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Molecular Biotechnology of Development and Growth in Fish Muscle

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The myotomal muscles are the main edible part of teleost fish. This short review summarises current information on myogenic genes of particular biotechnological interest from the perspective of growth regulation and food chemistry. The cellular and molecular mechanisms underlying myogenesis at different stages of the life-cycle are described. The importance of temperature as a factor regulating growth and tissue phenotype is discussed with reference to developmental plasticity and temperature acclimation responses. The applications of muscle biotechnology to fisheries and aquaculture are highlighted together with areas for future research.

KEYWORDS myoganesis; growth regulation; gene paralogues; developmental plasticity; temperature acclimation; myosin

1. Introduction

The central goal of finfish aquaculture is the production of myotomal muscle. It is therefore important to understand the interactions of fish nutrition and environment on muscle development and growth at the molecular and cellular level. Although it is likely that the functions of genes regulating muscle development and growth are generally conserved in fish relative to mammals, some important differences have been documented. Embryonic myogenesis in teleosts has several unique features relative to amniote relatives. For example, cells are specified to the muscle lineage much earlier in teleosts, during mid-gastrulation (at 70–75%

epiboly; Weinberg *et al*. 1996), which probably reflects the early requirement to generate body movement imposed by external fertilization. These first muscle cells, the adaxial cells, then migrate from the medial to lateral somite to develop into a layer of slow muscle fibres anatomically separated from the fast-twitch muscle, a trait maintained throughout adult stages. In addition, teleost somites develop an external cell layer that has some conserved molecular and cellular characteristics of the amniote dermomyotome (Hollway *et al*. 2007), which supplies myogenic precursors for postembryonic growth (Gros *et al*. 2005).

The main cultured fish are teleosts, which form more than 95% of the ray finned fishes (the Actinoptergyii) (Nelson 2006). The most important aquaculture species are found in some of the most successful teleost superorders including the Acanthopterygii (Perciformes, e.g. tilapia sp., sea bass [*Dicentrarchus labrax*], halibut [*Hippoglossus hippoglossus*]), Ostariophysi (cyprinids, e.g. the common carp [*Cyprinus carpio*]), Protacanthoptergyii (salmonids, e.g. rainbow trout [*Oncorhynchus mykiss*] and Atlantic salmon [*Salmo salar*]), and Paracanthopterygii (gadoids, e.g. Atlantic cod [*Gadus morhua*]). The recent sequencing of the green spotted pufferfish (*Tetraodon nigroviridis*) genome, which is most compact vertebrate genome known (~365 Mb; Brenner *et al*. 1993) provided compelling evidence for the long suspected whole genome duplication (WGD) event at the base of the Actinoptergyian lineage some 320–350 Mya (Van de Peer 2004). For example, ~1000 pairs of duplicated genes in *T. nigroviridis* and the tiger pufferfish (*Takifugu rubripes*) genome tended to have a single duplicated copy on a single distinct chromosome (Jaillon *et al*. 2004). Additionally, a striking pattern of double conserved synteny was observed in the *T. nigroviridis* genome, where two chromosomal segments were conserved relative to mammals (Jaillon *et al*. 2004). It has been estimated that around 15% of the duplicated genes from this basal WGD have been retained in extant species (Jaillon *et al*. 2004). Several teleost families, including the salmonids, have undergone a second WGD with an estimated ~50% of duplicated paralogues retained (Allendorf and Thorgaard 1984). The evolutionary history of each target species is therefore an important consideration in the goal of distinguishing conserved and divergent features of muscle growth regulation in teleosts, requiring rigorous phylogenetic analysis and careful characterisation of gene paralogues (e.g. Kerr *et al*. 2005; Macqueen and Johnston 2008a).

Significant progress has been made in understanding the main events occurring in embryonic myogenesis in a model species, the zebrafish *Danio rerio* L. (e.g. Devoto *et al*. 1996; Barresi *et al*. 2000; Hammond *et al*. 2007; Hollway *et al*. 2007; Stellabotte *et al*. 2007). The advantages of working with zebrafish in terms of its draft genome sequence, small size, short generation time and embryonic transparency, make it the organism of choice for investigating muscle development in teleosts. Further, the availability of multiple mutant lines with aberrant development including muscle growth, as well as the now routine use of morpholino antisense RNA to knockdown genes of interest, have made zebrafish the most important teleost as a comparative model for studying conserved molecular and cellular features of myogenesis across vertebrates.

2. Myogenic Genes of Biotechnological Interest

The short review in this section is focused on a sub-set of the genetic pathways regulating muscle growth that are of particular interest from an aquaculture biotechnology perspective.

2.1. The MyoD gene family

Myogenic regulatory factors (MRFs) are a family of four basic helix-loop-helix transcription factors that are highly conserved between mammals and fish and are required for muscle lineage determination (MyoD, Myf5, Mrf4 [also known as Myf6]) and for the initiation and stabilization of muscle differentiation (Myogenin, Mrf4) (Rudnicki *et al*. 1993; Hasty *et al*. 1993; Kassar-Duchossoy *et al*. 2004). These "master" transcription factors play a central role in regulating muscle development and growth (Tapscott, 2005). Each protein contains two conserved motifs, the basic region and the helix-loop-helix (HLH) domain. The HLH domain enables MRFs to form dimers with bHLH containing E-proteins allowing binding via the basic domains to a conserved CIS-acting site (with the sequence CANNTG) found in the regulatory regions of most muscle-specific genes. Further a motif towards the C-terminal of MRFs, the amphipathic Helix-3, which is most strongly conserved in MyoD and Myf5, partially conserved in Mrf4 and poorly conserved in Myogenin, underlies the different abilities of the family members to act as muscle determination factors (Bergstrom and Tapscott, 2001). Although the MRFs show significant redundancy, each gene has evolved a unique expression pattern and specialised role in myogenesis. *myod* and *myf5* are expressed prior to segmentation in adaxial cells in several teleost species (Weinberg *et al*. 1996; Temple *et al*. 2001; Tan and Du 2002; Cole *et al*. 2004). Morpholino "knockdown" of either gene in zebrafish had no effect on slow muscle formation, whereas double "knockdowns" lacked a viable myogenic program (Hammond *et al*. 2007). This result is reminiscent of mice mutants lacking functional genes for both Myf5 and MyoD, where the skeletal muscle lineage was entirely absent (Rudnicki *et al*. 1993) and indicates a conserved function in myogenic specification. However, unlike the situation in mammals, where Mrf4 can compensate for MyoD or Myf5 as a muscle specification factor (Kassar-Dushossoy *et al*. 2004), in zebrafish lacking Myf5 and MyoD expression, *mrf4* transcripts and muscle differentiation are absent (Hinits *et al*. 2007).

The teleost *myod* gene is conserved as duplicated copies in more than one lineage. For example, two salmonid MyoD paralogues were characterised in rainbow trout (*Oncorhynchus mykiss*) and named TmyoD and TmyoD2 (Rescan and Gauvry 1996). Further, several species of the Acanthopterygii have two paralogues, including the Gilthead seabream (Tan and Du 2002), Atlantic halibut (Galloway *et al*. 2006) and the tiger pufferfish (Macqueen and Johnston 2006). The Acanthopterygian MyoD paralogues, were, originally denoted as MyoD1 and MyoD2 (Tan and Du 2002). Conversely, the zebrafish genome contains a single copy of MyoD. More recently a third MyoD sequence was characterised that is commonly conserved in salmonids (Macqueen and Johnston 2006). Phylogenetic reconstruction by maximum likelihood clustered teleost MyoD proteins into two clades, the first containing the single MyoD protein of the Ostariophysi (i.e. zebrafish) as well as MyoD1 of the Acanthopterygii and all three salmonid MyoD sequences, whereas the second clade contained solely the MyoD2 protein of the Acanthopterygii (Macqueen and Johnston 2006). An investigation of the genomic neighbourhood encompassing *myod* across the vertebrates revealed that genes on the single *myod*-containing chromosome of tetrapods were retained in both zebrafish and Acanthopterygians in a compelling pattern of double conserved synteny (Macqueen and Johnston 2008a). Phylogenetic reconstruction of these *myod*-neighbouring genes using Bayesian and maximum likelihood methods supported a common origin for teleost paralogues following the split of the Actinopterygii and Sarcopterygii. These results strongly suggested that a *myod*-containing

chromosome was duplicated during the teleost WGD, but that the one of the duplicated copies was subsequently lost in the Ostariophysi (including zebrafish) and Protacanthopterygii (including salmonids) lineages (Macqueen and Johnston 2008a). We have proposed a consensus nomenclature in which the ancestral gene, as conserved in diploid tetrapods, should be named *myod* and teleost paralogues should be first identified by their orthology to the *myod1* or *myod2* genes that arose during the WGD and then more recently derived copies, such as those retained in salmonids, should be named within this framework as *myod1*(*a*/*b*/etc) or *myod*2(*a*/*b*/etc) (Macqueen and Johnston 2008a). Thus the single *myod* gene in zebrafish should be designated *myod1* and the salmonid genes, as *myod1a*, *1b* and *1c*.

In situ hybridisation was used to describe the expression pattern of all four MyoD family members and their known paralogues in the developing myotomal muscle of the tetraploid teleost, Atlantic salmon. Concurrent expression domains of slow muscle myosin light chain-1 (*smlc1*) and *pax7* (required for the maintenance of myogenic progenitor cells in mammals, Seale *et al*. 2000) were also recorded to place the information within the context of known muscle differentiation events. Adaxial myoblasts expressed *myod1a* prior to and during somitogenesis followed by *myod1c* (20 somite stage) and *mrf4* (25–30 somite stage), before they migrated laterally across the myotome, to form a single layer of slow muscle fibres (Macqueen *et al*. 2007). *myf5* was detected prior to somitogenesis in the presomitic mesoderm, but not in the adaxial cells in contrast to other teleosts studied (Macqueen *et al*. 2007). The expression of *myf5*, *myod1*b and myogenin was not confined to regions expressing *smlc1*, and transcripts were present in overlapping domains consistent with a role in fast muscle myogenesis (Macqueen *et al*. 2007). From the end of segmentation, each MRF was expressed to a greater or lesser extent in zones of new fibre production, the progenitors of which probably originated in the external cell layer, which expressed *pax7* (Macqueen *et al*. 2007). Interestingly, the combined expression fields of *myod1a*, *myod1b* and *myod1c* in salmon recapitulated the expression pattern of the single *myod1* paralogue in zebrafish (Fig. 1A). It is plausible that where multiple teleost *myod* paralogues are found, such as in salmonids, that each gene has become sub-functionalised according to the model of Force *et al*. (Force *et al*. 1999), with each paralogue regulated by a sub-set of the *cis*-acting elements found in the promoter region(s) of the ancestral *myod* gene (Rescan 2001; Macqueen and Johnston 2006). In line with this model, it is also possible that a second, as yet uncharacterised salmonid *myf5* paralogue has inherited the *cis*-acting regulatory elements governing the adaxial cell expression domain conserved in other teleosts (Fig. 1B). Since hundreds of skeletal muscle genes are regulated downstream of MRFs in mammals (Blais *et al*. 2005; Bean *et al*. 2005), the presence of multiple paralogues may provide additional levels of control and complexity of expression patterns providing some selective advantage leading to their retention in the genome.

2.2. Myostatin

Myostatin (*Mstn*, also known as GDF-8) is a member of the transforming growth factor-β gene family (TGF-β), which was discovered to be a potent negative regulator of muscle growth in mammals (McPherron *et al*. 1997). The skeletal muscles of *mstn* null mice contain a larger number of fibres of greater diameter than wild-type controls, increasing muscle mass by up to 3-fold (McPherron *et al*. 1997). Naturally occurring *mstn* mutations are known to contribute to heavily muscled phenotypes observed in cattle, sheep, dogs and even humans (reviewed in Lee 2007a). *Mstn*-null mice also show a significant reduction in fat storage

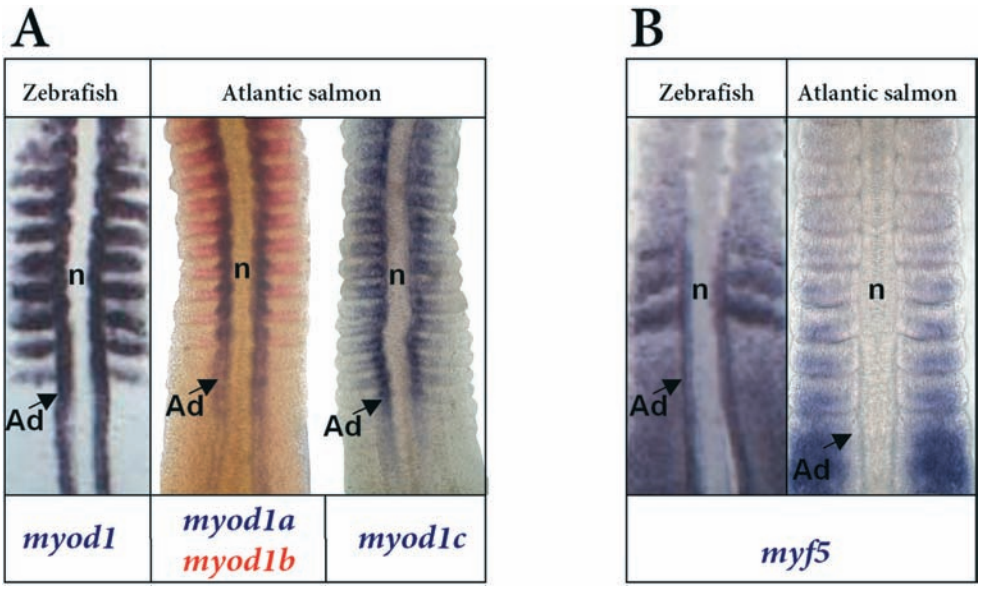

Fig. 1. (**A**) Embryonic expression of *myod1* paralogues in teleosts. Example images showing *in situ* hybridisation of either whole mount Atlantic salmon (30 somite stage (ss), right of box A) or zebrafish (10 ss, left of box A) embryos to specific complementary RNA probes to *myod1* (zebrafish) and *myod1a*, *myod1b* and *myod1c* (salmon). In salmon, the three *myod1* co-orthologues (*myod1a*, *myod1b*, *myod1c*) have distinct but overlapping expression domains, that together recapitulated the zebrafish *myod1* expression domain. This likely reflects the portioning of distinct *cis*-acting regulatory motifs conserved in the single zebrafish *myod1* gene, between the regulatory regions of the salmonid co-orthologues, following the duplication-degeneration-complementation (DDC) model (Force *et al.* 1999). (**B**) In zebrafish (10 ss, right of box B) as well as Acanthopterygian teleosts where the expression domain has been recorded, *myf5* transcripts accumulate strongly in the adaxial cells of the presomitic mesoderm and newly formed somites. In Atlantic salmon (30ss, right of box B) this *myf5* adaxial cell expression domain was absent at an equivalent stage of development, although the remaining expression pattern was comparable to zebrafish. We suggested that a second, as yet uncharacterised *myf5* paralogue is conserved in the salmonid lineage that fulfils this expression domain under the DDC hypothesis. Zebrafish panels were adapted from Hamade *et al.* (2006) with permission from Elsevier.

(McPherron and Lee 2002). These features of Mstn make it a most interesting biotechnological candidate for potential therapeutic applications in human muscle diseases (e.g. Nakatani *et al*. 2008), but also as a potential target to improve muscle growth by breeding or genetic engineering programs in agriculture (e.g. Pirottin *et al*. 2005) or aquaculture.

In mammalian cell culture, Mstn downregulates several MRFs (Langley *et al*. 2002) and prevents the transition of myoblasts from G1 to the S phase of the cell cycle (Thomas *et al*. 2000). Mstn is thought to inhibit myoblast proliferation and differentiation through a tumour growth factor-B pathway involving the activin receptor Act RIIB and the phosphorylation of Smads 2 and 3 (reviewed in Joulia-Ekaza and Cabello 2007). Transgenic mice expressing high levels of a dominant negative form of Act RIIB exhibited dramatic increases in muscle mass comparable to those seen in Mstn knockouts (Lee and McPherron 2001). Smad4 potentiates

Mstn signalling whereas Smad7 and Smurf1 are inhibitors (Joulia-Ekaza and Cabello 2007). Myogenin and the p21 cyclin-dependent kinase inhibitor are probably the major physiological targets of Mstn (Joulia *et al*. 2003). Promoter analysis of *mstn* in mammals reveals MyoD binding sites, glucocorticoid response elements and several FoxO boxes (Allen and Unterman 2007; Joulia-Ekaza and Cabello 2007). FoxO1 is of particular interest as an activator of *mstn* expression since it upregulated ubiquitin ligase expression, leading to protein degradation during muscle wasting (Allen and Unterman 2007).

The structure of the *mstn* gene has been elucidated in numerous teleost species because of its potential biotechnological interest (e.g. zebrafish, Xu *et al*. 2003; Atlantic salmon, Østbye *et al*. 2001; rainbow trout, Rescan *et al*. 2001 and Gilthead seabream, Maccatrozzo *et al*. 2001). The teleost gene has two introns and three exons that encode a 374–377 amino acid protein, which includes a signal peptide, conserved cysteine residues and a RPXXR proteolytic signal domain. Cleavage at the conserved RXXR region gives rise to the N-terminal latency associated peptide and a ~26 kD active processed peptide which is highly conserved across species. *Mstn* is conserved as two genes in most teleosts (*mstn1* and *mstn2*), as a result of the basal WGD (Maccatrozzo *et al*. 2001; Kerr *et al*. 2005). In salmonid teleosts, all the paralogues from the lineage-specific genome tetraploidization have been retained, as there are two paralogues per gene, namely *mstn1a/b* and *mstn2a/b* (*mstn2b* is a psuedogene) (Kerr *et al*. 2005; Garikipati *et al*. 2007). The ActRIIB receptor has also been cloned and characterised from Atlantic salmon and was found to be expressed in multiple tissues (Østbye *et al*. 2007).

There is somewhat contradictory evidence for *mstn* being a major negative regulator of muscle growth in teleosts as shown in mammals. In adult mammals the expression of *mstn* is almost exclusively restricted to muscle, whereas in teleosts expression occurs in a diverse range of tissues, including brain and ovaries (e.g. Østbye *et al*. 2001; Roberts and Goetz 2001; Garikipati *et al*. 2006). Such findings are consistent with a wider physiological role for *mstn* genes in teleosts. An interesting recent finding was that both *mstn*1 and *mstn*2 transcripts were upregulated 600-fold in the spleen of zebrafish exposed to overcrowding stress (Helterline *et al*. 2007). While most authors have generally been unable to detect embryonic *mstn* mRNA using *in situ* hybridisation (e.g. Xu *et al*. 2003; Kerr *et al*. 2005), Amali *et al*. (2004) reported ubiquitous expression of *mstn* in zebrafish embryos by this approach. Further, they reported that injecting morpholino mRNA for *mstn* into one to four cell zebrafish embryos caused later upregulation of MRFs and major changes in somite morphology consistent with a role in negative regulation of growth and myogenesis (Amali *et al*. 2004). However, the validity of Amali and co-workers findings has been questioned by other authors (Kerr *et al*. 2005; Helterline *et al*. 2007). Microinjection of double-stranded DNA (dsRNA) corresponding to the biologically active C-terminal myostatin domain of tilapia into zebrafish embryos was reported to increase body mass relative to controls as well as to augment muscle hypertrophy and hyperplasia (Acosta *et al*. 2005). In contrast, overexpression of the *mstn* propeptide (a negative regulator of the active peptide) in zebrafish produced only a relatively minor increase in muscle fibre number (~12%) and no change in fibre diameter or MRF expression (Xu *et al*. 2003). Furthermore, forced exercise in rainbow trout produced a marked hypertrophy of fast muscle fibres, but only a 6–7% decrease in myostatin active peptide relative to tank-rested controls (Martin and Johnston 2005). Taken together, these results do not provide strong evidence for a conserved

role for teleost *mstn* as a potent negative regulator of myogenesis, but certainly indicate a need for further functional studies.

2.3. Follistatin

Follistatin (*Fst*) is a secreted glycoprotein that is expressed in many mammalian tissues where it was first identified as a potent inhibitor of follicle stimulating hormone (Phillips and de Krestor 1998). Fst is an inhibitor of Mstn and Bone Morphogenic Proteins (BMPs) and a known regulator of amniote myogenesis (Amthor *et al*. 2002, 2004). Null *fst* mice showed retarded growth and reduced muscle mass (Matzuk *et al*. 1995) whereas mice over-expressing Fst showed significantly increased muscle fibre hyperplasia and hypertrophy relative to wild-type controls (Lee and McPherron 2001). In chick embryos, *fst* is expressed in myogenic progenitor cells of the dermomyotome and it antagonises the inhibitory effect of *Mstn* on transcription factors including *myoD* and *pax3* (Amthor *et al*. 1996, 2004). Fst protein was also shown to bind BMPs at a level that stopped BMP-induced apoptosis and promoted excessive muscle growth, with upregulation of *pax3* (Amthor *et al*. 2002). Furthermore, transgenic mice that lack a viable *Mstn* gene, but overexpress Fst display an even more exaggerated muscle-growth phenotype than *mstn* mutants (Lee 2007b).

In teleost fish, research with *fst* is limited from a myogenic perspective, despite it potential to manipulate muscle mass. We recently demonstrated by phylogenetic reconstruction (maximum likelihood) and comparative analyses of genomic synteny, that *fst* was duplicated during the teleost WGD and retained as two paralogues (*fst1* and *fst2*), in the Ostariophysi superorder (in zebrafish and in catfishes) but not in the Acanthopterygii or Protacanthopterygii (Macqueen and Johnston 2008b), which have a single gene (*fst1*). In adult salmon, zebrafish and seabream, *fst1* was shown to be expressed in multiple tissues, including fast and slow muscles (Macqueen and Johnston 2008b; Funkenstein *et al*. 2008), whereas zebrafish *fst2* showed a more restricted pattern and was virtually absent from fast muscles (Macqueen and Johnston 2008b). A potential conserved role for teleost *fst* in embryonic myogenesis was demonstrated, as *fst1* transcripts were expressed concurrently to *pax7* in presumptive myogenic precursors of the anterior somite, external cell layer and pectoral fin buds (Macqueen and Johnston 2008b).

2.4. The insulin-like growth factor system

In mammals, insulin-like growth factor-I (IGF-I) and IGF-II represent the main endocrine and autocrine regulators of skeletal muscle growth. IGF-I is synthesised in liver and other tissues including skeletal muscle. The liver expression of the *IGF-I* gene is controlled by the pituitary/growth hormone axis. Circulating levels of IGF-1 are regulated by six binding proteins (IGFBPs) in mammals and there are several receptor subtypes (Denley *et al*. 2005). IGF-I is synthesized as a pre-pro-peptide that is processed by proteolytic cleavage to produce the mature IGF-I and E-peptide, and both peptides are co-secreted. Multiple isoforms of pro-IGF-I have been identified from fish to human with differences only in their C-terminus E-peptides. IGF-I Ea and IGF-IEb splice variants have been described in rodents (Yang *et al*. 1996). IGF-IEa corresponds to the liver form of IGF-I whereas IGF-IEb, also referred to as mechano-growth factor (MGF), is produced locally in the muscle in response to mechanical stimuli (Goldspink 1999). IGFI-Eb differs from IGF-IEa by a 52-bp insert in exon 5, and also with respect to its promoter region (Yang *et al*. 1996; Goldspink 1999). In humans, there is evidence that following mechanical stress the IGF-pre-propeptide is initially spliced towards MGF (which is designated IGF-IEc,

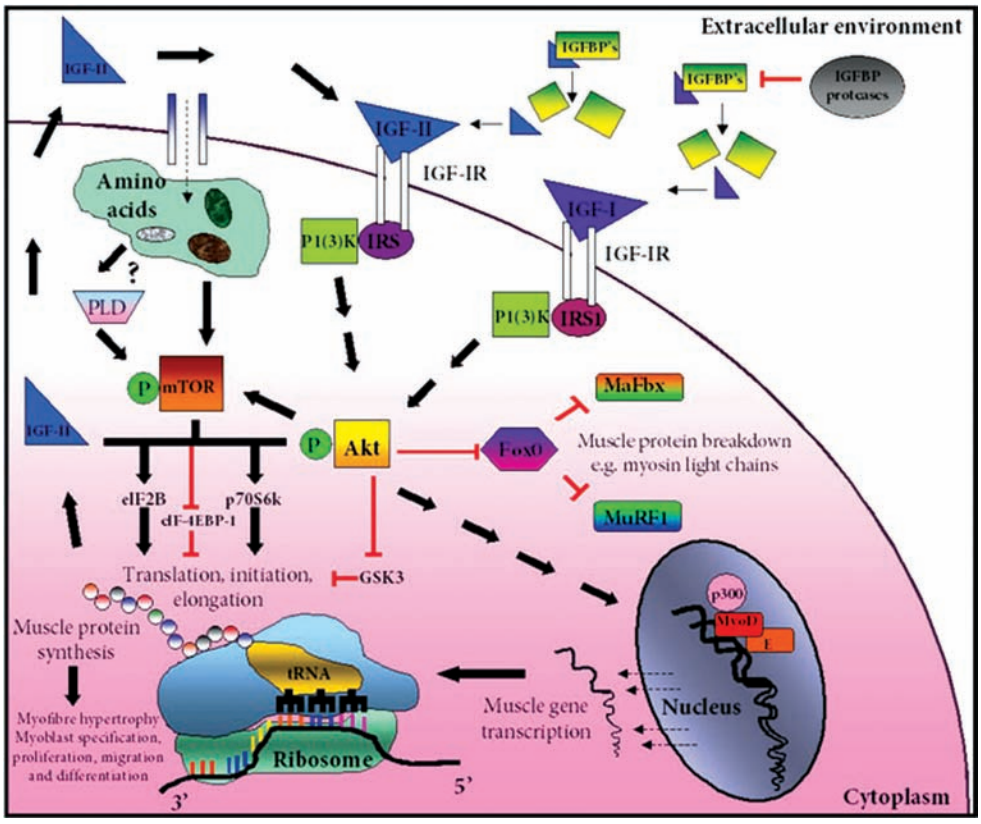

Fig. 2. A diagrammatic representation of the the IGF axis regulating muscle growth and atrophy through the mTOR/Akt/P70S6K signalling cascade (see text for abbreviations and details).

but is still equivelent to rodent MGF/IGF-IEb), resulting in the activation and proliferation of muscle progenitor cells (Ates *et al*. 2007) and the facilitation of myoblast migration (Mills *et al*. 2007). Subsequently, the IGF-pre-propeptide is spliced towards the IGF-I Ea splice variant, which is the main source of mature IGF-I required for myogenic differentiation and MGF is downregulated (Ates *et al*. 2007). Although IGF-IEa and MGF may have different roles in muscle growth (Yang and Goldspink 2002), much remains to be discovered about their regulation and function.

The signalling pathways that are activated by IGF-I and IGF-II in mammals have been studied using a combination of *in vivo* and cell culture models of muscle hypertrophy in combination with pharmacological and gene "knock-out" experiments (reviewed in Glass 2005) and are summarized in Fig. 2. Briefly, the binding of IGF-I to a membrane receptor triggers the activation of several intracellular kinases, including phosphatidylinositol-3-kinase (PI3K). PI3K phosphorylates the membrane phospholipids phoshatidylinositol-4,5-bis-phosphate to phoshatidylinositol-3,4,5-trisphosphate, creating a lipid binding site on the cell membrane for a serine/threonine kinase called Akt (or PKB-protein kinase B). The subsequent translocation of Akt to the membrane facilitates its phosphorylation and activation by PDK-1. Downstream targets of Akt include

the mammalian target of rapamycin (mTOR), which activates (p70S6K, elEF2) or represses (GSK3, glycogen synthase kinase 3, ef1–4EBP1 [aka PHAS-1]) key proteins regulating translation and protein synthesis (Fig. 2). Significantly, the IGF-1/PI3K/Akt signalling pathway also functions as a switch between protein synthesis and degradation, suppressing catabolic pathways by inhibiting the FoxO family of transcription factors preventing induction of the muscle-specific ubiquitin ligases MAFbx and MuRF1 (Fig. 2). The autocrine IGF-II transcription required for skeletal myocyte differentiation is regulated by mTOR and the availability of branched chain amino acids such as isoleucine (Erbay *et al*. 2003) (Fig. 2). Phosholipase D (PLD) has recently been demonstrated as an upstream regulator of mTOR and may provide a molecular link between amino acid availability and mTOR activity (Yoon and Chen 2008) (Fig. 2). Thus the mTOR-IGF axis provides a direct molecular link between nutritional levels and protein synthesis leading to muscle fibre growth.

Fish are ectotherms and in nature temperate species are often subject to marked seasonal fluctuations in food supply resulting in annual cycles of growth. It is therefore likely that many aspects of the IGF-system regulating growth in fish differ from those described for mammals. Nevertheless the main features of the growth hormone/IGF system appear to be highly conserved between fish and mammals. Numerous studies have characterised the coding sequences of teleost orthologues of IGF-I, IGF-II, IGF-receptors and IGFBPs (reviewed in Wood *et al*. 2005). Several teleost genes in the IGF system have paralogues that probably arose from genome duplication events. Four different isoforms of pro-IGF-I containing identical mature IGF-I but different lengths of E-peptides have been identified in rainbow trout (Shamblott and Chen 1993). However, the majority of teleost studies of IGF-I and IGF-II expression have not distinguished between different splice variants or simultaneously investigated IGF-receptor and IGF-binding expression which may be important in the regulation of the bioavailability and effective concentration of the hormones. Furthermore, it has not yet been established whether any of the teleost IGF-I splice variants responds to mechanical stimuli as in mammals. The relative importance of IGF-I and IGF-II expression may also be species dependent (Chauvigné *et al*. 2003; Peterson *et al*. 2004). Fasted fish reduce metabolic rate and can survive for long periods on energy stores whilst displaying negative growth. Refeeding following fasting results in a rapid growth spurt often called compensatory growth. In fasted rainbow trout, refeeding resulted in an 8- and 15-fold increase in IGF-I mRNA after 4 and 12 d respectively, but only a small and much slower increase in IGF-II expression (Chauvigné *et al*. 2003). Consistent with this finding, plasma *IGF-I* increased four days after refeeding in fasted trout, while plasma *IGF-II* concentration increased after one day (Gabillard *et al*. 2006). Further, IGF receptor Ia expression declined following refeeding whilst IGF receptor Ib transcript levels were unchanged (Chauvigné *et al*. 2003). Transcripts for *IGFBP4*, *IGFBP2*, *IGFBP5* and IGFBP-related protein 1 were also reduced in fasted trout, before being upregulated upon refeeding (Gabillard *et al*. 2006).

2.5. Calpain/calpastatin

The calpain–calpastatin system has been extensively studied in mammals because of its importance in regulating a wide range of physiological processes including protein turnover and growth (Goll *et al*. 2003), cell cycle progression, as well as myoblast differentiation, migration and fusion (Dedieu *et al*. 2004; Barnoy *et al*. 1996). There is also considerable biotechnological interest in these proteins because of their effects on meat quality. Proteolysis by these proteases

post mortem influences muscle texture with an inverse relationship between calpastatin levels and meat tenderization (Duckett *et al*. 2000). Calpains are calcium-dependent cysteine proteinases that are present in all cells. The ubiquitous μ- and m-calpains catalyse the limited proteolysis of cytoskeletal and membrane proteins and are regulated by Ca^{2+} concentration and the specific protein inhibitor calpastatin (Goll *et al*. 2003). μ-calpain (calpain 1) is active at μM calcium concentration and m-calpain (calpain 2) is active at mM calcium concentrations. Each calpain has a common 30 kDa regulatory subunit and a unique 80 kDa catalytic subunit. In typical calpains, the catalytic subunit has four domains: Domain I (autolytic activation), Domain II (cysteine catalytic site), Domain III (switch domain) and Domain IV (calmodulin-like calcium binding domain). Molecular cloning has also identified a large number (15 to date in mammals) of Calpains, some of which are predominantly tissue specific, including Calpain 3 (p94), that is expressed principally in skeletal muscle in mammals (Jones *et al*. 1999), and others that are expressed more ubiquitously (Goll *et al*. 2003). Calpain 3 mRNA transcripts are ten times more abundant in muscle than those of Calpains 1 and 2 and its genetic disruption underlies limb girdle muscular dystrophy type 2A (Goll *et al*. 2003). The purified protein is unstable on isolation and *in vivo* it is thought to be stabilised by interaction with titin, a giant 3.7 Mda cytoskeletal protein that spans the muscle half sarcomere from M to Z line (Duguez *et al*. 2006). It has also been suggested that calpain 3 plays a central role in sarcomere remodelling (Duguez *et al*. 2006).

Calpastatin is a specific inhibitor of calpains. In mammals, calpastatin has four homologous C-terminal inhibitory domains (I–IV) downstream of a non-inhibitory leader domain (L) and an N-terminal XL sequence (Goll *et al*. 2003). Several isoforms of calpastatin have been reported that are expressed in a muscle fibre type-specific fashion. The different isoforms arise from alternative splicing, different start sites of translation/transcription and different states of phosphorylation (reviewed in Sentandreu *et al*. 2002). Studies with transgenic mice that over-express human calpastatin have provided evidence that calpains regulate muscle glucose metabolism and muscle mass (Otani *et al*. 2004). Calpastatin transgenic mice showed decreased levels of the myocyte enhancer factors, MEF2A and MEF2D, and a significant hypertrophy of muscle mass (Otani *et al*. 2004). In another study, it was shown that overexpression of calpastatin prevented myoblast fusion and strongly downregulated myogenin expression (Barnoy *et al*. 2005).

The calpain/calpastatin system is poorly studied in fish compared with mammals. Calpain 1 and 2 have been partially purified from a number of aquaculture species. Recently, full-length cDNAs have been obtained for calpain 1 and 2 from the rainbow trout that show around 65% identity with mouse orthologues (Salem *et al*. 2005a). Starvation for 35 d in the rainbow trout resulted in the up-regulation of mRNA transcripts for calpain 1 (2.2-fold), calpain 2 (6.0-fold) and calpastatin (1.6-fold) (Salem *et al*. 2005a). These results indicate that season of harvest and pre-slaughter starvation period are likely to affect the calpain/calpastatin system and hence flesh texture and storage characteristics. In rainbow trout, two CAST isoforms have been reported, a long (CAST-L) and a short (CAST-S) form (Salem *et al*. 2005b). Fish CASTs have fewer repetitive inhibitory domains than found in mammals (one or two versus four).

Whereas tenderisation is a positive attribute in red meat, in fish softness represents a loss of quality and hence economic value. Verrex-Bagnis *et al*. (2002) used Western blotting to show that calpain 2 released α-actinin and desmin from myofibrils following *in vitro* degradation of

myofibrils. Calpain was shown to degrade troponin T and α-actinin in sea bass (Delbarre-Ladrat *et al*. 2004). The carboxyterminal region of dystrophin, a cytoskeletal actin binding protein, is highly sensitive to degradation by calpain 2 (Bonnal *et al*. 2001). Several studies have shown that during the pre-rigor period, cytoskeletal proteins are affected by the first proteolytic events. These cleavages disrupt connections between myofibrils and the extracellular matrix, induce segmentation of myofibrillar cores, and modify the rheological properties of the tissue (Bonnal *et al*. 2001). Dystrophin release has proved to be a very useful marker of the early events of proteolysis during fish storage.

3. Embryonic Myogenesis

The somites are transient embryonic structures common to all vertebrates that form from mesodermal tissue in a rostral to caudal wave. Somites give rise to the myotomes (the future skeletal muscle) and the sclerotome (the future skeleton), which is much reduced in teleosts compared to amniotes, a likely adaptation to the additional buoyancy provided by water relative to a terrestrial environment. Prior to segmentation two distinct cell types can already be distinguished morphologically and by their gene expression patterns. In the epithelial zebrafish somite, a four by five layer of cuboidal cells (the adaxial cells) reside adjacent to the notochord which differentiate into a single stack of twenty elongated cells that migrate radially through the somite to form a superficial layer of slow muscle fibres (Devoto *et al*. 1996). This migration of adaxial cells is driven by differential cell adhesion through the actions of Cadherin transmembrane proteins (Cortés *et al*. 2003). Undifferentiated adaxial cells express *myod1* and *myf5* (Weinberg *et al*. 1996; Coutelle *et al*. 2001) and their specification is regulated by Hedgehog (Hh) morphogens in a dose-dependent manner (Blagden *et al*. 1997; reviewed by Ingham and Kim, 2005). Another sub-population of adaxial derived cells, termed the muscle pioneers, remain in a medial position in the somite and express engrailed genes (Weinberg *et al*. 1996; Devoto *et al*. 1996). The cells of the lateral pre-somitic mesoderm of zebrafish were shown to differentiate into the embryonic fast muscle fibres (Devoto *et al*. 1996) and two main sub-populations have since been characterised. A population in the posterior-lateral epithelial somite requires Fibroblast growth factor 8 (*fgf8*) signalling for *myod1* expression and terminal differentiation (Groves *et al*. 2005). The other population arises medially within the somite and like the slow muscle pioneers, expresses engrailed genes, in response to Hh signalling (Wolff *et al*. 2003). Interestingly, in *fgf8* mutants, a residual medial fast fibre population was identified that was not dependent on Hh signalling and presumably is regulated through some other midline signal (Groves *et al*. 2005). Hh and Fgf8 signalling suppress Pax3/7 and promote the expression of *myf5* and *myod1* in specific muscle progenitor cell populations (Feng *et al*. 2006, Hammond *et al*. 2007). A combination of vital dye staining and lineage-tracking techniques in zebrafish were used to show that somite sub-domains are generated through a whole-somite rotation of 90° from the starting position, which begins during mid-somitogenesis and is complete by the end of the segmentation period (Hollway *et al*. 2007). Somite-rotation has been shown to require Sdf cytokine signalling (Hollway *et al*. 2007). The anterior component of the somite forms the external cell layer of Pax3/7 expressing cells (Hollway *et al*. 2007; Stellabotte *et al*. 2007). A sub-set of these cells migrate from the external cell layer to form fast muscle fibres in the late embryo and larval stages, whilst others remain in the external cell layer and are thought to be a source of myogenic progenitor cells for later stages of post-embryonic growth (Hollway

et al. 2007; Stellabotte *et al*. 2007). The external cell layer also provides progenitors used in the growth of the dermis and pectoral and dorsal fin muscles suggesting it has a functional role equivalent to the amniote dermomyotome (Hollway *et al*. 2007).

4. Postembryonic Myogenesis

In the late embryo and early larval stages, primary embryonic slow and fast muscle fibres are supplemented by fibres from discrete germinal zones, a process which has been termed stratified hyperplasia (SH) (Rowlerson and Veggetti 2001). Three anatomically distinct, but overlapping waves of SH have been observed in zebrafish, resulting in a continuous increase in the number of slow fibres per myotomal cross-section from hatching until the maximum adult length (~40 cm standard length, snout to caudal peduncle) (Lee and Johnston, unpublished results). Growth of the myotome also involves an increase in the length and diameter of fibres, a process that requires the absorption of myogenic progenitor cells to provide additional nuclei (reviewed in Johnston, 2006). Using mutant lines of zebrafish deficient in midline Hedgehog signalling it was shown that the first wave of SH does not require a scaffold of embryonic slow muscle fibres and proceeds independently of sonic Hh (Barresi *et al*. 2001). The origin of the myogenic precursors that fuel later phases of SH remains to be established, but could also be the external cell layer which persists in adult stages (Hollway *et al*. 2007; Stellabotte, *et al*. 2007).

Expansion in the number of fast muscle fibres with growth also proceeds via an initial phase of stratified hyperplasia (reviewed in Rowlerson and Veggetti 2001). The intensity and duration of this growth phase varies considerably between species, but usually involves the addition of new fast fibres at the dorsal and ventral surfaces of the myotome. Fate mapping studies have shown that a Pax7 expressing sub-set of the external cell population migrate through the embryonic slow muscle and give rise to fast muscle fibres in larvae (Stellabotte *et al*. 2007; Hollway *et al*. 2007). Within lineage-related clusters it was also possible to detect the original undifferentiated cell at the site of muscle fibre generation, indicating a stem cell self-renewal system (Stellabotte *et al*. 2007; Hollway *et al*. 2007) as proposed for mammalian satellite cells (Schultz 1996). SH is the only mechanism of fast fibre expansion reported in the guppy (*Poecilia reticulata*) (Veggetti *et al*. 1993) and in two out of the eight families of Antarctic notothenioid fishes (Harpagiferidae and Channichthyidae) (Johnston *et al*. 2003a). The adaptive radiation of the Notothenioidei has been associated with a general reduction in fibre number and an increase in fibre diameter from the basal to more derived genera, and this includes species that reach a large body size (~85 cm) (Johnston *et al*. 2003a).

The second and main mechanism for increasing fast fibre number in all other species examined, including zebrafish, is mosaic hyperplasia (MH). MH involves myogenic progenitor cells distributed throughout the myotome and results in successive waves of myotube production producing a characteristic mosaic pattern of fibre diameters. Myotube production in fast muscle only continues until the fish has attained ~40–45% of its maximum body length, with subsequent growth restricted to an expansion in the diameter and length of muscle fibres, and nuclear accretion (Weatherley *et al*. 1988; Johnston *et al*. 2003b). However, in response to injury and as a component of the repair response, new myotube production can be initiated even after the end of fibre recruitment (Rowlerson *et al*. 1997). In those species that show sexual dimorphism in body size, such as the Argentinean hake (*Merluccius hubbsi*) (Calvo 1989) and the Atlantic halibut (*Hippoglossus hippoglossus*),

the duration of fibre recruitment differs between male and female fish (Hagen *et al*. 2006), although the underlying mechanism is unknown. A genome-wide screen in the Tiger pufferfish (*Takifugu rubripes*) revealed a sub-set of genes that are specifically upregulated concomitant with the end of fibre recruitment in fast muscle, although whether any of these genes has a role in establishing the final fibre number (FFN) remains to be established (Fernandes *et al*. 2005). The origin of myogenic precursor cells required for MH has also not been determined, although the external cell layer must be a strong candidate. In this regard it is interesting to note that in zebrafish the external cell layer only contains at most a few dozen Pax7 positive cells per somite whereas in adult fish ~3,500 fast muscle fibres are eventually produced per myotomal cross-section. Furthermore fast muscle fibres with the maximum diameter (80 μm) contain ~1,700 nuclei per cm length (Lee and Johnston 2008). If the external cell layer is indeed the sole source of cells for juvenile and adult growth then this implies that a very small number of self-replicating stem cells gives rise to an enormous number of myogenic progenitors via some lineage restricting mechanism. The number of resident myogenic progenitors can be increased in fasted fish by feeding (Brodeur *et al*. 2003) and can also be manipulated by photoperiod regime (Johnston *et al*. 2003b). For example, in seawater stages of farmed Atlantic salmon that were transferred from short winter days to continuous light there was a transient 70% increase in the number of myogenic progenitors (c-met positive mononuclear cells) relative to controls (Johnston *et al*. 2003b). Continuous light treatment for 6 months over the winter/early spring was associated with longer term benefits in growth performance, as well as a higher final fibre number and greater levels of nuclear accretion in muscle fibres compared to fish subject to natural photoperiod (Johnston *et al*. 2003b).

5. Developmental Plasticity and Adult Growth

It has long been known that exposing fish eggs to different conditions of temperature and/or salinity fish influences a range of meristic characteristics such as vertebral number in the adult stage (Hempel and Blaxter 1961). Later it was discovered that egg incubation temperature affected the number and size distribution of myotomal muscle fibres in Atlantic salmon alevins at hatching (Stickland *et al*. 1988). Since this first paper there have been numerous further studies on the developmental plasticity of muscle growth at different temperatures and/or oxygen levels in a wide range of species (reviewed in Johnston 2006). Most of these studies have been restricted to the larval or early juvenile stages and have typically involved just two embryonic treatments. Recently, we demonstrated that in Atlantic salmon, altering temperature treatments from fertilisation until the embryonic eye became completely pigmented (the "eyed stage") was sufficient to affect growth trajectory over the entire lifecycle (Macqueen *et al*. 2008). Furthermore, embryonic temperature set the final number of fast muscle fibres per myotomal cross-section (FFN) and altered fibre size distribution and the myonuclear content of individual muscle fibres in adult fish (Johnston *et al*. 2003c; Macqueen *et al*. 2008) (Fig. 3). For the Salmobreed (Norway, A/S) strain, FFN showed a distinct optimum for the 5°C treatment, and was reduced at higher or lower embryonic temperatures (Fig. 3). Experiments showing embryonic environment can affect adult growth can only be explained by direct temperature effects on embryonic tissues, such as the external cell layer. The period to eye pigmentation encompasses the window in which the external cell layer is formed (Hollway *et al*. 2007; Macqueen *et al*. 2008). Undifferentiated *pax7* expressing

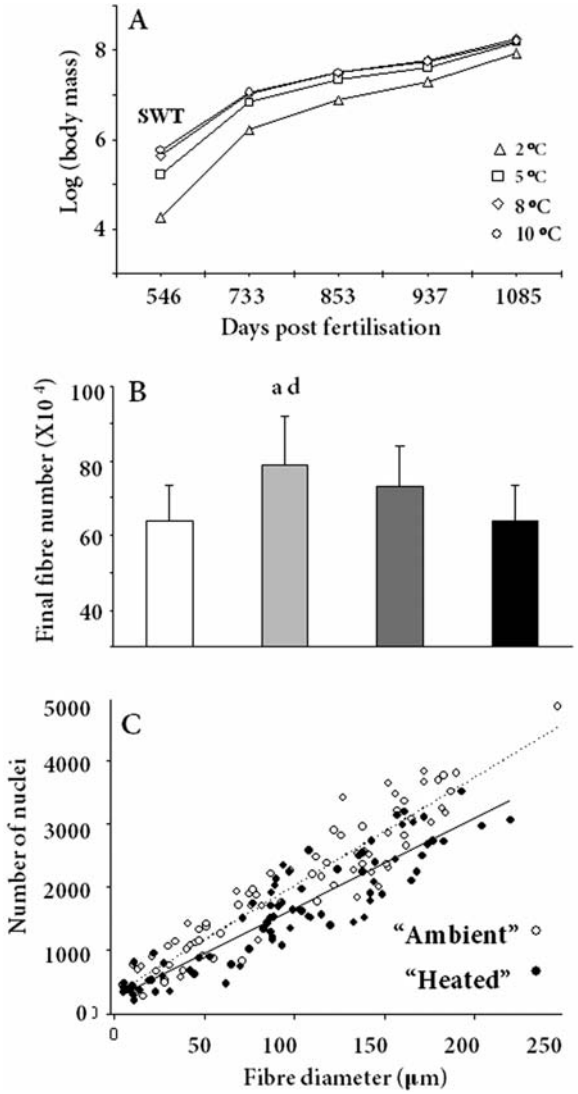

Fig. 3. Embryonic temperature modulates growth and the final muscle fibre number in adult Atlantic salmon (*Salmo salar* L.). (**A**) Shows the different seawater growth trajectories of Atlantic salmon reared for a short embryonic developmental window at either 2, 5, 8, or 10°C and then provided equal growth opportunity. While 10 and 8°C treatments were significantly heavier at seawater transfer (SWT), strong compensatory growth was observed at lower temperatures, although 2°C fish did not attain the body sizes reached by 5, 8 and 10°C treatments. (**B**) Shows the norm of reaction response of the final number of fast myotomal muscle fibres in adult Atlantic salmon from the same experiment as A. The final number of fibres showed an optimum at 5°C and was reduced at either higher or lower temperatures by a maximum of 17%. a d indicates a significant difference ($p < 0.001$) compared to 2 and 10°C respectively. (A, B) From Macqueen *et al.* 2008). (**C**) Shows the nuclear content of fast myotomal muscle fibres of a range of diameters in adult Atlantic salmon reared from fertilisation to smolitification at either ambient temperature or in water heated by 1–3°C and then provided equal growth opportunity. The muscle fibres of fish from the ambient treatment had significantly more nuclei at equivalent fibre diameters ($p < 0.001$). From Johnston *et al.* (2003c).

MPCs remain in the external cell layer of adult zebrafish and are a possible source of myogenic progenitors for MH (Hollway *et al*. 2007). In zebrafish there is an inverse relationship between Pax7 and MRFs in terms of protein expression. Ablation of *myod1* and *myf5* by morpholino antisense RNA in zebrafish increased the number of Pax7 expressing cells (Hammond *et al*. 2007). In Atlantic salmon, we have shown heterochronies in MRF expression (*myf5* and *mrf4*) with respect to developmental stage for different temperature treatments to the "eyed stage" (Macqueen *et al*. 2007), a finding paralleled at the mRNA and protein level in rainbow trout (Xie *et al*. 2001). A plausible working hypothesis is that temperature alters the timing and intensity of expression fields for MRF transcripts with downstream consequences for the number of Pax7 expressing anterior cells. Since the external cell layer MPCs are self-renewing (Hollway *et al*. 2007; Stellabotte *et al*. 2007), an increase in the number derived from the anterior somite could have long-term consequences for muscle fibre recruitment and nuclear accretion. Whatever the mechanism, the finding that a brief period during embryogenesis can "set" the adult final fibre number without significantly compromising somatic growth is of practical significance to the fish farming industry since muscle fibre density has been shown to be an important flesh quality trait affecting texture (Johnston *et al*. 2000). Given the pervasive effects of embryonic environment on adult growth it is also entirely possible that developmental plasticity has long-term effects on other tissues controlling feeding, foraging and growth e.g. the appetite centre in the hypothalamus and/or various endocrine/neuroendocrine tissues.

6. Temperature Acclimation Responses

Temperate fish living in habitats with stable seasonal temperature cues can modify their swimming performance following several weeks acclimation to a new temperature regime (Fry and Hart 1948). The mechanisms underlying the plasticity of locomotory performance are complex and vary between species, but are typically reversible in juvenile and adult stages (Johnston and Temple, 2002). Proximal mechanisms include changes in the relative amounts of cellular organelles (Johnston and Maitland 1980) and altered expression of hundreds of genes (Gracey *et al*. 2004). In the goldfish *Carassius auratus* (Johnston *et al*. 1975) and common carp *C. carpio* (Heap *et al*. 1985) changes in muscle myofibrillar ATPase activity constitute a major element of the acclimation response. The common carp has become a model species for the study of temperature acclimation in fish (Watabe 2002). Studies with fully activated skinned fibres found that maximum tension and shortening speed increased at low temperatures in both fast and slow muscles following a period of cold acclimation (Johnston *et al*. 1985). Changes in the expression of myosin heavy chain (MYH) isoforms play a key role in the plasticity of myofibrillar ATPase activity and contractile properties with temperature acclimation (Hwang *et al*. 1990; Watabe *et al*. 1992; Guo *et al*. 1994). Three distinct MYH cDNAs have been cloned from the fast muscle of the common carp acclimated to either 10°C or 30°C (Imai *et al*. 1997; Hirayama and Watabe 1997). The relative proportions of each isoform varied with acclimation temperature (Imai *et al*. 1997) (Fig. 4); the 10°C-type MYH (MYHF10) and the 30°C-type MYH (MYHF30) cDNAs were the predominant transcripts in 10°C- and 30°C-acclimated fish, respectively. A third cDNA (intermediate-type) was expressed over a relatively broad temperature range (MYHFint) (Imai *et al*. 1997).

Myosin heavy chain expression in fast muscle also varies with acclimation temperature in the medaka *Oryzias latipes* (Liang *et al*. 2007). The complete medaka *MYH*

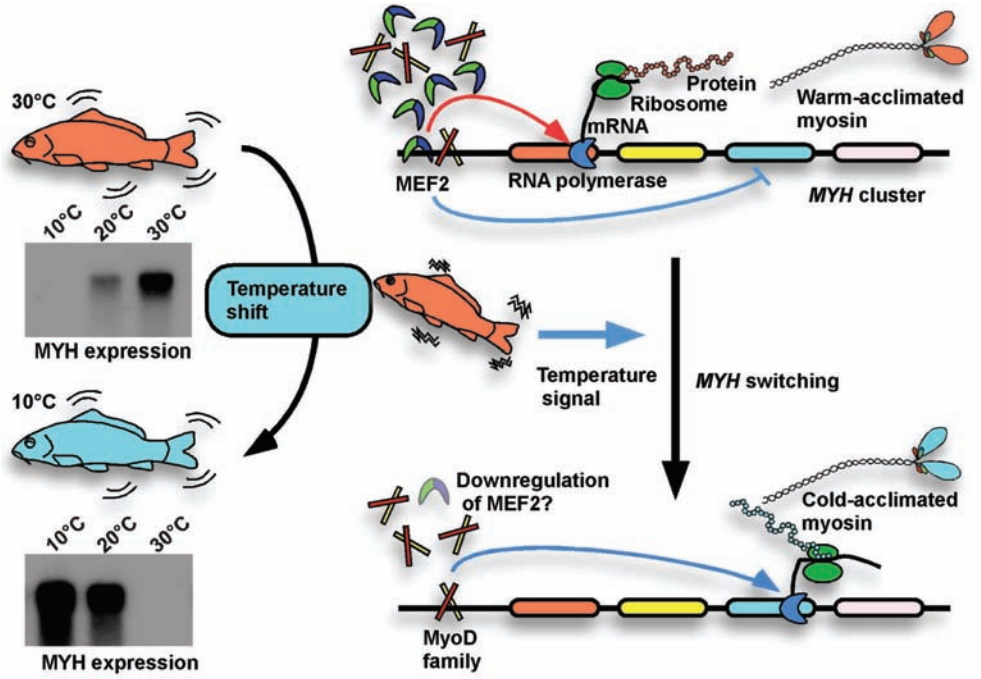

Fig. 4. The expression of fast muscle myosin heavy chain genes with temperature acclimation in the common carp (*Cyprinus carpio* L.) are regulated by members of the MyoD and MEF2 gene families.

(*mMYH*) locus of 219 kbp revealed a cluster of 11 tandemly arrayed *mMYH*s, in which eight genes are transcribed and three are pseudogenes. Expression analysis revealed that two genes were highly expressed in medaka acclimated to either 10 or 30°C whereas there was comparatively low expression level of three other genes exclusively in 30°C-acclimated fish. The ~6 kb 5′-flanking region of the predominantly expressed *MYH*s at 10°C (*mMyH10*) and 30°C (*mMyH30*) contained various *cis*-elements that were putative binding sites for transcription factors such as MyoD, myocyte enhancer factor 2 (MEF2) and nuclear factor of activated T cells (NAFTc). The truncation of the MEF2 binding site located at –966 to –957 in the 5′-flanking region of *mMYH10* resulted in the activation of gene expression at 10°C; a result confirmed using a mutation construct (Liang *et al*. 2008). An E box site for MyoD family members was located at –613 to –607 in *mMYH10*, and was found to be responsible for transcriptional activity. In contrast, the MEF2 binding site located at –960 to –951 of *mMYH30* was involved in the activation of this gene at 30°C. Thus, the MEF2 binding site is crucial for the temperature-dependent expression of *mMYH*s (Fig. 4).

7. Applications and Perspectives for Future Research

An integrated understanding of the genetic pathways regulating muscle development and growth is important for the identification of particular genes that have large phenotypic effects on production and quality traits in aquaculture species. Natural population variation in these key genes will provide the basis for future programs involving

marker-assisted selection to produce superior strains with advantageous characteristics. Such studies may also identify gene and protein biomarkers that could have utility in new types of growth trial, enabling the cost effective development of novel diets and the identification of nutritional components with strong biological effects. Research on the "cross-talk" between regulatory systems controlling growth of the muscle, skeleton and immune system are likely to be of particular importance in understanding abnormal development leading to the relatively high incidence of body deformities observed in some aquaculture species. Although such studies are in their infancy, progress is expected to be rapid, particularly given the adoption of new high throughput sequencing technologies leading to the completion of more fish genomes and transcriptomes to the draft level. This chapter has not considered the role of microRNAs in muscle development and growth, as there are currently few relevant teleost studies. MicroRNAs are conserved small regulatory RNAs that regulate the stability and translation of hundreds of gene targets. The role of microRNAs in fish growth will be an important topic for future research. The finding that embryonic environment has profound affects on adult muscle growth through mechanisms of developmental plasticity is of direct relevance for the production of juvenile fish for on growing. Future research should also focus on maternal or cross-generational effects of broodstock environment on larval and juvenile muscle growth that may act via maternally transmitted mRNA transcripts in the yolk.

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