every 6h by anaesthetizing in 0.1% (m/v) tricaine (MS-222), puncturing the chorion with a hypodermic needle, and fixing in 4% (m/v) paraformaldehyde in phosphate buffered saline (PBS). After 12h of fixation the embryos were washed once in PBS and stored at -80°C in 100% methanol.

Plasmid clones and cRNA probes

The *MyoD*, *myogenin* and *Myf-5* clones used were supplied by Professor Shugo Watabe (University of Tokyo) and are described by Kobiyama *et al.* (1998). 10°C-type, intermediate-type, and 30°C-type *MyHC* were also supplied by Professor Watabe and are described by Imai *et al* (1997). The two embryonic-type *MyHC* clones (*Eggs22* and *Eggs24*) were supplied by Professor Geoff Goldspink (Royal Free and University College Medical School) and are described by Ennion *et al.* (1999).

DIG-labelled cRNA probes were constructed from linear plasmids as in chapter 4. Details of plasmids, restriction endonucleases and transcriptases are shown in Table 1.

In-situ hybridization

Five embryos of equivalent developmental stages from each sample were selected per cRNA probe. *In-situ* hybridization was carried out using the procedure described in chapter 4. Photographs were taken on a Leica MZ7.5 binocular microscope using darkfield illumination using a Ziess Axiocam imaging system.

RNA dot-blotting

Total RNA was extracted from the trunk muscle of hatched larvae (the head, tail and yolk sac were removed) using Tri-reagent. RNA dot-blots were performed by spotting 2.5μg of total RNA in 0.5μl water onto nitrocellulose (Hybond-N^+), and fixing at 120°C in an oven for 30 mins. A 30 min prehybridization was carried out in 50% (v/v) formamide, 0.1% (m/v) N-lauroylsarcosine, 0.02% (m/v) SDS, 2% (v/v) sheep serum at 65°C, before addition of probe at 100ng/ml. After hybridization overnight at 65°C, the blots were washed 2×15 min in 2×SSC, 0.1% (m/v) SDS at room temperature, followed by 2×15 min in 0.5×SSC, 0.1% SDS at 65°C. Membranes were blocked in 1% (v/v) sheep serum, 100mM maleic acid, 150mM NaCl, pH 7.5 for 1h, before addition of an alkaline phosphatase conjugated anti-DIG antibody (Roche) at a dilution of 1/100,000. After a 30 min incubation in the antibody solution, membranes were washed 2×15 min in 100mM maleic acid, 150mM NaCl, pH 7.5, 0.3% (v/v) Tween-20. Detection was achieved using a 1:100 dilution of the chemiluminescent substrate CSPD (Roche), in 100mM Tris-HCl, 100mM NaCl, pH 9.5 followed by exposure to X-ray film.

Table 1. Details of myogenic regulatory factor and myosin heavy chain clones, and modifying enzymes used.

Gene name	GenBank accession no.	Clone length (nt)	Plasmid	Sense endonuclease	Antisense endonuclease	Sense Transcriptase	Antisense Transcriptase
MyoD	AB012882	1221	pBluescript SK$^-$	XhoI	SpeI	T3	T7
Myogenin	AB012881	855	pBluescript SK$^-$	NotI	XhoI	T7	T3
Myf-5	AB012883	500	pBluescript SK$^-$	NotI	XhoI	T7	T3
MyHC Eggs22	AJ009735	161	pBluescript$^+$	EcoRI	HindIII	T3	T7
MyHC Eggs24	AJ009734	152	pBluescript$^+$	EcoRI	HindIII	T3	T7
MyHC 10℃-type	D50474	232	pBluescript SK$^-$	NotI	XhoI	T7	T3
MyHC Intermediate-type	D50475	523	pBluescript SK$^-$	NotI	XhoI	T7	T3
MyHC 30℃-type	D50476	254	pBluescript SK$^-$	NotI	XhoI	T7	T3

Phylogenetic Analysis of MRF sequences

A phylogenetic analysis was undertaken using full-length amino acid sequences of vertebrate MRFs taken from the GenBank database (NCBI, Bethesda, USA). An initial multiple alignment was constructed using the Clustal algorithm in Lasergene (DNAstar Inc.) which was then improved by eye. A neighbour-joining (NJ) tree was constructed in PHYLIP (Felsenstein, 1995) and bootstrapped 1000 times to provide statistical support. Parsimony analysis was carried out using PAUP (Swofford, 2002).

Results

Somitogenesis began almost immediately following epiboly as described by Verma *et al.* (1970) and Penáz *et al.* (1983), at 22 hours in the 18°C group and 12 hours in the 25°C group. Somites were formed at ~1 per hour (18°C) and ~2 per hour (25°C) to a final number of 38 or 39 (both groups). Time until 50% hatching was 120h at 18°C and 55h at 25°C. At hatching, embryos measured 3.72 ± 0.39mm (st. dev.), and there was no significant difference between temperature groups (t-test, $P > 0.05$, n = 20 fish per group)

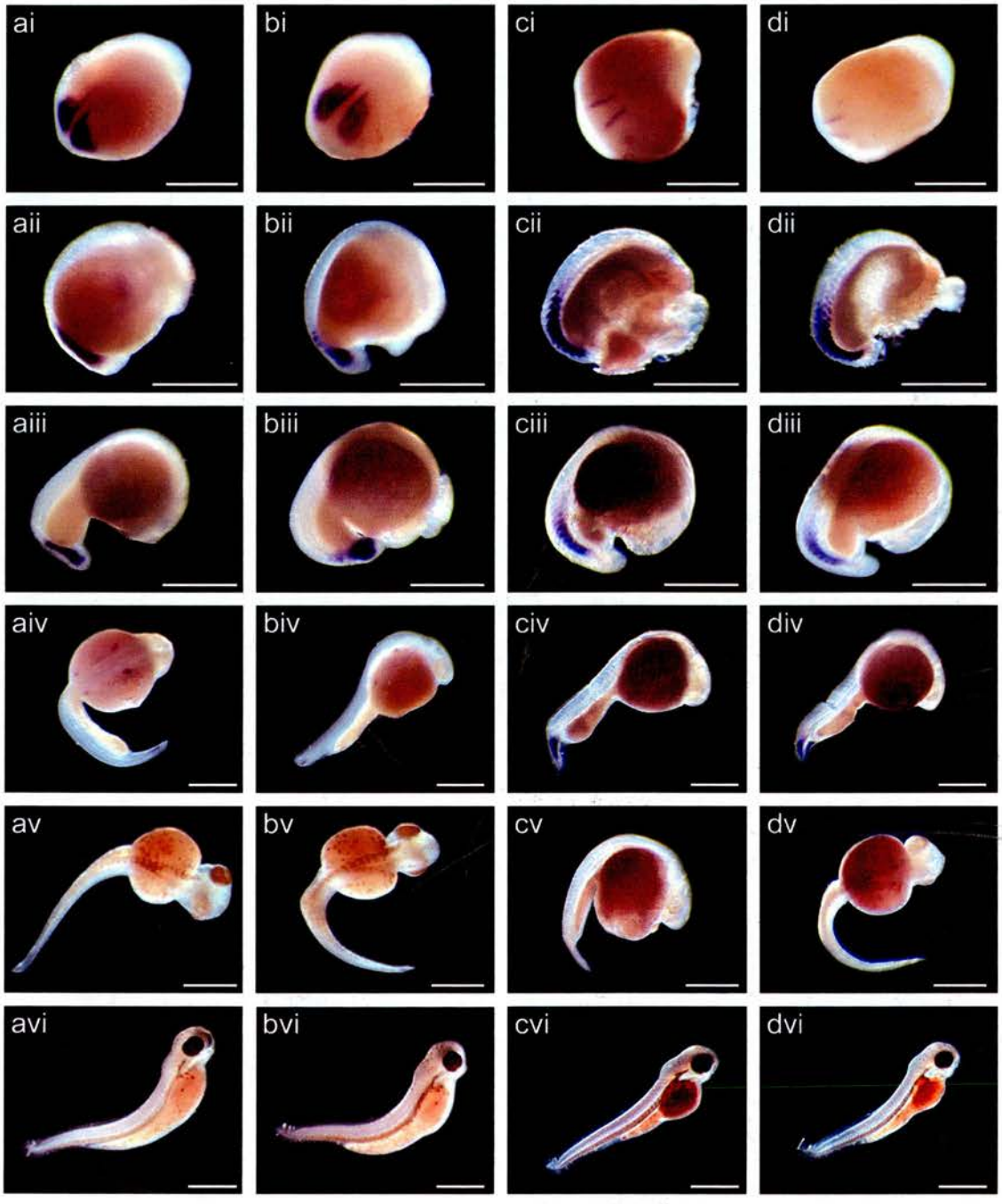
Expression of *MyoD* and *Myf-5* occurred simultaneously following epiboly in the pre-somitic mesoderm. *MyoD* was expressed in a pair of bilaterally symmetrical strips corresponding to the position of the adaxial cells (Fig. 1ci, 1di), adjacent to the notochord. *Myf-5* was also expressed in the adaxial cells, but as development proceeded, transcripts spread further laterally into the mesoderm. Prior to the appearance of the first somite furrows, *Myf-5* expression could be seen very faintly in 2 bands corresponding to the cellular fields of the first somites (Fig. 2a). As soon as each somite formed however, expression of *Myf-5* was down-regulated. In contrast,

Fig. 1. (Opposite and overleaf). Expression of myogenic regulatory factors and embryonic *myosin heavy chain* isoforms in common carp embryos reared at 18 and 25°C during development. Scale bars – 1mm.

a. *Myf-5* 18°C
b. *Myf-5* 25°C
c. *MyoD* 18°C
d. *MyoD* 25°C
e. *Myogenin* 18°C
f. *Myogenin* 25°C
g. *MyHC Eggs 22* 18°C
h. *MyHC Eggs 22* 25°C
i. *MyHC Eggs 24* 18°C
j. *MyHC Eggs 24* 25°C

Roman numerals indicate different stages of development

i Completion of epiboly, prior to somite formation
ii ~15-somite stage
iii ~23-somite stage
iv ~30-somite stage
v completion of somitogenesis (38 or 39 somites)
vi recently hatched larvae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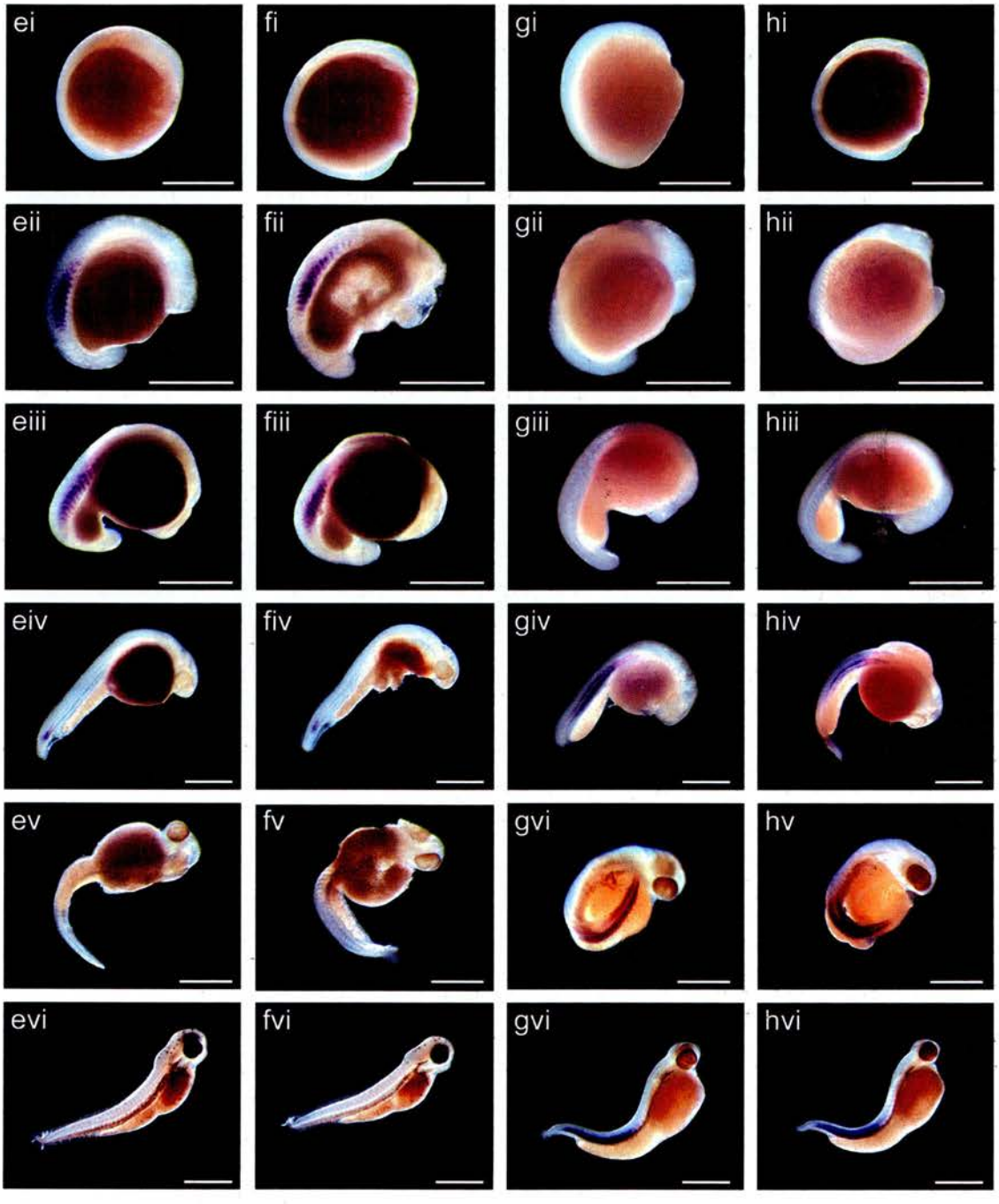
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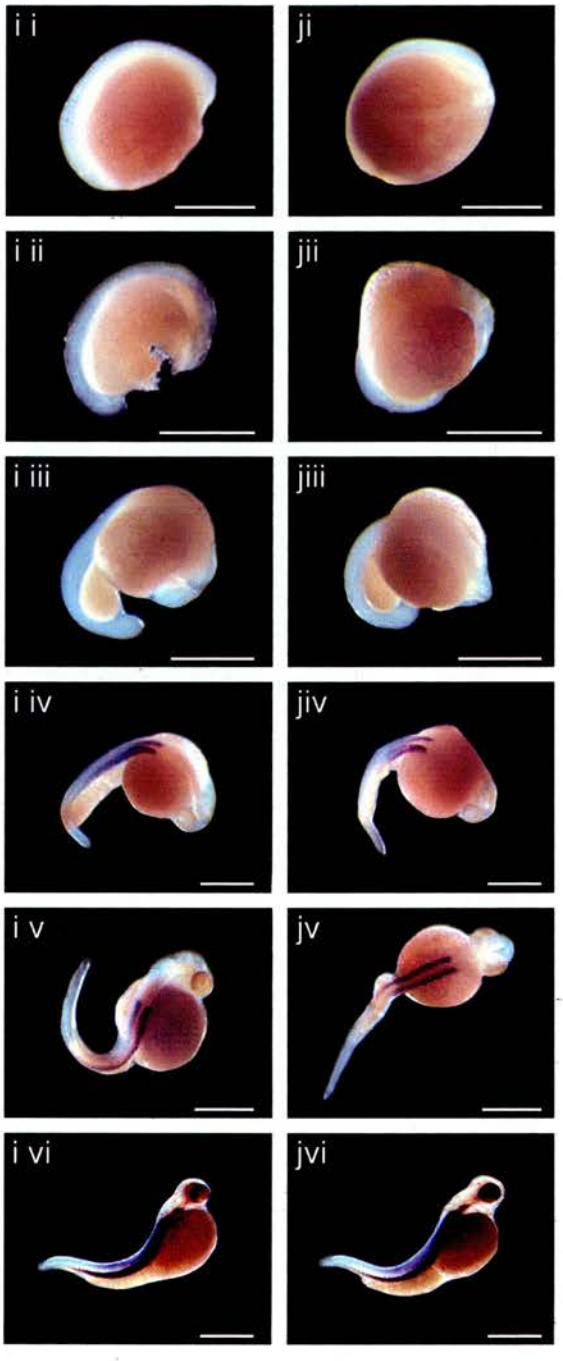
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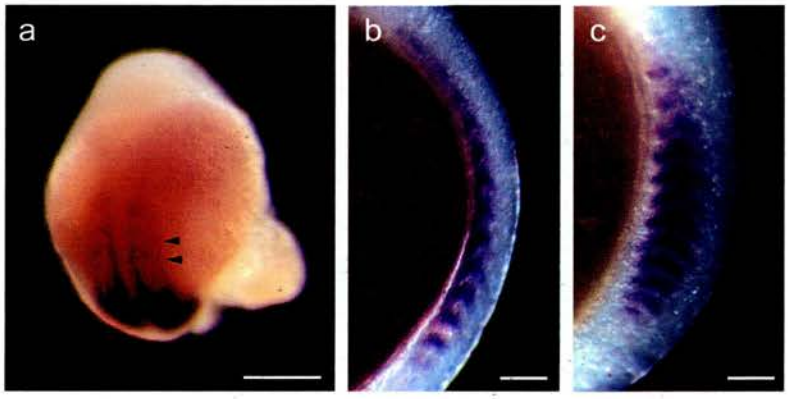


Fig. 2a. *Myf-5* expression in two presomitic bands immediately prior to the onset of somitogenesis. Scale bar - 500μm

Fig 2b. *MyoD* expression in the first ~12 somites. Scale bar - 100μm.

Fig 2c. *Myogenin* expression in ~12 somites (17-somite stage embryo). Scale bar - 100μm.

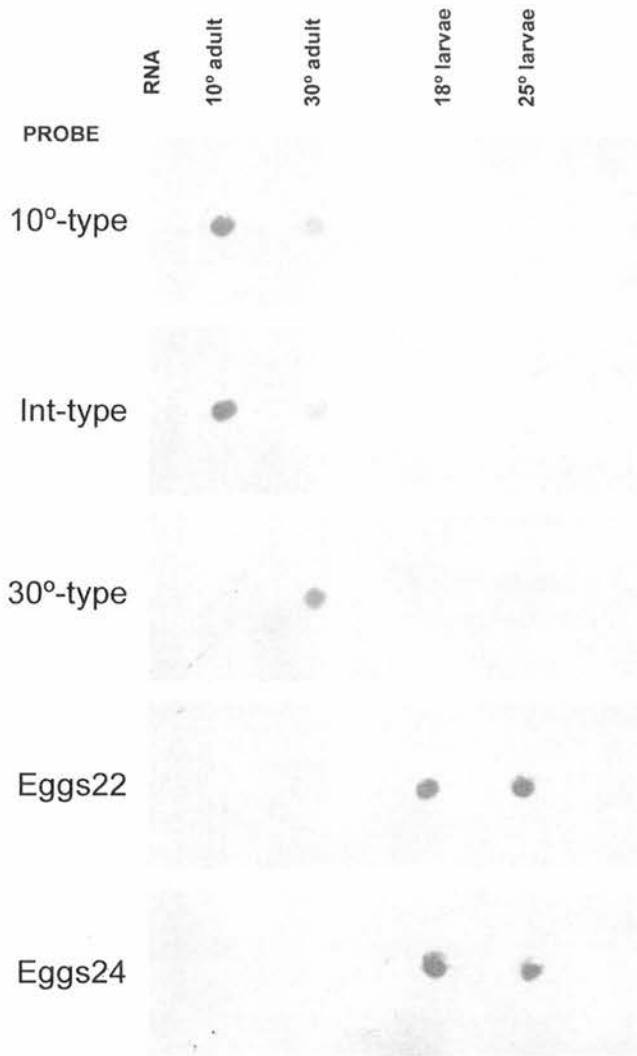


Fig. 3. RNA dot blots showing expression of myosin heavy chain isoforms in larvae grown at 18°C and 25°C, and in adult (10cm) fish acclimated to 10°C and 30°C

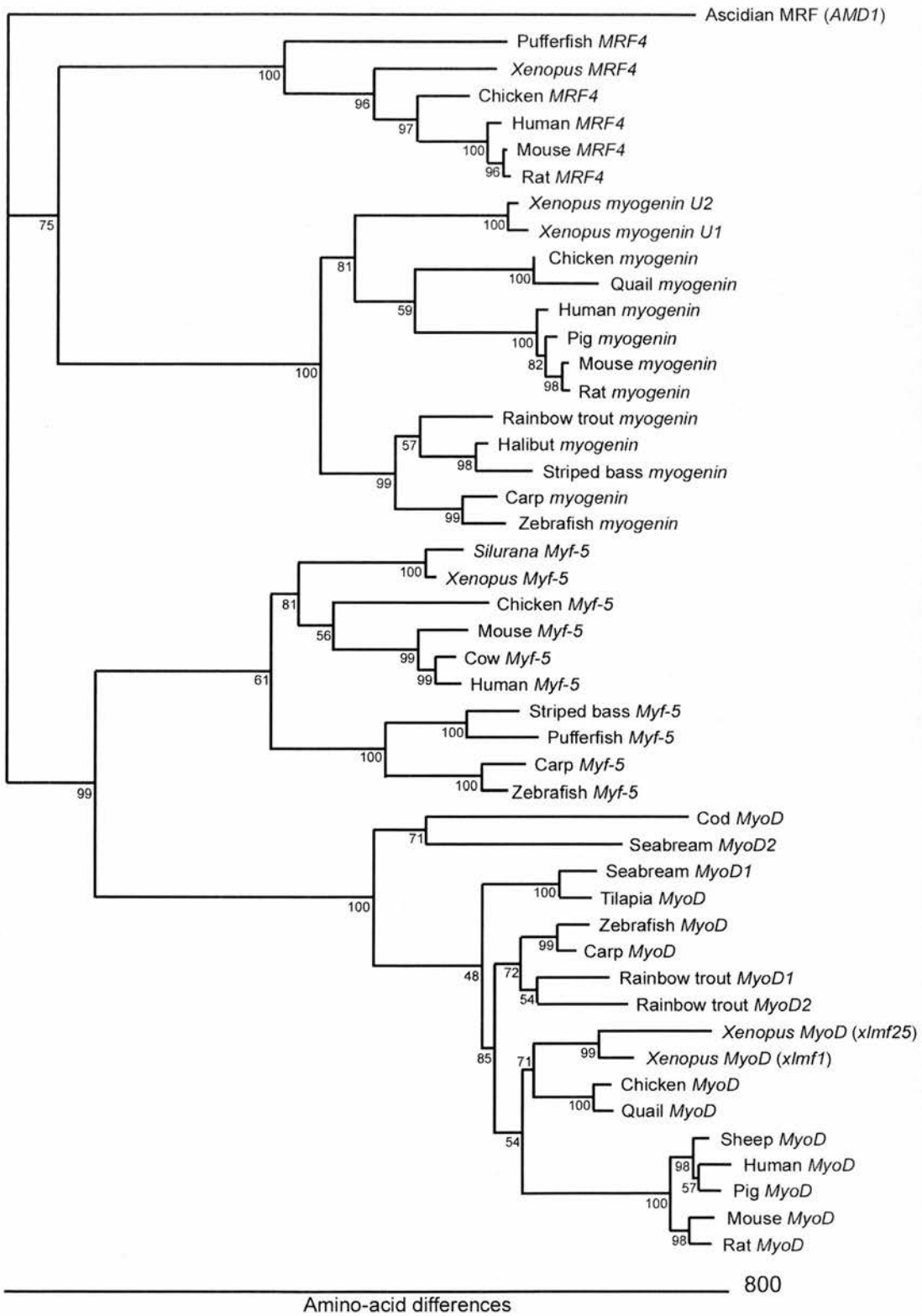


Fig. 4. Neighbour-joining tree of vertebrate myogenic regulatory factors with an Ascidian outgroup. Node numbers refer to the percentage of 1000 bootstrap pseudoreplicates supporting a clade. Branch lengths are proportional to the number of amino-acid subsitutions. Accession numbers are given in appendix II.

expression of *MyoD* persisted as the somites were formed (Figs. 1c, 1d) The dynamics of expression were such that at any time during somitogenesis, the newest ~12 somites stained positive for *MyoD* (Fig. 2b).

Myogenin expression was switched on in the somites later than *Myf-5* and *MyoD* (Figs 1e-f). The extent of staining lagged behind that of *MyoD* by ~5 somites and ~12 were stained at any one time. The expression patterns of all three transcripts gave the appearance of a rostral-caudal wave, lead by *Myf-5*, and followed by *MyoD* and *myogenin* respectively (Figs 1a-f). No differences were seen between 18°C and 25°C groups, relative to developmental stage.

The embryonic forms of *MyHC*, *Eggs22* and *Eggs24*, were first expressed at the 25-30 somite stage beginning in the anterior-most somites and progressing caudally (Figs 1g-j). After the completion of somitogenesis, *Eggs22* transcripts became concentrated in the caudal somites, whereas *Eggs24* predominantly stained the anterior somites. Expression persisted post-hatch, but was much reduced. No differences were seen between 18°C and 25°C groups with respect to developmental stage (Fig.1g-j). No expression of 10°C-type, intermediate-type and 30°C-type *MyHC* isoforms were seen at any stage. Positive dot blots using RNA isolated from fast muscle of 10 and 30°C acclimated adult (10cm total length) carp, alongside negative blots from the 18°C and 25°C incubated post-hatch larvae provided a positive control for the *in-situ* results (Fig. 3).

The neighbour-joining tree separated the four MRFs in relation to the outgroup Ascidian sequence (Fig. 4, accession numbers are given in appendix II). Within genes, clades broadly reflected evolutionary relationships, and the majority of the bootstrap values were high (>90%). Further support was given by comparison with the tree from parsimony analysis, which was almost identical, albeit with less resolution (i.e.

the relationships between the mammalian, avian and amphibian *MyoD* branches were unable to be resolved by this technique, see Appendix II). Importantly, the *Xenopus MyoD* and *myogenin* paralogues clustered together, as did the trout *MyoD* paralogues. In contrast, the seabream sequences were more highly divergent.

Discussion

The expression patterns of carp *MyoD* and *myogenin* more closely resembled those of the zebrafish than those of other teleosts studied to date. In the trout, *MyoD* expression extends laterally outwards from the adaxial cells relatively late in development, after the somites acquire their chevron shape, whereas in the other fish species studied (zebrafish [Weinberg *et al.*, 1996], herring [Temple *et al.*, 2001], seabream [Tan and Du, 2002]) adaxial cell expression of *MyoD* occurs across the somites soon after their formation. Furthermore, expression of *myogenin* persisted for longer in the somites than expression of *MyoD*, unlike in the herring, where the reverse is the case (Temple *et al.*, 2001). Expression of carp *Myf-5* also resembled that seen in the zebrafish, although in the zebrafish a more clearly defined banding pattern is seen in the presomitic mesoderm prior to somite formation, with at least five *Myf-5*-positive presomitic bands (Coutelle *et al.*, 2001). Similarities between expression patterns might be expected between the carp and zebrafish given that they are taxonomically closely related, both belonging to the family Cyprinidae. At the current time there are no other studies of teleost *Myf-5* expression for further comparison. *MRF4* expression has not been studied in any teleost to date, although a genomic clone has recently been isolated from the pufferfish *Fugu rubripes* (Carvajal *et al.*, 2001; Fig. 4).

The expression patterns of the embryonic *MyHC* isoforms *Eggs 22* and *Eggs 24* also showed no difference in timing of expression between temperature groups, and the timing of expression was broadly similar to that described by Ennion *et al.* (1999). However, the finding that the adult 10°C-type, intermediate-type and 30°C-type *MyHC*s were not expressed, even as the embryonic forms disappeared was significant, although not altogether unexpected. Other *MyHC* isoforms must be present to bridge the gap, either further embryonic forms, adult forms, or forms specific to the larval stages. Embryonic *MyHC* isoforms have been described in a variety of other species including human (Karsch-Mizrachi *et al.*, 1989; Eller *et al.*, 1989) rat (Strehler *et al.*, 1986), chicken (Molina *et al.*, 1987; Hofmann, 1988) and *Xenopus* (Radice and Malacinski, 1989). However, the myosin heavy chain multigene family in the carp is particularly large. Kikuchi *et al.* (1999) recently isolated 29 different genomic clones, more than twice the number present in humans (Soussi-Yanacostas *et al.*, 1993; Kikuchi *et al.*, 1999). Such diversity in carp myosin genes probably reflects the need for different molecular characteristics during the life cycle, as a result of allometric scaling relationships and temperature acclimation (Imai *et al.*, 1997; Ennion *et al.*, 1999; Kikuchi *et al.*, 1999; Cole and Johnston, 2001).

The neighbour-joining tree for the MRF family is shown in figure 4. The topology supports the notion proposed by Atchley *et al.* (1994), that all four members evolved from a common ancestor by gene duplication. After an initial duplication, each lineage divided again, one giving rise to *Myf-5* and *MyoD*, and the other giving rise to *myogenin* and *MRF4*. However, despite the fact that *MRF4* is most closely related to *myogenin*, in the human and pufferfish the *MRF4* gene is most closely associated spatially with *Myf-5*. In human, *Myf-5* and *MRF4* are located on chromosome 12, with their start codons only 8.5kb apart (Patapoutian *et al.*, 1993)

and in pufferfish they are even closer together, with their start codons differing by less than 5kb (genomic clone encoding *Myf-5* and *MRF4*, GenBank accession no. AJ308546). It is possible that the functions of the two genes demand that they respond to the same control regions, or that their close proximity is essential for their autoregulation, a hypothesis that is supported by the fact that in all of the three *MRF4* knockout mice constructed, *Myf-5* function is also affected (Summerbell *et al.*, 2002).

Recently, the view of the MRFs as a discrete family of four transcription factors has been clouded by the discovery of paralogous forms, which have diverged in function in some species. Rescan and Gauvry (1996) isolated a second form of *MyoD* from the trout, and demonstrated different expression patterns using *in-situ* hybridization. *MyoD1* was expressed in the adaxial cells of the unsegmented mesodermal plate and in the developing somites. *MyoD2* expression however, was initiated later and was limited to the posterior compartment of the somite. Similarly, in *Xenopus*, paralogous forms of both *MyoD* and *myogenin* have been isolated. One *MyoD* transcript (*xlmf25*) is expressed as a maternal mRNA in the early embryo, whilst the other (*xlmf1*) is activated from the zygotic genome near to the beginning of somitogenesis (Scales *et al.*, 1990; Scales *et al.*, 1991). Of the *myogenin* transcripts, one (*XmyogU2*) is expressed during embryogenesis, whilst the other (*XmyogU1*) is exclusive to the adult skeletal muscle (Charbonnier *et al.*, 2002).

The expression of paralogous genes is common in organisms such as trout and *Xenopus*, both of which have undergone recent genome duplication events and are in a state of pseudotetraploidy (Hughes and Hughes, 1993; Allendorf and Thorgaard, 1984; Rescan, 2001). However, the non-tetraploid gilthead seabream also differentially expresses two parologous forms of *MyoD* (Tan and Du, 2002). In this

case, the sequence identity of the two forms is lower than for the tetraploid organisms (Fig. 4), suggesting a more ancient duplication event.

No parologous forms of MRF family genes have been isolated from any of the tetrapod lineage with the exception of the tetraploid *Xenopus*, and paradoxically, despite the availability of whole genome shotgun sequences, in the zebrafish or pufferfish. The dynamics of teleost genome evolution is extremely complex, with evidence for specific genome duplication events remaining a contentious issue (Meyer and Schartl, 1999; Meyer and Malago-Trillo, 1999; Taylor *et al.*, 2001a; Taylor *et al.*, 2001b; Robinson-Rechavi *et al.*, 2001a; Robinson-Rechavi *et al.*, 2001b). In any case, whether at the whole genome or more regional level, teleost genomes are characterized by a high rate of duplication followed by substantial gene loss (Smith *et al.*, 2002; Robinson-Rechavi *et al.*, 2001c; Sibthorpe 2002). Further characterizing the molecular evolution of the MRF family in relation to function remains a challenging but potentially rewarding task.

Chapter 6: General discussion.

The results presented in chapters 2-5 demonstrate a broad approach to the question of the effect of temperature on muscle development in teleosts, bringing together anatomy with environmental physiology and molecular genetics. Chapter 2 concentrated on embryonic development in the Atlantic cod *Gadus morhua* at a single temperature (7°C), towards the centre of the normal developmental range, and was necessary to establish a "base line" on which to build subsequent chapters. The first staging series for the cod was produced over 100 years ago (Sars, 1876), but since then little detailed observation has been carried out, partly due to the technical difficulties associated with raising eggs and larvae successfully. However, there have been major advances in teleost embryology in general over the last decade, mainly due to focus on model species such as the zebrafish *Danio rerio* and the medaka *Oryzias latipes* (see Ishikawa, 2000; Dooley and Zon, 2000; Wakamatsu *et al.*, 2001; Amatruda *et al.*, 2002). Chapter 2 provided a re-examination of cod development in the light of these studies, and attention was able to be placed on the timing and development of specific tissues and organ systems. Currently, interest in the developmental biology of cod is undergoing a renaissance due to the development of cod as an aquaculture species. Concomitant with the development of the new industry is a wave of applied research on growth and development (Galloway *et al.*, 1998; Galloway *et al.*, 1999a), and the effects of biotic and abiotic factors on growth efficiency (Hansen *et al.*, 2001), and final flesh quality (Morias *et al.*, 2001). The new staging series will undoubtedly prove a valuable resource in these studies, as well as in the day-to-day staging of embryos in commercial hatcheries.

Chapter 3, in which the relative timing of developmental events was investigated in relation to rearing temperature, had its origins in early studies of characteristics of wild fish stocks. In species such as Atlantic herring *Clupea harengus*, a rich diversity of stocks can be identified on the basis of spawning place and time, and counts of meristic characters such as the number of fin rays, vertebrae, keeled scales or gill rakers (Parrish and Saville, 1967; Rosenberg and Palman, 1962). Variation in these characters comprises both heritable and environmentally induced components (Ali and Lindsay, 1974). Recently, muscle cellularity has been shown to be particularly sensitive to temperature during the embryo stages in a number of species, but directional responses are complex varying between stocks and species (Stickland *et al.*, 1988; Vieira and Johnston, 1992; Brookes and Johnston, 1994; Hanel *et al.*, 1996; Matschak *et al.*, 1998; Galloway *et al.*, 1998; Galloway *et al.*, 1999b). In addition to meristic variation, a variety of intraspecific developmental heterochronies have been documented in laboratory studies. For instance in the herring, rostral to caudal progression of myofibril synthesis has been shown to occur at earlier somite stages as temperature is increased (Johnston *et al.*, 1995), and the development of unpaired fins and associated musculature occurs at shorter body lengths at higher temperatures (Johnston *et al.*, 1997). Fukuhara *et al.* (1990) showed that in the Japanese flounder *Paralichthys olivaceus* the appearance of the pectoral fins, development of the mouth, and deposition of eye pigment occurred relatively earlier at higher temperatures. Similarly hatching has been shown to occur at different developmental stages depending on the ambient temperature in salmon (Pavlov, 1984), plaice (Ryland, 1975), turbot (Gibson and Johnston, 1995), and tambaqui (Vieira and Johnston, 1999). In chapter 3, embryonic development in the cod was generally found to be highly canalized with respect to temperature differences

compared to other species. However, two significant findings were that epiboly was delayed relative to somite stage at higher temperatures, and that white muscle fibre number was positively correlated with rearing temperature. It is possible that these factors are interlinked, through differential proliferation of presomitic myogenic cells and/or their relative exposure to inductive signals. This is a hypothesis that requires the cloning of candidate cDNAs to be readily testable, using techniques such as time-lapse confocal microscopy. Other aspects of development (e.g. appearance of the otic placode, gut lumen, otic vesicle, lens of the eye, otoliths and swim bladder) remained unchanged, although the timing of the first heart-beat started earlier at higher temperatures, possibly as an adaptive response to the decreasing oxygen solubility of water with increasing temperature. The low degree of developmental heterochrony seen between temperature groups in chapter 3 may have been due to the fact that the study was carried out within the normal thermal range for the species. As a general rule the frequency of detrimental defects increases at the very limits of temperature tolerance (Stockard, 1921), and it is likely that more subtle responses occur with decreasing proximity to these limits. It remains possible however that certain temperature-induced effects on phenotype, including effects on muscle fibre cellularity are adaptive, rather than simply detrimental effects occurring under sub-optimal conditions. For example, if a larva becomes more "fit" at a higher temperature as a result of expressing a particular phenotype, this would constitute a valid selection pressure for the development of an adaptive response. The evolutionary significance of such effects are difficult to prove experimentally. Swain (1992a and 1992b), studied the effect of vertebral number on burst swimming activity in sticklebacks *Gasterosteus aculeatus*, in relation to predation by sunfish *Lepomis gibbosus*. It was found that selection operated not on total vertebral number as expected, but on the

ratio of anterior to caudal vertebrae. As larval length increased, the optimal vertebral ratio decreased, reversing the direction of phenotypic selection on the same component of fitness, with only a slight change in larval size. Although complex, such modeling studies are clearly essential if we are to elucidate the potential advantages or disadvantages of the effect of temperature on physiological and/or behavioural traits.

Despite the temperature induced variation in fibre number seen in chapter 3, it was shown in chapter 4 that expression of muscle-specific protein (MSP) genes was invariant. This again reflected the fact that physiology is highly canalized within the normal developmental temperature range. Even so, a comparative view of the timing of expression of muscle specific genes with those of the zebrafish (Xu *et al.*, 2000) was important in its own right. In addition, sequence analysis of the clones revealed further evidence that *troponin I* (*TnI*) is more heterologous in fish than in mammals (Jackman *et al.*, 1998), and that the three isoforms known are not strictly orthologous to the mammalian fast, slow and cardiac isoforms. Similarly a cDNA encoding an isoform of *troponin C* (*TnC*) was isolated exhibiting highest sequence identity to the *Xenopus* slow/cardiac form, but which was expressed exclusively in the trunk muscle. It was noted that the propensity of the muscle-specific mRNAs to follow distinct activation patterns that proceed in a fixed temporal order, could facilitate their use as species- and developmental-stage specific markers. Surveys of pelagic egg abundance have been used to estimate spawning biomass in stock assessments (Armstrong *et al.*, 2001), and it is suggested that a high-throughput, ISH-based screen could provide the basis of a fast and accurate method of sample identification and staging.

Chapter 5 investigated the effect of the "master regulator" genes of muscle development in relation to temperature in the common carp *Cyprinus carpio*. In contrast to previous studies in rainbow trout *Oncorhynchus mykiss* (Xie *et al.*, 2001)

and sea bass *Dicentrarchus labrax* (Wilkes *et al.*, 2001), no differences in the timing of myogenic regulatory factors were seen between temperature groups. The expression patterns of the MRFs were most similar to those seen in the zebrafish, rather than the trout (Delalande and Rescan, 1999), where *MyoD* expression remains limited to the adaxial domain of the somite for a prolonged period subsequent to somite development, or the herring (Temple *et al.*, 2001) where expression of *myogenin* persists for longer in the embryonic somites than expression of *MyoD*. *Myf-5* expression also resembled that seen in the zebrafish (Chen *et al.*, 2001; Coutelle *et al.*, 2001) although expression in the presomitic mesoderm prior to somite formation was less extensive.

Teleost genomics is currently a minefield for inferences of gene ancestry. Analysis of the *Hox* genes, which play pivotal roles in specifying the body plans of a wide range of vertebrates, have provided evidence for two genome duplication events, one before and one after the radiation of the jawless (agnathan) fishes more than 400 million years ago (Amores *et al.*, 1998). Based on analysis of zebrafish and medaka, which appear to have seven or eight *Hox* gene complexes rather than the expected four, it has been proposed that a further whole genome duplication event occurred approximately 360 million years ago, after the sarcopterygian lineage (which leads to the lungfishes, coelacanths and all land vertebrates), radiated from the actinopterygian lineage (which leads to the ray-fined fishes) (Zardoya and Meyer, 1999; Naruse *et al.*, 2000). Species on the sarcopterygian branch (which also leads to *Homo sapiens*), are all likely to have only four *Hox* gene complexes, except in the case of some polyploid tetrapods such as *Xenopus*. It has been proposed that new redundant genes produced by major duplication events provide the raw material for radiative evolution (Ohno, 1970; Sidow, 1996; Holland, 1999). A whole genome duplication event however,

does not provide the only possible explanation for the abundance of duplicate genes in the actinopterygian lineage. Robinson-Rechavi *et al.* (2001a) compared families of orthologous genes which had been characterized in a minimum of three actinopterygian orders, excluding duplications older than the actinopterygian/sarcopterygian split. They concluded that most of the duplicates arose after the divergence of major fish groups, supporting a counter hypothesis of a high rate of local duplications, rather than a whole genome event. In the MRF phylogeny presented in chapter 5, the picture is still very much incomplete. The two *MyoD* paralogues isolated from trout (Rescan and Gauvry, 1996) are most likely the product of a recent duplication, since compared to other teleosts, the salmonids are in a state of pseudotetrapoidy, much like the tetrapod *Xenopus* (Hughes and Hughes, 1993). However, two *MyoD* paralogues have also been isolated from the gilthead seabream *Sparus aurata* which are more highly divergent, suggesting an earlier duplication. The cod *MyoD* cDNA isolated in chapter 4 clustered with the seabream *MyoD2* paralogue, and showed a similar, limited expression pattern suggesting that a second form may also exist in the cod. Seabream *MyoD2* remains however, the only paralogous MRF to be isolated from a non-tetraploid teleost despite whole genome shotgun sequences being available for both zebrafish and pufferfish (*Fugu rubripes*)

It is perhaps surprising that despite these sequencing projects, the gene/genome duplication argument still rages. Our ability to solve this particular question easily is limited by the vast amount of information which has become available in such a short time, and the current "lag" in the power of bioinformatics techniques to utilize it. Another important factor is the heterogeneity of teleost genomes; the pufferfish has the most compact genome of any vertebrate (400mb) compared to 1700mb in the zebrafish (Elgar *et al.*, 1999). The sequencing of many

other fish genomes is now underway, including the green-spotted pufferfish *Tetraodon nigroviridis*, rainbow trout, medaka *Oryzias latipes* and tilapia *Oreochromis niloticus*. Data from these projects, alongside increases in the power of bioinformatics will undoubtedly provide enlightening insights into teleost gene and genome evolution.

A final thought, as we approach the post-genomic era. Muscle development in particular is characterized by multiple transcripts spliced from the same gene, and post-translational modifications of the proteins. The MRF family themselves can auto- and cross-regulate their own transcription, and form protein-dimers with enhancer or inhibitor proteins, meaning that theoretically they can perform multiple tasks depending on their immediate cellular environment. Genome sequencing may well provide us with the "instructions to build an organism" but the secrets of the genome are inextricably linked with those of the transcriptome and the proteome, each of which interacts with the environment over time, to produce the visible phenotype.

Appendix I

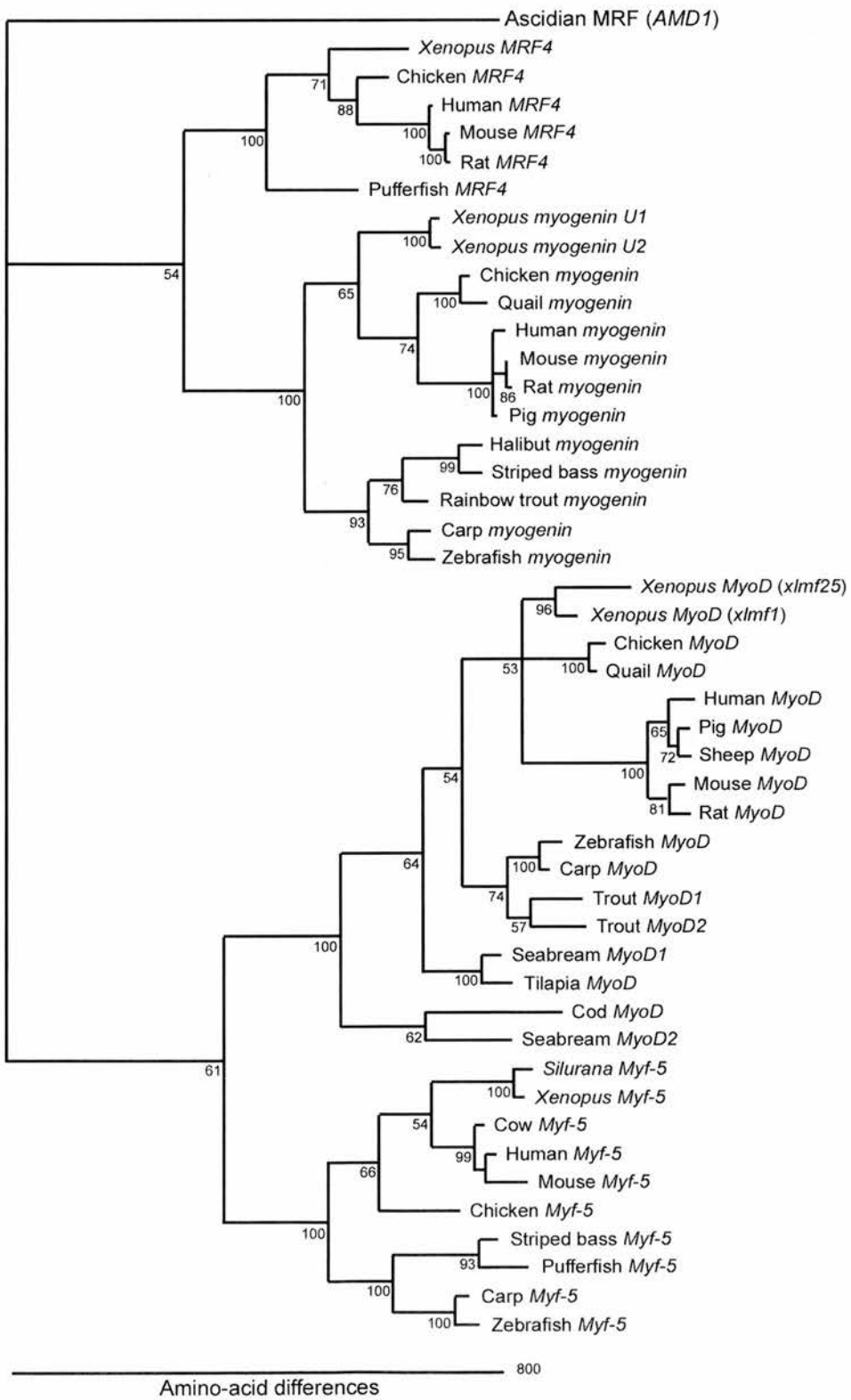
3' Race	*MyoD*		*α-actin*		*MyHC*		*TnT*		*TnI*		*CK-M*		*TnC*		*L15*	
Step	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)
Initial Denaturing	94	2:00	94	2:00	94	2:00	/	/	94	2:00	94	2:00	94	1:00	/	/
Denaturing	94	2:00	94	2:00	94	2:00	/	/	94	2:00	94	2:00	94	1:00	/	/
Annealing	62	0:45	54	0:45	56	1:00	/	/	54	0:45	58	1:30	56	0:45	/	/
Extension	72	1:00	72	0:45	72	1:00	/	/	72	1:00	72	1:30	72	1:00	/	/
Final Extension	72	5:00	72	5:00	72	7:00	/	/	72	7:00	72	7:00	72	5:00	/	/
Cycles	35		30		35		/		35		30		30		/	
Rounds	3		1		1		/		1		1		1		/	

5' Race	*MyoD*		*α-actin*		*MyHC*		*TnT*		*TnI*		*CK-M*		*TnC*		*L15*	
Step	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)
Initial Denaturing	94	2:00	94	2:00	/	/	94	2:00	94	2:00	/	/	/	/	/	/
Denaturing	94	2:00	94	2:00	/	/	94	2:00	94	2:00	/	/	/	/	/	/
Annealing	65	1:00	61	1:00	/	/	56	1:00	58	1:00	/	/	/	/	/	/
Extension	72	1:00	72	1:20	/	/	72	1:20	72	1:00	/	/	/	/	/	/
Final Extension	72	5:00	72	5:00	/	/	72	5:00	72	5:00	/	/	/	/	/	/
Cycles	30		30		/		35		30		/		/		/	
Rounds	1		1		/		1		1		/		/		/	

Expression Analysis	*MyoD*		*α-actin*		*MyHC*		*TnT*		*TnI*		*CK-M*		*TnC*		*L15*	
Step	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)
Initial Denaturing	94	2:00	/	/	/	/	/	/	/	/	/	/	/	/	94	2:00
Denaturing	94	2:00	/	/	/	/	/	/	/	/	/	/	/	/	94	2:00
Annealing	63	1:00	/	/	/	/	/	/	/	/	/	/	/	/	55	1:00
Extension	72	1:30	/	/	/	/	/	/	/	/	/	/	/	/	72	1:00
Final Extension	72	7:00	/	/	/	/	/	/	/	/	/	/	/	/	72	7:00
Cycles	35		/		/		/		/		/		/		35	
Rounds	1		/		/		/		/		/		/		1	

Appendix II

Parsimony tree of vertebrate myogenic regulatory factors with an Ascidian outgroup. Node numbers refer to the percentage of 100 bootstrap pseudoreplicates supporting a clode. Branch lengths are proportional to the number of amino-acid substitutions.



Accession numbers of MRF sequences

Ascidian MRF (AMD1)	D13507
Xenopus MRF4	S84990
Chicken MRF4	D10599
Human MRF4	NM_002469
Mouse MRF4	NM_008657
Rat MRF4	NM_013172
Pufferfish MRF4	AJ308546
Xenopus myogenin U1	AY046531
Xenopus myogenin U2	AY046532
Chicken myogenin	D90157
Quail myogenin	L15473
Human myogenin	NM_002479
Mouse Myogenin	D90156
Rat myogenin	NM_017115
Pig myogenin	U14331
Halibut myogenin	AJ487982
Striped bass myogenin	AF463526
Rainbow trout myogenin	Z46912
Carp myogenin	AB012881
Zebrafish myogenin	NM_131006
Xenopus MyoD xlmf1	M31116
Xenopus MyoD xlmf25	M31118
Chicken MyoD	L34006
Quail MyoD	L16686
Human MyoD	NM_002478
Pig MyoD	U12574
Sheep MyoD	X62102
Mouse MyoD	XM_124916
Rat MyoD	M84176
Zebrafish MyoD	NM_131262
Carp MyoD	AB012882
Rainbow trout MyoD1	X75798
Rainbow trout MyoD2	Z46924
Seabream MyoD1	AF478568
Seabream MyoD2	AF478569
Tilapia MyoD	AF270790
Cod MyoD	AF329903
Silurana Myf-5	AY050251
Xenopus Myf-5	X56738
Cow Myf-5	M95684
Human Myf-5	NM_005593
Mouse Myf-5	XM_192677
Chicken Myf-5	X73250
Striped bass Myf-5	AF463525
Pufferfish Myf-5	AJ308546
Carp Myf-5	AB012883
Zebrafish Myf-5	NM_131576

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