The Journal of Experimental Biology 213, 200-209
Published by The Company of Biologists 2010
doi:10.1242/jeb.033126

Dietary lipid levels have a remarkable impact on the expression of growth-related genes in Senegalese sole (*Solea senegalensis* Kaup)

C. Campos[1,2], L. M. P. Valente[2,3], P. Borges[2], T. Bizuayehu[1] and J. M. O. Fernandes[1,*]

[1]Faculty of Biosciences and Aquaculture, Bodø University College, NO-8049 Bodø, Norway, [2]CIIMAR–Centro Interdisciplinar de Investigação Marinha e Ambiental, Rua dos Bragas, 177, 4050-123 Porto, Portugal and [3]ICBAS–Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, 4099-003 Porto, Portugal

*Author for correspondence (jorge.fernandes@hibo.no)

Accepted 7 October 2009

SUMMARY

In Senegalese sole (*Solea senegalensis* Kaup), growth is negatively correlated to dietary lipid levels. To understand the molecular basis of this effect a molecular toolbox of 12 genes, including *fgf6*, *fst*, *mstn1*, *myf5*, *mrf4*, *myod1*, *myod2*, *myog*, *myHC*, *mylc2*, *igf1r* and *insr*, was developed. The expression profiles of these genes were investigated in white muscle and liver of fish fed with three dietary lipid levels (4%, 12% and 20%). The expression of *igf-I* and *igf-II* was also examined. *MRFs* and *myosins* were only expressed in the muscle and, except for *myf5*, the general trend was a decrease in expression with an increase in dietary lipids. *Fgf6* was identified for the first time in liver and its expression augmented in hepatic tissues with increasing dietary lipid levels. A similar tendency was observed for *mstn1* and *igf-I*. The opposite was observed for *igf1r* expression in muscle and liver. *Myog*, *mrf4*, *mylc2* and *igf1r* were highly correlated with growth and nutrient utilisation indices. In addition to its practical implications, this work provides a valuable contribution towards our understanding of the genetic networks controlling growth in teleosts.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/213/2/200/DC1

Key words: *Solea senegalensis*, dietary lipids, muscle, growth factors, somatotropic axis, myogenic regulatory factors.

INTRODUCTION

Understanding the regulation of growth in fish is of particular importance for successful aquaculture production of any given species. In carnivorous marine fish, lipids rather than carbohydrates have been used as a major non-protein energy source, as it can lead to protein sparing (Cowey et al., 1975; Dias et al., 1998). In several flatfish species, an increase in the dietary lipid content or a decrease in the protein/fat ratio was shown to have a negative effect on growth or feed efficiency, as reported for the Senegalese sole *Solea senegalensis* (Kaup) (Borges et al., 2009), the turbot *Psetta maxima* (Regost et al., 2001) or the flounder *Paralichthys olivaceus* (Temminck and Schlegel) (Lee et al., 2000). It is known that the composition of the diet, and particularly dietary lipids, can influence the endocrine system and alter the expression of genes of the somatotropic axis (Benedito-Palos et al., 2007; Cameron et al., 2007). But most of the studies on the influence of nutrition in teleost gene expression have focused on the type of amino acids and fatty acids or on the fast and refeeding regimes (Benedito-Palos et al., 2007; Bower et al., 2008; Chauvigné et al., 2003; Hagen et al., 2009; Johansen and Overturf, 2006; Montserrat et al., 2007; Panserat et al., 2008; Rescan et al., 2007), and there is little or no knowledge on the effects of different dietary lipid contents in the regulation of growth genes.

Growth in teleosts consists mainly in the deposition of protein, and is regulated by genes within the somatotropic/IGF (insulin-like growth factor) axis and by different families of growth factors (De-Santis and Jerry, 2007). The growth control at the muscle level is mediated by various genes, namely the myogenic regulatory factors (MRFs), growth factor-like members of the fibroblast growth factor family (FGFs), and the transforming growth factor-β (TGF-β) (De-Santis and Jerry, 2007; Tan and Du, 2002). Striated muscle tissues have myosin as the major structural component. Both heavy (MyHC) and light chains (MyLC) of myosin exist as multiple isoforms that are tissue and/or developmental stage-specific (Funkenstein et al., 2007; Ikeda et al., 2007). The MRFs (MyoD, Myf5, Mrf4 and Myog) are a family of highly conserved proteins that are transcription factors for myogenic genes (for a review, see Bergstrom and Tapscott, 2001). The existence of paralogues and even splice variants of the master transcription factor *myoD* add a new dimension to the myogenic programme. For example, the tiger pufferfish (*Takifugu rubripes* Temminck and Schlegel) expresses alternatively spliced transcripts of *myoD1* splice variants, which are differentially expressed with temperature during early development (Fernandes et al., 2007). Myostatin (*mstn* or *gdf-8*) acts as a potent negative regulator of skeletal muscle mass by depressing the number of myoblasts and the degree of fibre enlargement, and in teleosts it is likely to regulate growth of non-muscle tissues and even influence the development of immune cells (Helterline et al., 2007; Lee and McPherron, 2001; Terova et al., 2006; Xu et al., 2003). Myostatin action is antagonised by follistatin (*fst*), an activin-binding protein that is involved in muscle growth and development (Lee and McPherron, 2001). Members of the FGF family are potent moderators of critical phases of muscle development. In particular, fibroblast growth factor 6 (*fgf6*) may play a crucial role because it has an expression profile essentially restricted to developing and adult skeletal muscle (Terova et al., 2006), and is likely to be associated with the prolonged muscle hyperplasia in fish (Rescan, 1998). Components of the somatotropic axis as insulin-like growth factors (*igf-I* and *igf-II*) are single-chain polypeptides with structural homology to pro-insulin that play a key role in the regulation of metabolism and mytogenic processes (De-Santis and Jerry, 2007; Duan, 1998). Most of Igf's biological actions are mediated by the

receptor for Igf-I, which is present in a wide variety of cell types (Jones and Clemmons, 1995). The number of insulin and Igf-I receptors (*insr* and *igf1r*, respectively) appears to be physiologically regulated in a similar way, with both undertaking changes in number according to the fish nutritional status (Mommsen, 2001; Planas et al., 2000).

The Senegalese sole *S. senegalensis* is a common species of high commercial value in Southern Europe and a promising candidate for commercial scale aquaculture (Dinis et al., 1999; Imsland et al., 2003). Recently, it has been shown that dietary lipid levels have a dramatic influence on the growth of Senegalese sole juveniles (Borges et al., 2009) but the molecular basis of this effect is not known. Therefore, the aims of the present study were: (i) to clone and characterise the Senegalese sole orthologues of key genes known to be correlated with growth and development [fibroblast growth factor 6 (*fgf6*), follistatin (*fst*), myostatin 1 (*mstn1*), myogenic factor 5 (*myf5*), muscle regulatory factor 4 (*mrf4*), two paralogues of the myoblast determination protein (*myod1* and *myod2*), myogenin (*myog*), myosin heavy chain (*myHC*), myosin light chain 2 (*mylc2*), Igf-I receptor (*igf1r*) and insulin receptor (*insr*)], (ii) to identify optimal reference genes for nutrition studies quantifying gene expression, and (iii) to investigate how the expression of these target genes in muscle and liver tissues is influenced by the dietary lipid content.

MATERIALS AND METHODS

Feeding experiment and sample collection

The Senegalese sole feeding experiment took place at the CIIMAR facilities (University of Porto, Porto, Portugal) and included diets with different lipids levels (4%, 12% and 20% dry matter, DM) (Table 1). Crude protein levels were identical for all diets and approximately 56% DM. Triplicate groups of 20 fish with a mean initial body mass of 9.9±1.8 g were distributed among 15 fibreglass tanks (50 cm × 35 cm) for each treatment. Each tank was supplied with filtered, heated (20±1°C) seawater (30‰). Water temperature, O_2, salinity, pH and nitrogenous compounds were monitored regularly during the entire trial, and fish were exposed to an artificial photoperiod of 12 h:12 h light:dark. At the beginning and end of the experiment individual fish masses were recorded. Fish were fed *ad libitum* by automatic feeders and the ration offered was adjusted daily based on the feed losses in each tank. The experiment was performed in accordance with the European Economic Community animal experimentation guidelines directive (86/609/EEC). At the end of the trial, 24 h after their last meal, individual animals were weighed and humanely killed with a sharp blow to the head, following the FELASA category C recommendations. For each diet, samples of fast muscle and liver were carefully macrodissected from eight fish. Tissues were snap frozen in liquid nitrogen and stored at –80°C for three months prior to RNA extraction and cDNA synthesis.

Table 1. Ingredients and proximate composition of the experimental diets with different levels of dietary lipids (4%, 12%, 20%)

	Dietary treatments		
	D4	D12	D20
Ingredients (%)			
Fish meal low temperature	37	37	37
CPSP G	1	3.5	3.5
Squid meal	5	5	5
Soybean meal 48	16	14	9.5
Corn gluten	12.5	12	9
Wheat meal	23	14.5	8
Wheat gluten	3	3.5	9
Gelatin	2	2	2
Fish oil	0	8	16.5
Choline chloride	0.1	0.1	0.1
Lutavit C35	0.03	0.03	0.03
Lutavit E50	0.05	0.05	0.05
Mineral† and vitamin mix*	0.25	0.25	0.25
Betaine	0.07	0.07	0.07
Proximate composition			
Dry matter (DM) (%)	91.30	92.20	92.61
Ash (% DM)	8.48	8.30	7.82
Crude protein (% DM)	57.02	56.82	56.06
Crude fat (% DM)	4.07	13.67	22.50
Gross energy (kJ g^{-1} DM)	20.56	22.87	24.58

*Vitamins (mg or i.u. kg^{-1} diet): vitamin A, 8000 i.u.; vitamin D3, 1700 i.u.; vitamin K3, 10 mg; vitamin B12, 0.02 mg; vitamin B1, 8 mg; vitamin B2, 20 mg; vitamin B6, 10 mg; folic acid, 6 mg; biotin, 0.7 mg; inositol, 300 mg; nicotinic acid, 70 mg; pantothenic acid, 30 mg; vitamin E (Lutavit E50), 300 mg; vitamin C (Lutavit C35), 500 mg; betain (Betafin S1), 500 mg.

†Minerals (g or mg kg^{-1} diet): Mn (manganese oxyde), 20 mg; I (potassium iodide), 1.5 mg; Cu (copper sulphate), 5 mg; Co (cobalt sulphate), 0.1 mg; Mg (magnesium sulphate), 500 mg; Zn (zinc oxide) 30 mg; Se (sodium selenite) 0.3 mg; Fe (iron sulphate), 60 mg; Ca (calcium carbonate), 2.15 g; dibasic calcium phosphate, 5 g; KCl, 1 g; NaCl, 0.4 g.

RNA extraction and cDNA synthesis

Approximately 100 mg of Senegalese sole muscle or liver was placed into Lysing Matrix D tube (QBiogene/Medinor, Oslo, Norway) containing QIAzol (Qiagen, Nydalen, Sweden) and homogenised for 40 s at 6000 r.p.m. using the MagNA Lyser instrument (Roche, Mannheim, Germany). Total RNA was extracted according to the Tri reagent method (Sigma, Oslo, Norway) and treated with the gDNA wipeout buffer supplied with the QuantiTect reverse transcription kit (Qiagen) for 5 min to remove any trace genomic DNA contamination. Assessment of RNA quality was performed by agarose gel electrophoresis on a 1.2% (w/v) agarose gel containing SYBR safe DNA gel stain (Invitrogen, Carlsbad, CA, USA). RNA samples were then quantified with a Nanodrop spectrophotometer (Nanodrop Technologies/Saven Werner, Kristiansand, Norway). Absorbance ratios (260/280 nm) were greater than 1.9, indicating high purity RNA. One microgram of total RNA was used to synthesise cDNA with the QuantiTect reverse transcription kit (Qiagen). The reverse transcription was performed for 30 min at 42°C, and following a 3 min incubation at 95°C to inactivate the Quantiscript reverse transcriptase, the cDNA was requantified using the Nanodrop spectrophotometer.

Gene cloning and sequencing

Igf-I and *igf-II* had already been cloned in Senegalese sole and these sequences were retrieved from the NCBI database (GenBank accession numbers AB248825 and AB248826, respectively). For all of the remaining target genes (*fgf6*, *fst*, *mstn1*, *myf5*, *mrf4*, *myod1*, *myod2*, *myog*, *myHC*, *mylc2*, *igf1r*, *insr*), BLAST similarity searches against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed to identify their orthologues in other teleost species. CLUSTALW alignments (http://align.genome.jp/) were performed and degenerate primers were designed against the most conserved regions of the sequences (Table 2). *Netprimer* (http://www.premierbiosoft.com/netprimer) was used to estimate the melting temperatures of the primers and to investigate the presence of potential dimers and hairpins. Following amplification by polymerase chain reaction (PCR), the products of interest were analysed by agarose gel electrophoresis, excised from the gel with

Table 2. List of degenerate primers used to clone target genes

Gene	Forward sequence (5′→3′)	Reverse sequence (5′→3′)	Accession	Size (bp)
myf5	CMTCCCARGTCTACTACGACA	CTSCCRGGGGAGAAGGTG	FJ515910	604
mrf4	TGATGGACCTTTTTGAGAC	CGATGGAGGASAGRCRSAG	EU934042	471
myog	GCTTTTYGAGACCAACCC	GGAATGTCCAYWGGAAAGGC	EU934044	734
myod1	CTVBTGAAGCCGGAYGAC	CCGTCATGCCRTCGGAGCAG	FJ009109	497
myod2	TAYGATGACCCCTGCTTC	CYACGATGCTGGACARAC	FJ009108	569
mstn1	CAGTRGTTCTGAGYGASCARG	ACCATGGMGGGGATCTTGC	EU934043	932
fst	CCATCATGTTTRGGATGCTG	CTGGGATATGTGGTGTTGTC	EU934045	889
fgf6	AAGGYTCCTCAYCAGTATG	CATAWWCKDGGRAGGAARTG	FJ009110	616
myHC	TCTGCTGMAGAGTGCTGAAAC	GGCTCTCTCMAGGTCCATAC	FJ515911	599
mylc2	GGYTCCTCCAAYGTGTTCTC	TGTGATGACGTAGCAGATKTKC	FJ515912	412
Igf1r	CATCAGYTCGAAYAGCATGTC	CARCCMACCCTGGTCATC	FJ515914	480
insr	GRATAGAGTTYCTCAAYGAAGC	GACGCATYTTRGGRTTGWACTG	FJ515913	598

The GenBank accession numbers and expected amplicon sizes are also indicated.

the QIAquick gel extraction kit (Qiagen) and cloned onto a pCR4-TOPO® plasmid vector (Invitrogen). Cycle sequencing reactions were performed with T3 or T7 primers using the ABI prism BigDye (v.3.1) Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA) and sent for sequencing at the University Hospital North Norway (Tromsø, Norway). DNA sequences were analysed with CodonCode Aligner v.2.0.6 (CodonCode Corporation, Dedham, MA, USA) and their identity determined by BLASTN similarity searches against the NCBI database.

Quantitative real-time PCR (qPCR)

For the cloned target genes, specific primers were designed for qPCR (see Table S1 in supplementary material). Primers for the reference genes were based on *S. senegalensis* sequences retrieved from the NCBI database (Table 3). These primers do not distinguish between known splice variants of the target genes. The genomic sequences of orthologous genes from other teleost species were retrieved from Ensembl (http://www.ensembl.org/) and aligned to the experimental cDNA sequences from Senegalese sole with Spidey (http://www.ncbi.nlm.nih.gov/spidey), in order to predict their intron/exon structure. Whenever possible, primers that span at least one intron/exon border were designed to avoid amplification of potential contaminating genomic DNA. The cDNAs were diluted 50× prior to using them as templates for the qPCR reactions. Quantification of gene expression was performed by qPCR with SYBR Green chemistry (Qiagen) on a LightCycler® 480 (Roche), as detailed elsewhere (Fernandes et al., 2008). The 10 µl reactions were prepared in 96-well plates and included 4 µl of 50×-diluted cDNA template, 1 µl of each primer pair at 5 µmol l^{-1} and 5 µl of QuantiTect SYBR Green containing ROX as reference dye (Qiagen). After sealing the plates with adhesive optical film (Roche), samples were denatured for 15 min at 95°C and then subjected to 45 cycles of amplification, according to the following thermocycling profile: denaturation for 15 s at 94°C, annealing for 20 s at 60°C and extension for 20 s at 72°C. Specificity of the qPCR reaction and the presence of primer dimers were checked by examining the melting curves generated with a dissociation protocol from 65°C to 97°C. Five-point standard curves of a 5-fold dilution series (1:1–1:625) from pooled cDNA were used for PCR efficiency calculation. All samples were run in duplicate and minus reverse transcriptase, and no template controls were included in all plates. A positive plate control was also used. Cycle threshold (C_T) values were determined using the fit-point method using the LightCycler® 480 software with a fluorescence threshold arbitrarily set to 0.2.

Statistical analysis

Evaluation of expression stability for the five potential reference genes (Table 3) was done using the statistical application *geNorm* (Vandesompele et al., 2002). Expression of target genes was evaluated by the relative quantification method as reported in Fernandes et al. (Fernandes et al., 2008). Differences in the expression level of candidate genes with tissue and diet were examined by a one-way analysis of variance (ANOVA) with Holm–Sidak *post-hoc* tests using the SigmaStat statistical package (Systat software, London, UK). Significance levels were set at $P<0.05$. When the data did not meet the normality and/or equal variance requirements, a Kruskal–Wallis one-way ANOVA on ranks with a Dunn's test for *post-hoc* comparisons was performed instead. A Pearson's product–moment correlation (Zar, 1996) was used to compare mean normalised expression data *versus* growth parameters (Borges et al., 2009): daily growth index (DGI), protein efficiency ratio (PER), feed conversion ratio (FCR) and body lipid and protein gains (g kg^{-1} MBM day^{-1}), using the SigmaStat software.

Gene expression data were further subjected to unsupervised hierarchical cluster analysis with GEPAS 4.0 Suite software (http://gepas.bioinfo.cipf.es/), using Pearson's correlation coefficient as a similarity measurement. The silhouette index in ETE Tree Viewer was used to check the quality of each partition.

Table 3. List of the reference gene primers used for real-time PCR

Gene	Accession	Forward sequence (5′→3′)	Reverse sequence (5′→3′)	Size (bp)	*E* (%)	R^2
Actb2	DQ485686	GAAGATGACCCAGATTATGTTTG	CGGAGTCCATGACGATACCAG	119	96	0.997
HSP90AB	AB367527	CATCACCCTGTTTGTGGAGAAG	CAATCTTTGGCTTGTCCTCTG	119	95	0.999
Ubq	AB291588	TCTGCGTGGTGGTCTCATC	TGACCACACTTCTTCTTGCG	135	89	0.984
rpS4	AB291557	CTGCTGGATTCATGGATGTG	GGCAGTGATGCGGTGGAC	100	95	0.999
Eef1a1	AB326302	ATTGGCGGCATTGGAACA	CATCTCCACAGACTTGACCTC	116	94	0.999

For each gene, its GenBank accession number, amplicon size, amplification efficiency (*E*) and correlation coefficient (R^2) of the calibration curve are indicated.

RESULTS

Growth performance

A detailed analysis of the influence of dietary lipids on growth rate and nutrient utilisation has been reported in a paper directly linked to the present study (Borges et al., 2009). In brief, there was an overall decrease in fish growth with a corresponding increase in dietary lipid levels. Sampled fish fed on a diet containing 4% lipids had the highest growth performance ($P<0.05$), which was significantly higher than those fed on a diet containing 20% lipids but was not significantly different from those fed on a diet with 12% lipids.

Cloning of key myogenic genes from *S. senegalensis*

Partial coding sequences of *myf5* (604 bp), *mrf4* (471 bp), *myog* (734 bp), *myod1* (497 bp), *myod2* (569 bp), *mstn1* (932 bp), *fst* (889 bp), *fgf6* (616 bp), *myHC* (599 bp), *mylc2* (412 bp), *igf1r* (480 bp) and *insr* (598 bp) were obtained by homology cloning of Senegalese sole genes against other teleost species (see Table 2 for primers, amplicon size and GenBank accession numbers). *igf-I* and *igf-II* had already been cloned in *S. senegalensis* (Funes et al., 2006), so their public sequences were used. The amino acid identity of Senegalese sole putative proteins with other vertebrate species is presented as an identity matrix in Table S2 (see Table S2 in supplementary material).

Fst, Mstn-1 and Fgf6

Senegalese sole Fst and Mstn1 putative proteins consisted of 295 and 310 amino acids, respectively. CLUSTALW multiple sequence alignments of *S. senegalensis* Fst and their orthologues in other vertebrate species revealed higher amino acid identity values among fish species compared with mammals and birds (see Table S2 and Fig. S1 in supplementary material). Mstn1 protein alignments showed that the domain TGF-β is extremely conserved across different taxa (see Fig. S2 in supplementary material). *Solea senegalensis* Mstn1 shared 88% identity with *P. olivaceus* and 89% with *Dicentrarchus labrax* (Linnaeus) but lower identity values were observed when comparing with non-teleost species (see Table S2 in supplementary material). The Fgf6 putative protein has 204 residues and has 92% and 85% of amino acid identity with *D. labrax* and *Oncorhynchus mykiss* (Walbaum), respectively (see Table S2 and Fig. S3 in supplementary material).

MRFs

From the *S. senegalensis MRFs* mRNA sequences, partial putative proteins were found to consist of 200 (Myf5), 156 (Mrf4), 165 (MyoD1), 189 (MyoD2) and 244 (Myog) amino acid residues. CLUSTALW multiple alignments revealed a generally very high amino acid identity with other teleost species but relatively lower identity values when sequences from *Gallus gallus* (Linnaeus), *Mus musculus* (Linnaeus) or *Homo sapiens* (Linnaeus) were compared (see Table S2 and Figs S4–S8 in supplementary material).

MyHC and Mylc2

The putative proteins of Senegalese sole MyHC and Mylc2 consist of 199 and 137 amino acids residues, respectively, and revealed an exceptionally high degree of conservation throughout different vertebrate taxa, showing values above 90% of amino acid identity with other teleosts, and above 80% with species like *G. gallus*, *M. musculus* or *H. sapiens* (see Table S2 and Figs S9 and S10 in supplementary material).

IR and Igf1R

Igf1R and IR partial putative proteins were found to consist of 160 and 199 amino acid residues, respectively. Multiple CLUSTALW alignments showed that both receptors are extremely conserved among different vertebrate taxa, particularly within teleost species (see Table S2 and Figs S11 and S12 in supplementary material).

Expression profiles of candidate housekeeping genes

All samples had C_T values within the linear dynamic range of the corresponding calibration curves and there was no amplification in the no-template controls. Of the five potential housekeeping genes (*Actb2*, *HSP90AB*, *Ubq*, *rpS4* and *Eef1a1*, see Table 3 for details), *Eef1a1* and *rpS4* were selected by *geNorm* as being the most stable and suitable gene pair for Senegalese sole muscle studies, and were therefore used to normalise the target gene expressions (Fig. 1A). For liver gene expression analysis, *Eef1a1* and *Ubq* were found to be the most stable genes (Fig. 1B). However, the *geNorm* output showed that including one additional reference gene (*Actb2*) improved the reliability of the normalisation factor for the liver (Fig. 1B), because the values for the pairwise variation between two sequential normalisation factors were above the threshold value of 0.15.

Relative expression of target genes

Similarly to what was observed for the reference genes, all samples had C_T values that were within the linear dynamic range of the calibration curves, and the no-template controls produced no amplification. Analysis of relative expression of target genes in fish fed with diets containing 4%, 12% and 20% lipids (D4, D12 and D20, respectively) revealed a number of significant differences in the transcript levels between diets (Figs 2 and 3).

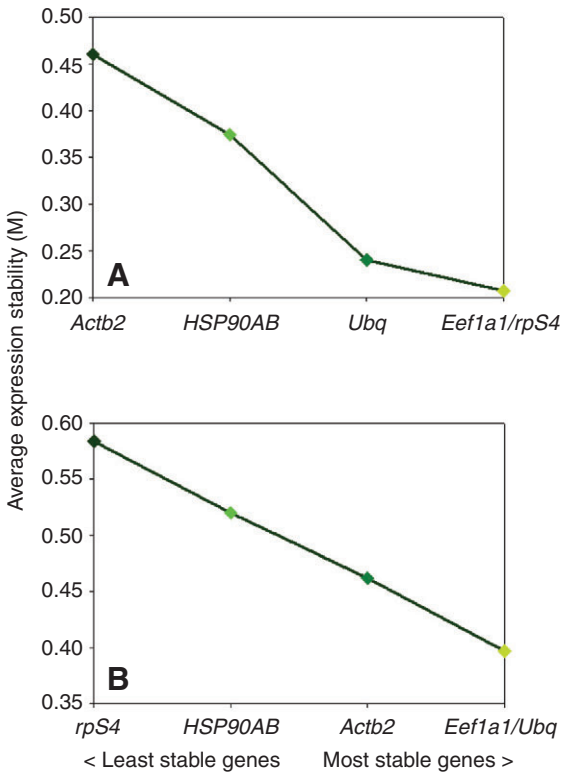


Fig. 1. Ranking of reference genes for muscle (A) and liver (B) according to their expression stability in Senegalese sole juveniles fed with different diets. Mean expression stability values were calculated by *geNorm*. The most stable reference genes for muscle studies were *Eef1a1* and *rpS4*, and for liver were *Eef1a1*, *Ubq* and *Actb2*.

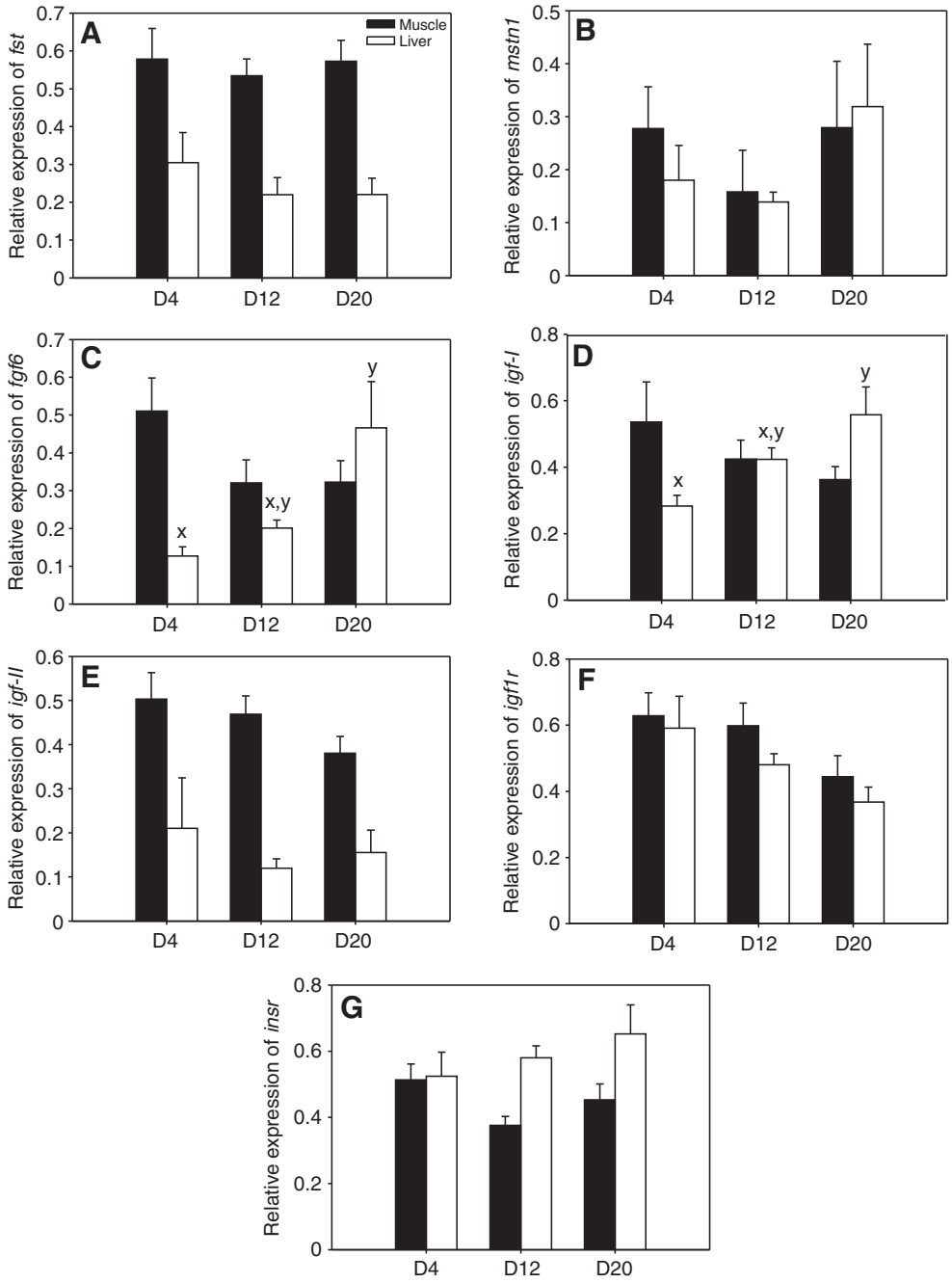


Fig. 2. Relative expression of *fst* (A), *mstn1* (B), *fgf6* (C), *igf-I* (D), *igf-II* (E), *igf1r* (F) and *insr* (G) in fast muscle (black bars) and liver (white bars) of Senegalese sole juveniles fed with different diets (D4, D12 and D20). Transcripts were quantified by qPCR and normalised using the geometric average of suitable reference genes. Data are expressed in arbitrary units. Error bars indicate the standard error of the mean for each treatment (N=8). Different superscript letters indicate significant differences among dietary treatments (a, b, c for muscle; x, y, z for liver).

fst expression decreased moderately from D4 to D12 (Fig. 2A). *mstn1* did not show significant differences among the diets but the highest expression values were revealed in D20 both liver and muscle (Fig. 2B). Relative expression of *fgf6* showed an increase by 3.7-fold in liver transcript levels from D4 to D20, with a significant difference between D4 and D20 ($P<0.05$, Fig. 2C). *igf-I* increased approximately 2-fold from D4 to D20 in liver tissues ($P<0.05$) but no significant differences were seen in the muscle, although a slight decrease in expression was observed from D4 to D20 (Fig. 2D). For *igf-II* no significant differences were observed among the diets, even if a small decrease was seen from D4 to D20 in the muscle (Fig. 2E). *igf1r* showed a descending trend expression from D4 to D20, both in the liver and muscle (Fig. 2F). Relative expression of the *insr* did not significantly differ among diets (Fig. 2G).

myf5 did not differ significantly among treatments, although the highest expression value in muscle was observed in D20 and the lowest in D4 (Fig. 3A). In muscle tissues, *mrf4* mRNA levels decreased considerably from D4 to D12, and from D12 to D20 ($P<0.05$) (Fig. 3B). The paralogues *myod1* and *myod2* showed no significant differences among the diets (Fig. 3C,D). However, there was a small decrease in expression from D4 to D12 and to D20, although for *myod2* the relative expression in fish fed with 20% lipids was slightly superior than those fed with 12% lipids (Fig. 3D). *myog* expression profiles did not reveal significant differences among treatments but showed a downward trend in relative expression from D4 to D20 (Fig. 3E). *myHC* did not show any significant differences among diets but the highest expression value was observed in D4 (Fig. 3F). For *mylc2*, a significant decrease by 1.5-fold in transcript levels was seen between D4 and D20 ($P<0.05$, Fig. 3G).

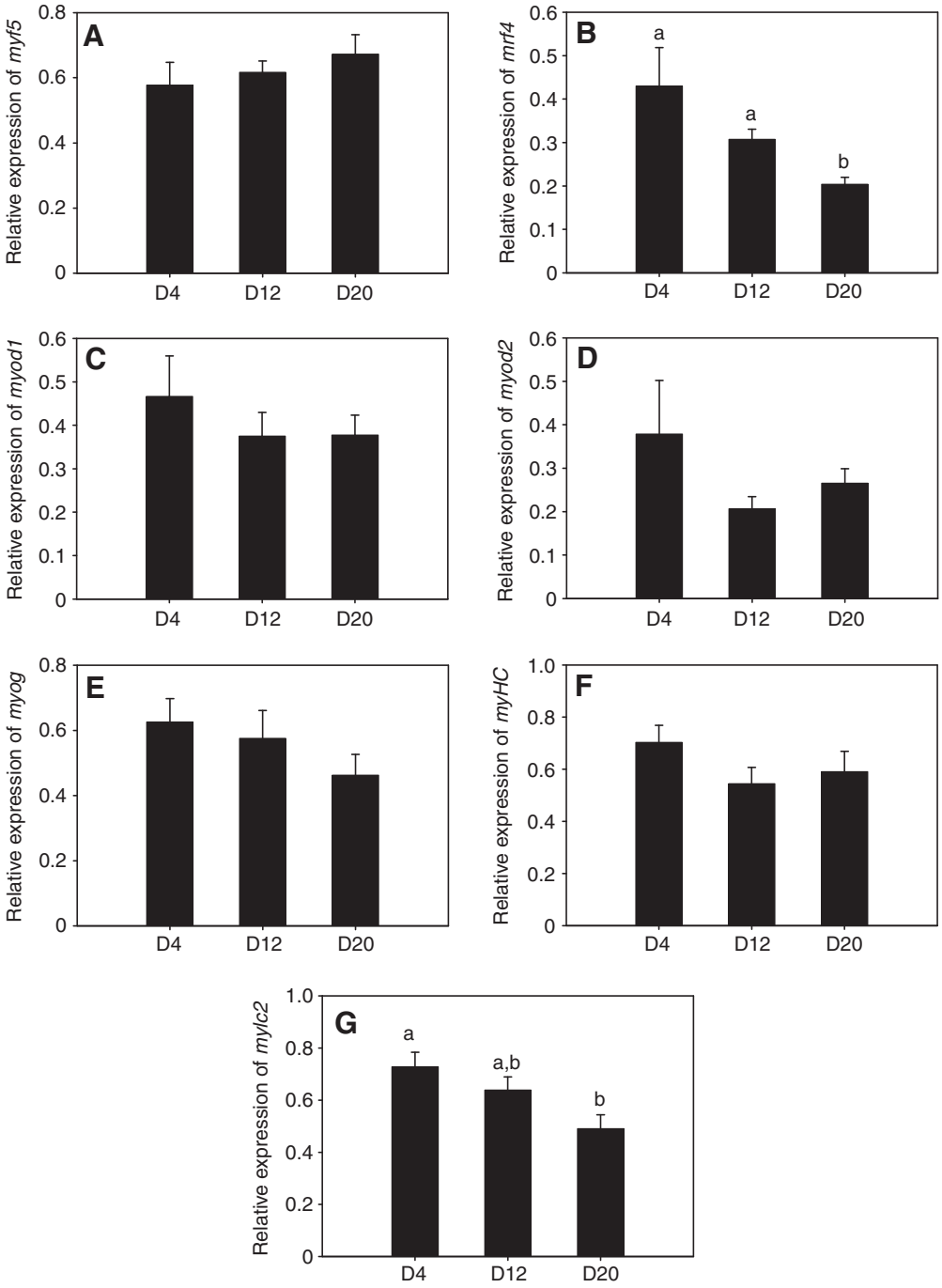


Fig. 3. Relative expression of *myf5* (A), *mrf4* (B), *myod1* (C), *myod2* (D), *myog* (E), *myHC* (F) and *mylc2* (G) in fast muscle of Senegalese sole juveniles fed with different diets (D4, D12 and D20). Transcripts were quantified by qPCR and normalised using the geometric average of suitable reference genes. Data are expressed in arbitrary units. Error bars indicate the standard error of the mean for each treatment (N=8). Different superscript letters indicate significant differences among dietary treatments (a, b, c for muscle; x, y, z for liver).

Gene expression and growth parameters

fgf6 expression in muscle was positively correlated with PER (P<0.05, R=0.77) and negatively correlated with FCR (P<0.05, R=–0.79) (Table 4). Protein gain was positively correlated with *myog* (P<0.05, R=0.90) and *mrf4* (P<0.05, R=0.86) expression in the muscle. DGI was also positively correlated with *myog* (P<0.05, R=0.95) and *mrf4* (P<0.05, R=0.92) expression, as well as PER (P<0.05, R=0.79 for *myog*, and P<0.05, R=0.84 for *mrf4*). *mylc2* expression in the muscle was positively correlated with body protein gain (P<0.05, R=0.76) and PER (P<0.05, R=0.76) but negatively correlated with lipid gain (P<0.05, R=–0.82) and FCR (P<0.05, R=–0.84). A significant but negative correlation existed between total lipid gain and *myHC* expression in the muscle (P<0.05, R=–0.78) (Table 4).

Positive correlations were found between body protein gain and *igf1r* expression in the liver (P<0.05, R=0.89) and also with DGI (P<0.05, R=0.80) as well with PER (P<0.05, R=0.88); however, a negative correlation occurred between *igf1r* expression in the liver and FCR (P<0.05, R=–0.91) (Table 4).

The relationships between *igf-I* expression in muscle and DGI or protein gain were not significant (R=0.68 and R=0.66, respectively). *mylc2* was not significantly correlated with DGI but had a high correlation value (R=0.70).

Hierarchical clustering of gene expression

Hierarchical clustering of gene expression was used as a visualisation tool to identify expression patterns among replicates. Genes were clustered vertically according to the similarity in their expression

across different diets (Fig. 4). In fast muscle, *mstn1* was placed in a separate node relative to all of the other genes, indicating a distinct expression pattern whereas the MRFs *mrf4*, *myog* and *myod1* were clustered together (Fig. 4A). In the liver, two main clusters were obtained: one with *igf1r*, *igf-II* and *fst*, and another containing with *fgf6*, *igf-I*, *insr* and *mstn1*, in which *msnt1* occupied a superior hierarchical position (Fig. 4B).

DISCUSSION

Understanding the underlying mechanisms of growth in fish has been a major focus for an effective and successful aquaculture production, and the nutritional impact on gene regulation has been receiving increased attention in aquatic research (for reviews, see Duan, 1998; Johnston et al., 2008). The present study focused on the nutritional modulation of the expression of key growth related genes in Senegalese sole. Differences in growth caused by dietary lipid content have been reported for this species where high-fat diets increased whole-body fat as well as dietary treatments affecting tissue lipid content (Dias et al., 2004). Moreover, the lipogenic pathway is more susceptible to regulation by lipid intake than the glycolytic pathway (Dias et al., 2004). The complete report of the present experiment concerning growth parameters is detailed in Borges et al. (Borges et al., 2009).

Choosing appropriate housekeeping genes is an essential step prior to any gene expression study. Our study examined five potential reference genes that have been tested in flatfish (Fernandes et al., 2008; Infante et al., 2008), and according to the *geNorm* application, *Eef1a1* and *rpS4* were selected for accurate normalisation of target gene expression in muscle whereas *Eef1a1*, *Ubq* and *Actb2* were selected for normalisation of gene expression in liver.

The present work identified 12 new partial coding sequences for key genes involved in growth and development in *S. senegalensis* (Table 2), and their putative proteins showed high conservation across various teleost species (see Table S2 in supplementary material). In particular, *myod1* and *myod2* paralogues were also identified in Senegalese sole. When comparing with non-teleost protein sequences, the myosins, Igf1R and IR partial proteins of Senegalese sole were the most conserved genes (see Table S2 in supplementary material), consistent with the fact that they share structural features and functional similarities with fish and other vertebrate counterparts (Elies et al., 1999; Ikeda et al., 2007; Planas et al., 2000). The Mrf4 partial amino acid sequence revealed a reasonably high identity with mammals and birds, and follistatin also showed high identity across taxa, as previously found for the gilthead seabream *Sparus aurata* (Linnaeus) (Funkenstein et al., 2009). The other Mrfs had a low degree of conservation across taxa, similar to what was reported for other teleosts (Gregory et al., 2004; Johansen and Overturf, 2005); however, the specific domain, bHLH was always highly conserved (see Figs S4–S8 in supplementary material), which is consistent with other studies (De-Santis and Jerry, 2007). Fgf6 partial sequence was less conserved when compared with non-teleost species, and it showed similar identity with *M. musculus*, *H. sapiens* and *O. mykiss* (Rescan, 1998). The *mstn* transcript was identified as *mstn1*, and the TGF-β specific domain of the protein was exceptionally conserved (see Fig. S2 in supplementary material), suggesting a high degree of conservation in gene function.

Growth factors such as Mstn have been identified as key peptides in the regulation of myogenesis in vertebrates by repressing skeletal muscle growth through inhibiting both muscle cell hypertrophy and hyperplasia (Lee and McPherron, 2001; Terova et al., 2006; Thomas et al., 2000; Xu et al., 2003). In teleosts, including the flatfish *P. olivaceus*, the ubiquitous expression of *mstn* suggests different physiological functions other than skeletal muscle growth and it proposed that *mstn* might even influence the development of immune cells (Funkenstein et al., 2009; Gregory et al., 2004; Zhong et al., 2008). In the present study, no significant differences were observed for the *mstn1* expression profile among the treatments but the highest expression was seen in fish from D20 for both muscle and liver (Fig. 2B), supporting the idea that Mstn limits growth and can be nutritionally regulated. Mstn has been shown to be involved in compensatory growth induced by refeeding and upregulated during fasting in sea bass (Terova et al., 2006). However, in the present work, fish from D12 showed the lowest mRNA signal and, apparently with this intermediate diet, growth could be less restrained by *mstn1* and more controlled by other growth factors.

No correlation was found between *S. senegalensis* growth and *fst* expression, but a negative correlation between total lipid gain and *fst* expression in the liver may indicate that liver metabolism

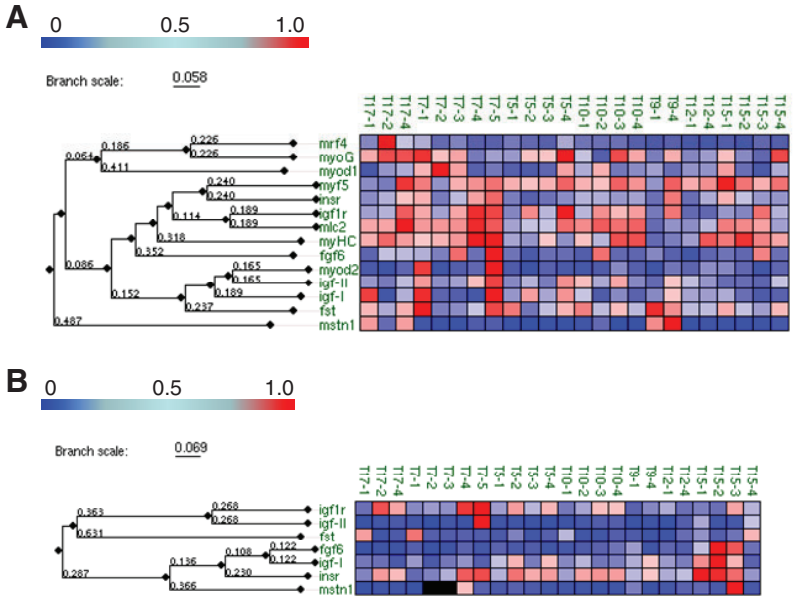


Fig. 4. Unsupervised hierarchical clustering analysis of the genes in muscle (A) and liver (B) according to the similarity in their expression across different diets. Each row represents the expression pattern of a single gene across diets and each column corresponds to a single sample. Expression levels are represented by a colour tag, with red representing the highest levels and blue the lowest levels of expression. Missing values are indicated by black squares.

Table 4. Correlations between gene expression in muscle (M) and liver (L) and growth parameters in Senegalese sole

Gene	Tissue	DGI*			PER†			FCR‡			Protein gain (g kg^{-1} MBM day^{-1})			Lipid gain (g kg^{-1} MBM day^{-1})		
		D4	D12	D20	D4	D12	D20	D4	D12	D20	D4	D12	D20	D4	D12	D20
		1.22^{a}± 0.16	0.94^{b}± 0.02	0.81^{b}± 0.08	1.70^{a}± 0.08	1.06^{b}± 0.06	0.83^{c}± 0.07	1.04^{c}± 0.05	1.66^{b}± 0.09	2.16^{a}± 0.18	2.00^{a}± 0.09	1.61^{b}± 0.08	1.35^{b}± 0.15	0.39± 0.001	0.52± 0.01	0.56± 0.09
fgf6	M				P<0.05, R=0.77			P<0.05, R=–0.79						P<0.05, R=–0.90		
fst	L															
myog	M		P<0.05, R=0.95			P<0.05, R=0.79			P<0.05, R=–0.84			P<0.05, R=0.90				
mrf4	M		P<0.05, R=0.92			P<0.05, R=0.84			P<0.05, R=–0.82			P<0.05, R=0.86				
igf1r	L		P<0.05, R=0.80			P<0.05, R=0.88			P<0.05, R=–0.91			P<0.05, R=0.89		P<0.05, R=–0.91		
mylc2	M					P<0.05, R=0.76			P<0.05, R=–0.84			P<0.05, R=0.76		P<0.05, R=–0.82		
myHC	M													P<0.05, R=–0.78		

DGI, daily growth index; PER, protein efficiency ratio; FCR, feed conversion ratio; MBM, mean body mass.
a,b,c Mean values within a row with unlike superscript letters were significantly different (P<0.05).
*DGI 100 × [(final body mass)$^{1/3}$ – (initial body mass)$^{1/3}$]/days.
‡FCR‡ dry feed intake/mass gain.
†PER‡ weight gain/crude protein intake.

is affected by lipid intake, and the importance of this protein in growth. Fst is known to inhibit Mstn but it is likely that the changes in *mstn1* expression were not sufficient to promote any adjustment in *fst* mRNA signal.

fgf6 was found to be expressed in both muscle and liver of Senegalese sole (Fig. 2C). To our knowledge, this is the first time that this fibroblast growth factor was detected in hepatic tissue. Moreover, its expression in the liver was upregulated by 3.7-fold from fish fed with D4 to D20 (Fig. 2C). The prolonged muscle hyperplasia in fish is associated with the continuous expression of *fgf6* until the adult stage, and in trout, accumulation of *fgf6* transcripts was seen in muscle, testis and brain tissues but not expressed in liver tissue (Rescan, 1998). In our study, *fgf6* expression in *S. senegalensis* fast muscle showed a decrease (although not significant) with an increase in dietary lipid and, interestingly, it was positively correlated with PER but negatively with FCR, suggesting an important role of *fgf6* in growth regulation. However, the significant increases in the transcript levels in hepatic tissues are not easily explained, because this is the first time that *fgf6* was reported in the liver. As pointed out by Rescan (Rescan, 1998), *fgf6* transcription in tissues other than muscle suggests a conservation of the regulatory elements within the promoter driving the tissue-specific expression.

In fish, nutritional status has an influence in the *igf-I* expression patterns (Duan, 1998), and *igf-I* mRNA levels have been extensively shown to be regulated by starvation and/or different diet composition, highlighting the complexity of physiological regulation of growth in fish (Benedito-Palos et al., 2007; Cameron et al., 2007; Chauvigné et al., 2003; Duan, 1998; Hagen et al., 2009; Montserrat et al., 2007). Increases in muscle *igf-I* transcript levels after refeeding were observed in the Atlantic halibut *Hippoglossus hippoglossus* (Linnaeus) (Hagen et al., 2009), in the muscle and liver of seabream *S. aurata* (Benedito-Palos et al., 2007) and it was upregulated in the switching to fast growth in the Atlantic salmon *Salmo salar* L. (Bower et al., 2008). In the present study, *igf-I* expression in the liver increased significantly from D4 to D20, whereas a slight decrease in the muscle was observed (Fig. 2D). Hepatic transcript levels were found to be positively correlated with fish growth in coho salmon *Oncorhynchus kisutch* (Duan et al., 1995) and in Nile tilapia, *Oncorhynchus niloticus* (Cruz et al., 2006) but our findings disagreed with these results, because significantly higher hepatic mRNA levels were found in fish with the lowest growth rate (D20) (Fig. 2). The positive relationships between *igf-I* expression in the muscle with DGI and protein gain may also indicate the importance of this growth factor in fish muscle growth. An opposite trend was observed for the *igf1r* signal in the muscle and liver, in which there was a decrease in expression from D4 to D20 (Fig. 2F). Moreover, the significant correlations obtained between *igf1r* expression in hepatic tissue and protein/lipid gain, DGI, PER and FCR highlight the relationship between *igf1r* expression and growth, as well as how this receptor is influenced by nutritional factors. Our results for *igf1r* expression patterns, even though not significantly different, are in line with those reported by Elies et al. (Elies et al., 1999) in turbot, where a higher signal was observed in muscle tissues, suggesting a positive correlation with the continuous growth of fish. In our study, a general downregulation of *igf-II* was observed from D4 to D20, showing a parallel with the decrease in fish growth (Fig. 2E). Fasting and refeeding experiments showed that the abundance of *igf-II* in the muscle increased during fasting in Atlantic halibut (Hagen et al., 2009). In *S. aurata*, *igf-II* mRNA levels in the skeletal muscle were downregulated upon replacement of fish oil by vegetable oil in the diets, and total

replacement of fish oil resulted in a decrease in growth (Benedito-Palos et al., 2007). Our results seem to support the hypothesis that in fish Igf-II may have an important role in growth throughout life, and its expression is upregulated in conditions were growth is promoted.

The number of insulin receptors can undergo changes in number, and it is known that the typical low number of insulin receptors in fish tissues is upregulated after an increase in the circulating insulin levels in order to augment the tissue response to insulin (Mommsen, 2001; Parrizas et al., 1994; Planas et al., 2000). In the present study, it was found that the amount of hepatic *insr* transcripts showed a non-significant but regular increase that paralleled the increase in dietary lipid levels whereas in the muscle a small transcript decline between D4 and the diet with highest fat content was observed (Fig. 2G). Fish, particularly carnivorous species, show insulin resistance-like metabolic behaviour (Moon, 2001). In mammals, levels of storage triglyceride in skeletal muscle are inversely related to insulin action (Storlien et al., 1997), and high fat diets have been positively related with hyperinsulinemia, hyperglycaemia and with decreases in the responsiveness of muscle glucose transporter GLUT4 to insulin (Hansen et al., 1998; Kim et al., 2000; Storlien et al., 1997). The ascending trend in *insr* transcript signal in Senegalese sole liver tissue from D4 to D20 seems to indicate the response of the liver to a great increase of insulin in the blood stream. However, plasma insulin was not measured in our study, and differences in *insr* expression across the diets were not significant, so one can only speculate about what was observed in the expression trends.

Except for *myf5*, the expression patterns of the *MRFs* revealed a general descending trend from fish fed with D4 to D20, and were only expressed in the muscle (Fig. 3A–E). The significant correlations obtained between *mrf4* and *myog* expression with DGI, PER, FCR and protein gain probably point to the importance of these two *MRFs* in the growth and protein accretion processes. *myog* and *mrf4* act in the terminal differentiation and fusion of myotubes, and *mrf4* is also a determination gene (Johansen and Overturf, 2005; Kassar-Duchossoy et al., 2004; Pownall et al., 2002), so the lower growth of the fish from D20 may be related with less myoblast specification and proliferation. Studies in rainbow trout showed that starvation had a dramatic impact in *myog* mRNA abundance in the muscle, with a great decrease in 30 days of starvation and an increase with refeeding (Johansen and Overturf, 2006). In our study, *myf5* mRNA abundance in the muscle slightly increased from fish fed with 4% lipids to 20% lipids (Fig. 3A). This opposite trend is not easy to explain, and little is known on the nutritional regulation of MRFs. However in mice, Myf5 is involved in muscle homeostasis and in acute and chronic regenerative myogenesis (Gayraud-Morel et al., 2007), so perhaps the trend in *myf5* expression that we observed in Senegalese sole muscle could be related with an attempt to sustain growth in adverse conditions. Expression of *myoD* paralogues was not significantly different among treatments, although the fish from D4 had the highest amount of transcripts (Fig. 3C,D), which can be related with the findings for the Antarctic plunder fish *Harpagifer bispinis*, where the expression of *myoD* was enhanced by feeding, suggesting an activation in the proliferation of myogenic progenitor cells (Brodeur et al., 2003).

Senegalese sole *myHC* and *mylc2* were only expressed in the muscle (Fig. 3F,G). In *mylc2* transcript levels, a significant decrease was seen between D4 and D20 (Fig. 3G), which could be related with fish growth performance, supported by the positive correlations between PER and total protein gain with *mylc2* expression in the muscle, and the negative correlation between FCR and lipid gain with *mylc2* expression (Table 4). Nevertheless, little is known about the specific regulation of MyLC (Funkenstein et al., 2007). *myHC* expression did not show any significant variation among diets but was negatively correlated with total body lipid gain (Table 4). In the Atlantic salmon it was found that *myHC* expression was highly correlated with protein accretion, and its expression could be used as a potential marker for fish growth (Hevrøy et al., 2006) but in Senegalese sole *mylc2* seems a more proper molecular marker for growth.

Hierarchical clustering of genes in fast muscle separated *mstn1* from all the other genes (Fig. 4A). Its position in the tree is in agreement with the hypothesis that Mstn1 may act in a superiorly hierarchical position relatively to other growth factors (Fig. 4A), repressing skeletal muscle growth in the smaller fish. The two main clusters obtained in hepatic tissue were in line with the gene expression results, as one group was upregulated from D4 to D20, and the other group showed a slight decreasing trend in expression (Fig. 4B).

Our findings strongly suggest that dietary lipid level has a great impact on the expression of genes related with growth in *S. senegalensis*. Transcripts in the liver and muscle showed different changes according to dietary lipid content and gene expression in these tissues seemed to be differently regulated with respect to nutritional status, as revealed by the gene clustering analysis. Importantly, the expression of *mylc2* and *mrf4* in fast muscle was significantly influenced by dietary lipids, and their expression levels correlated with several growth parameters, suggesting that they could be useful molecular markers for growth in Senegalese sole. Although not exhaustive, this study was a first approach to determine nutritional gene regulation in Senegalese sole. In addition to insulin, it would be of great interest in the future to examine for genes such as somatostatin and growth hormone. It is also essential to investigate the nutritional regulation of growth-related genes during ontogeny of Senegalese sole is also required.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the technical assistance provided by Marion Nilsen (Bodø University College, Norway). This work was financed by a grant from the Research Council of Norway to J.F. C.C. was supported by a grant from the EU COST-LarvaNet Action (Ref. 01/2008-COST-STSM-FA0801-3610).

REFERENCES

Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J. A., Kaushik, S. and Perez-Sanchez, J. (2007). Combined replacement of fish meal and oil in practical diets for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): Networking of systemic and local components of GH/IGF axis. *Aquaculture* **267**, 199-212.

Bergstrom, D. A. and Tapscott, S. J. (2001). Molecular distinction between specification and differentiation in the myogenic basic helix-loop-helix transcription factor family. *Mol. Cell. Biol.* **21**, 2404-2412.

Borges, P., Oliveira, B., Casal, S., Dias, J., Conceição, L. and Valente, L. M. P. (2009). Dietary lipid level affects growth performance and nutrient utilization of Senegalese sole (*Solea senegalensis*) juveniles. *Brit. J. Nutr.* **3055**, 17-53.

Bower, N. I., Li, X., Taylor, R. and Johnston, I. A. (2008). Switching to fast growth: the insulin-like growth factor (IGF) system in skeletal muscle of Atlantic salmon. *J. Exp. Biol.* **211**, 3859-3870.

Brodeur, J. C., Calvo, J. and Johnston, I. A. (2003). Proliferation of myogenic progenitor cells following feeding in the sub-antarctic notothenioid fish *Harpagifer bispinis*. *J. Exp. Biol.* **206**, 163-169.

Cameron, C., Moccia, R., Azevedo, P. A. and Leatherland, J. F. (2007). Effect of diet and ration on the relationship between plasma GH and IGF-1 concentrations in Arctic charr, *Salvelinus alpinus* (L.). *Aquac. Res.* **38**, 877-886.

Chauvigné, F., Gabillard, J. C., Weil, C. and Rescan, P. Y. (2003). Effect of refeeding on IGFI, IGFII, IGF receptors, FGF2, FGF6, and myostatin mRNA expression in rainbow trout myotomal muscle. *Gen. Comp. Endocrinol.* **132**, 209-215.

Cowey, C. B., Adron, J. W., Brown, D. A. and Shanks, A. M. (1975). Studies on nutrition of marine flatfish-metabolism of glucose by plaice (*Pleuronectes platessa*). *Br. J. Nutr.* **33**, 219-231.

Cruz, E. M. V., Brown, C. L., Luckenbach, J. A., Picha, M. E., Bolivar, R. B. and Borski, R. J. (2006). Insulin-like growth factor-I cDNA cloning, gene expression and potential use as a growth rate indicator in Nile tilapia, *Oreochromis niloticus*. *Aquaculture* **251**, 585-595.

De-Santis, C. and Jerry, D. R. (2007). Candidate growth genes in finfish – where should we be looking? *Aquaculture* **272**, 22-38.

Dias, J., Alvarez, M. J., Diez, A., Arzel, J., Corraze, G., Bautista, J. M. and Kaushik, S. J. (1998). Regulation of hepatic lipogenesis by dietary protein/energy in juvenile European seabass (*Dicentrarchus labrax*). *Aquaculture* **161**, 169-186.

Dias, J., Rueda-Jasso, R., Panserat, S., da, Conceicao, L. E. C., Gomes, E. F. and Dinis, M. T. (2004). Effect of dietary carbohydrate-to-lipid ratios on growth, lipid deposition and metabolic hepatic enzymes in juvenile Senegalese sole (*Solea senegalensis*, Kaup). *Aquac. Res.* **35**, 1122-1130.

Dinis, M. T., Ribeiro, L., Soares, F. and Sarasquete, C. (1999). A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. *Aquaculture* **176**, 27-38.

Duan, C. (1998). Nutritional and developmental regulation of insulin-like growth factors in fish. *J. Nutr.* **128**, 306-314.

Duan, C., Plisetskaya, E. M. and Dickhoff, W. W. (1995). Expression of insulin-like growth factor I in normally and abnormally developing coho salmon (*Oncorhynchus kisutch*). *Endocrinology* **136**, 446-452.

Elies, G., Duval, H., Bonnec, G., Wolff, J., Boeuf, G. and Boujard, D. (1999). Insulin and insulin-like growth factor-1 receptors in an evoluted fish, the turbot: cDNA cloning and mRNA expression. *Mol. Cell. Endocrinol.* **158**, 173-185.

Fernandes, J. M. O., Kinghorn, J. R. and Johnston, I. A. (2007). Differential regulation of multiple alternatively spliced transcripts of *MyoD*. *Gene.* **391**, 178-185.

Fernandes, J. M. O., Mommens, M., Hagen, O., Babiak, I. and Solberg, C. (2008). Selection of suitable reference genes for real-time PCR studies of Atlantic halibut development. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **150**, 23-32.

Funes, V., Asensio, E., Ponce, M., Infante, C., Canavate, J. P. and Manchado, M. (2006). Insulin-like growth factors I and II in the sole *Solea senegalensis*: cDNA cloning and quantitation of gene expression in tissues and during larval development. *Gen. Comp. Endocrinol.* **149**, 166-172.

Funkenstein, B., Skopal, T., Rapoport, B., Rebhan, Y., Du, S. J. and Radaelli, G. (2007). Characterization and functional analysis of the 5 ' flanking region of myosin light chain-2 gene expressed in white muscle of the gilthead sea bream (*Sparus aurata*). *Comp. Biochem. Physiol. D Genomics & Proteomics* **2**, 187-199.

Funkenstein, B., Rebhan, Y. and Skopal, T. (2009). Molecular cloning and characterization of follistatin in the gilthead sea bream, *Sparus aurata*. *Mol. Biol. Rep.* **36**, 501-511.

Gayraud-Morel, B., Chretien, F., Flamant, P., Gomes, D., Zammit, P. S. and Tajbakhsh, S. (2007). A role for the myogenic determination gene Myf5 in adult regenerative myogenesis. *Dev. Biol.* **312**, 13-28.

Gregory, D. J., Waldbieser, G. C. and Bosworth, B. G. (2004). Cloning and characterization of myogenic regulatory genes in three Ictalurid species. *Anim. Genet.* **35**, 425-430.

Hagen, Ø., Fernandes, J. M., Solberg, C. and Johnston, I. A. (2009). Expression of growth-related genes in muscle during fasting and refeeding of juvenile Atlantic halibut, *Hippoglossus hippoglossus* L. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **152**, 47-53.

Hansen, P. A., Han, D. H., Marshall, B. A., Nolte, L. A., Chen, M. M., Mueckler, M. and Holloszy, J. O. (1998). A high fat diet impairs stimulation of glucose transport in muscle. Functional evaluation of potential mechanisms. *J. Biol. Chem.* **273**, 26157-26163.

Helterline, D. L., Garikipati, D., Stenkamp, D. L. and Rodgers, B. D. (2007). Embryonic and tissue-specific regulation of myostatin-1 and -2 gene expression in zebrafish. *Gen. Comp. Endocrinol.* **151**, 90-97.

Hevrøy, E. M., Jordal, A. E. O., Hordvik, I., Espe, M., Hemre, G. I. and Olsvik, P. A. (2006). Myosin heavy chain mRNA expression correlates higher with muscle protein accretion than growth in Atlantic salmon, *Salmo salar*. *Aquaculture* **252**, 453-461.

Ikeda, D., Ono, Y., Snell, P., Edwards, Y. J., Elgar, G. and Watabe, S. (2007). Divergent evolution of the myosin heavy chain gene family in fish and tetrapods: evidence from comparative genomic analysis. *Physiol. Genomics* **32**, 1-15.

Imsland, A. K., Foss, A., Conceicao, L. E. C., Dinis, M. T., Delbare, D., Schram, E., Kamstra, A., Rema, P. and White, P. (2003). A review of the culture potential of *Solea solea* and *S. senegalensis*. *Rev. Fish Biol. Fish.* **13**, 379-407.

Infante, C., Matsuoka, M. P., Asensio, E., Canavate, J. P., Reith, M. and Manchado, M. (2008). Selection of housekeeping genes for gene expression studies in larvae from flatfish using real-time PCR. *BMC Mol. Biol.* **9**, 28.

Johansen, K. A. and Overturf, K. (2005). Sequence, conservation, and quantitative expression of rainbow trout Myf5. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **140**, 533-541.

Johansen, K. A. and Overturf, K. (2006). Alterations in expression of genes associated with muscle metabolism and growth during nutritional restriction and refeeding in rainbow trout. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **144**, 119-127.

Johnston, I. A., Macqueen, D. J. and Watabe, S. (2008). Molecular biotechnology of development and growth in fish muscle. In *Fisheries for Global Welfare and Environment, 5th World Fisheries Congress* (ed. K. Tsukamoto, T. Kawamura, T. Takeuchi, T. D. Beard Jr and M. J. Kaiser), pp. 241-262. TERRAPUB: Tokyo.

Jones, J. I. and Clemmons, D. R. (1995). Insulin-like growth factors and their binding proteins: biological actions. *Endocr. Rev.* **16**, 3-34.

Kassar-Duchossoy, L., Gayraud-Morel, B., Gomes, D., Rocancourt, D., Buckingham, M., Shinin, V. and Tajbakhsh, S. (2004). Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. *Nature* **431**, 466-471.

Kim, J. Y., Nolte, L. A., Hansen, P. A., Han, D. H., Ferguson, K., Thompson, P. A. and Holloszy, J. O. (2000). High-fat diet-induced muscle insulin resistance: relationship to visceral fat mass. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **279**, 2057-2065.

Lee, S. J. and McPherron, A. C. (2001). Regulation of myostatin activity and muscle growth. *Proc. Natl. Acad. Sci. USA* **98**, 9306-9311.

Lee, S. M., Cho, S. H. and Kim, K. D. (2000). Effects of dietary protein and energy levels on growth and body composition of juvenile flounder *Paralichthys olivaceus*. *J. W. Aquac. Soc.* **31**, 306-315.

Mommsen, T. P. (2001). Paradigms of growth in fish. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **129**, 207-219.

Montserrat, N., Gabillard, J. C., Capilla, E., Navarro, M. I. and Gutierrez, J. (2007). Role of insulin, insulin-like growth factors, and muscle regulatory factors in the compensatory growth of the trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **150**, 462-472.

Moon, T. W. (2001). Glucose intolerance in teleost fish: fact or fiction? *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **129**, 243-249.

Panserat, S., Kolditz, C., Richard, N., Plagnes-Juan, E., Piumi, F., Esquerre, D., Medale, F., Corraze, G. and Kaushik, S. (2008). Hepatic gene expression profiles in juvenile rainbow trout (*Oncorhynchus mykiss*) fed fishmeal or fish oil-free diets. *Br. J. Nutr.* **100**, 953-967.

Parrizas, M., Banos, N., Baro, J., Planas, J. and Gutierrez, J. (1994). Up-regulation of insulin binding in fish skeletal-muscle by high insulin levels. *Regul. Peptides* **53**, 211-222.

Planas, J. V., Mendez, E., Banos, N., Capilla, E., Castillo, J., Navarro, I. and Gutierrez, J. (2000). Fish insulin, IGF-I and IGP-II receptors: a phylogenetic approach. *Am. Zool.* **40**, 223-233.

Pownall, M. E., Gustafsson, M. K. and Emerson, C. P. (2002). Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Ann. Rev. Cell Develop. Biol.* **18**, 747-783.

Regost, C., Arzel, J., Cardinal, M., Robin, J., Laroche, M. and Kaushik, S. J. (2001). Dietary lipid level, hepatic lipogenesis and flesh quality in turbot (*Psetta maxima*). *Aquaculture* **193**, 291-309.

Rescan, P. Y. (1998). Identification of a fibroblast growth factor 6 (FGF6) gene in a non-mammalian vertebrate: continuous expression of FGF6 accompanies muscle fiber hyperplasia. *Biochim. Biophys. Acta* **1443**, 305-314.

Rescan, P. Y., Montfort, J., Ralliere, C., Le Cam, A., Esquerre, D. and Hugot, K. (2007). Dynamic gene expression in fish muscle during recovery growth induced by a fasting-refeeding schedule. *BMC Genomics* **8**, 438.

Storlien, L. H., Kriketos, A. D., Jenkins, A. B., Baur, L. A., Pan, D. A., Tapsell, L. C. and Calvert, G. D. (1997). Does dietary fat influence insulin action? *Ann. N. Y. Acad. Sci.* **827**, 287-301.

Tan, X. and Du, S. J. (2002). Differential expression of two MyoD genes in fast and slow muscles of gilthead seabream (*Sparus aurata*). *Dev. Genes Evol.* **212**, 207-217.

Terova, G., Bernardini, G., Binelli, G., Gornati, R. and Saroglia, M. (2006). cDNA encoding sequences for myostatin and FGF6 in sea bass (*Dicentrarchus labrax*, L.) and the effect of fasting and refeeding on their abundance levels. *Domest. Anim. Endocrinol.* **30**, 304-319.

Thomas, M., Langley, B., Berry, C., Sharma, M., Kirk, S., Bass, J. and Kambadur, R. (2000). Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. *J. Biol. Chem.* **275**, 40235-40243.

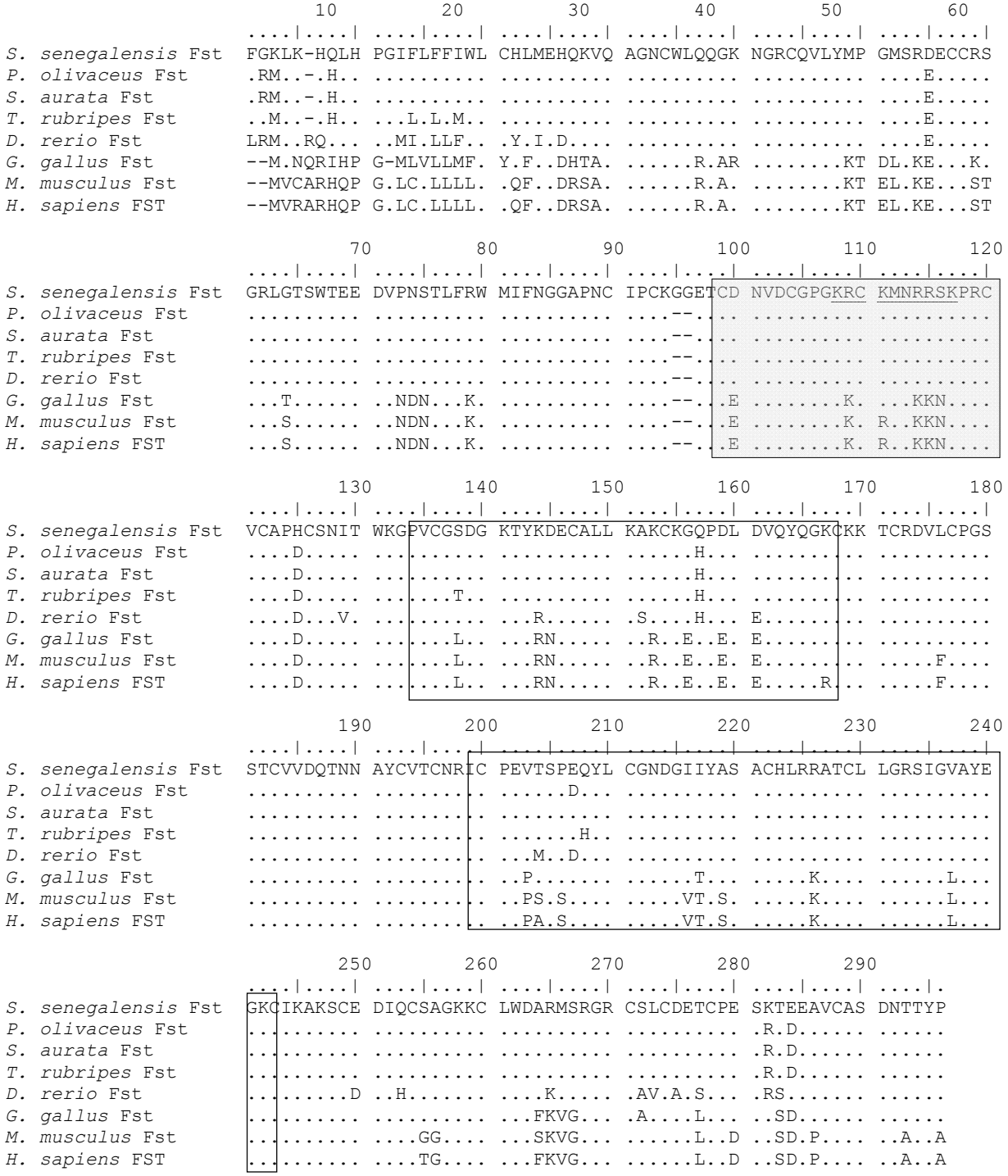
Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, RESEARCH0034.

Xu, C., Wu, G., Zohar, Y. and Du, S. J. (2003). Analysis of myostatin gene structure, expression and function in zebrafish. *J. Exp. Biol.* **206**, 4067-4079.

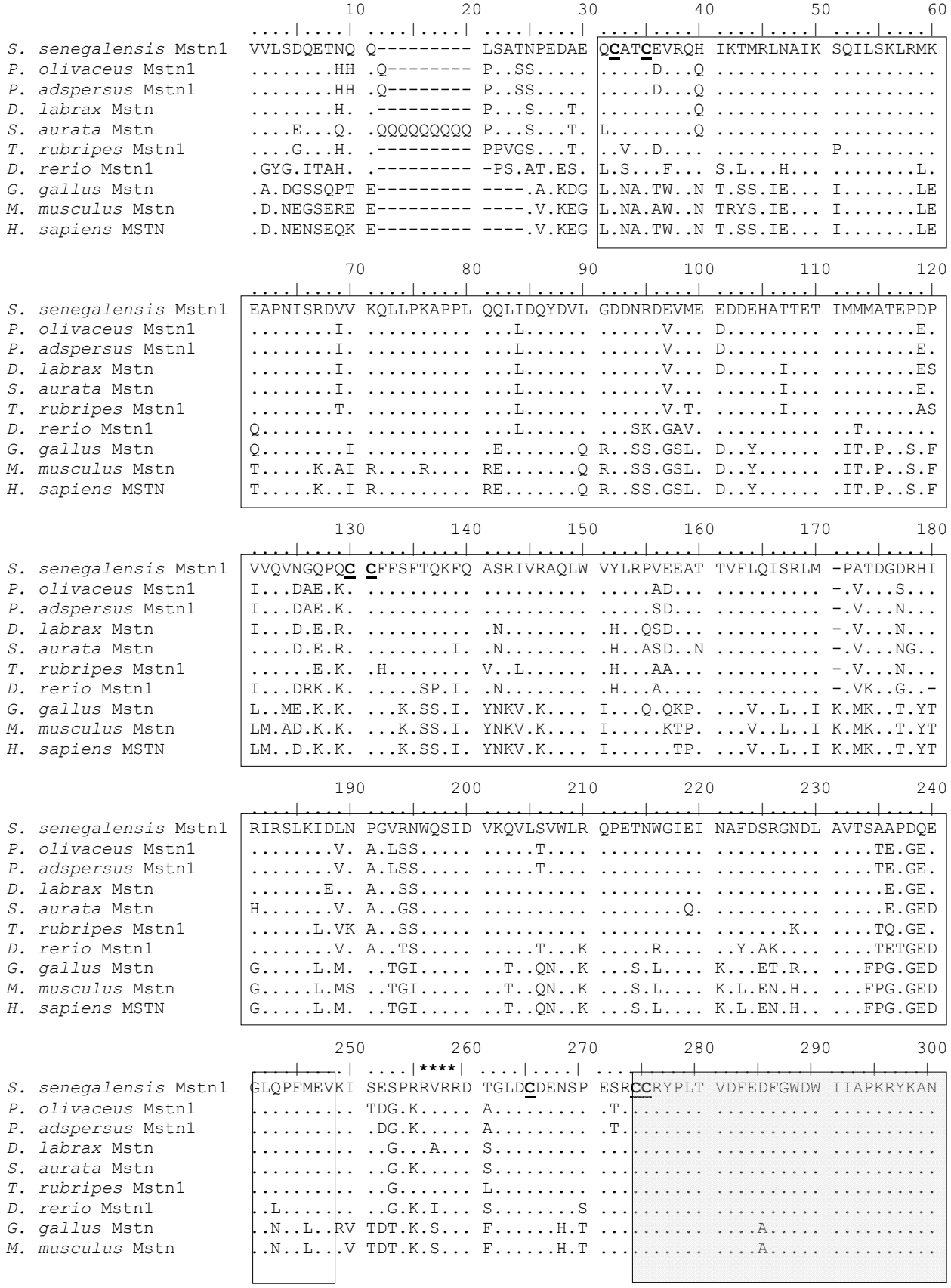
Zar, J. H. (1996). *Biostatistical Analysis*, 662 pp. Prentice-Hall: New Jersey.

Zhong, Q. W., Zhang, Q. Q., Chen, Y. J., Sun, Y. Y., Qi, J., Wang, Z. G., Li, S., Li, C. M. and Lan, X. (2008). The isolation and characterization of myostatin gene in Japanese flounder (*Paralichthys olivaceus*): Ubiquitous tissue expression and developmental specific regulation. *Aquaculture* **280**, 247-255.

Supplementary Figure S1



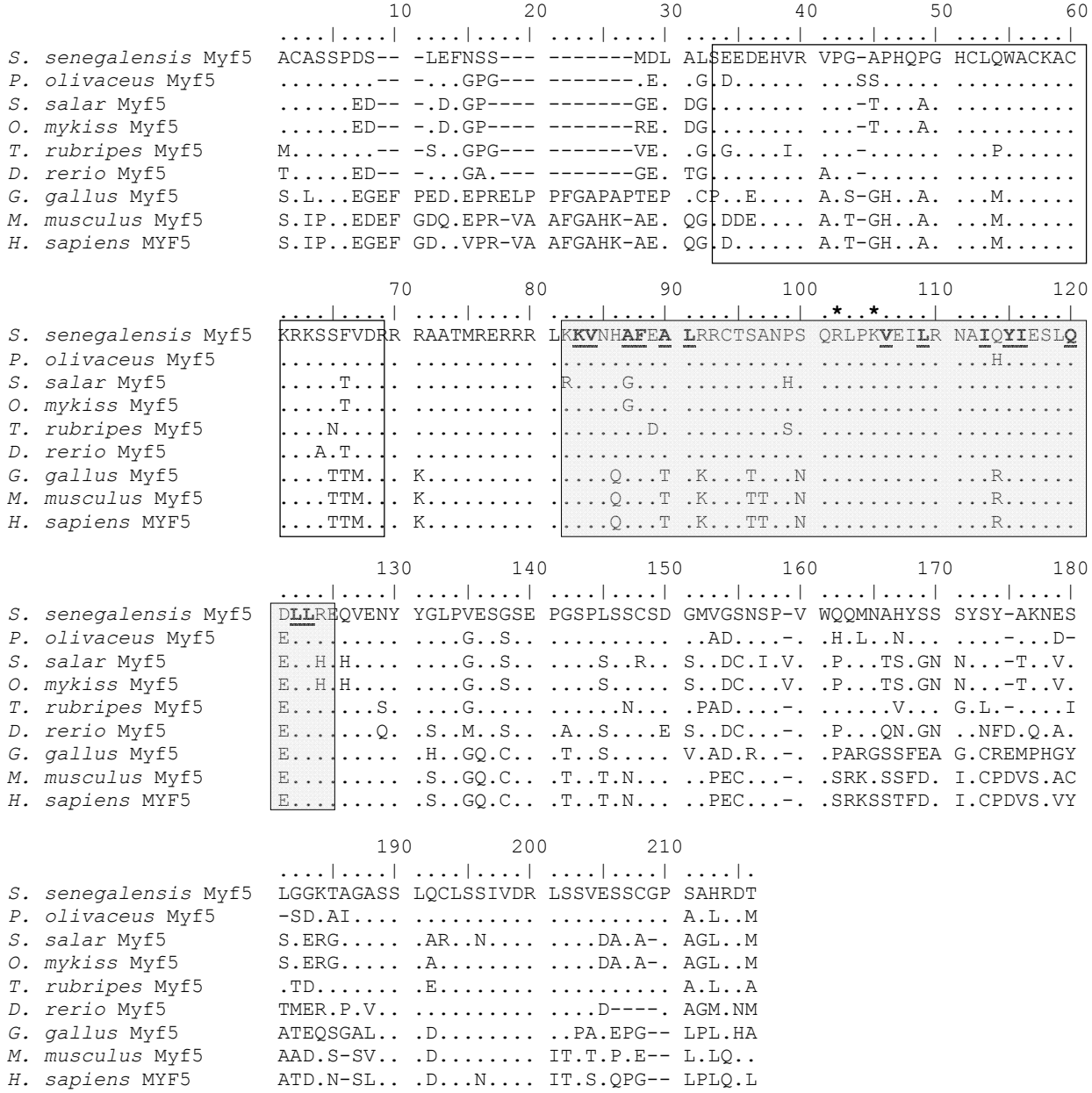
Supplementary Figure S2



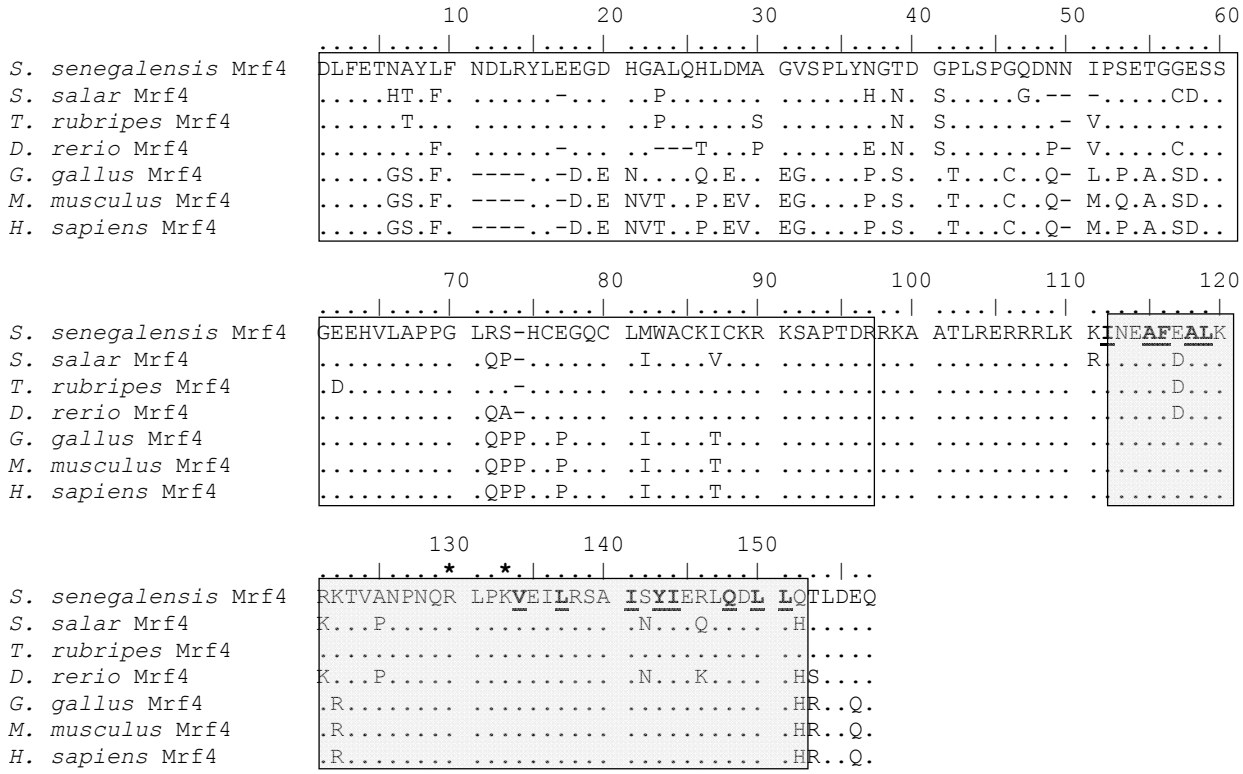
```
H. sapiens MSTN          ..N..L...V TDT.K.S... F......H.T .......... ....A..... ..........

                                    310        320
                                    ....|....| ....|...|
S. senegalensis Mstn1    YCSGECEYMH LQKYPHTHLV
P. olivaceus Mstn1       .......... ..........
P. adspersus Mstn1       .......... ..........
D. labrax Mstn           .......... ..........
S. aurata Mstn           .......... ..........
T. rubripes Mstn1        .......... ..........
D. rerio Mstn1           ......D..Y ..........
G. gallus Mstn           .......FVF ..........
M. musculus Mstn         .......FVF ..........
H. sapiens MSTN          .......FVF ..........
```

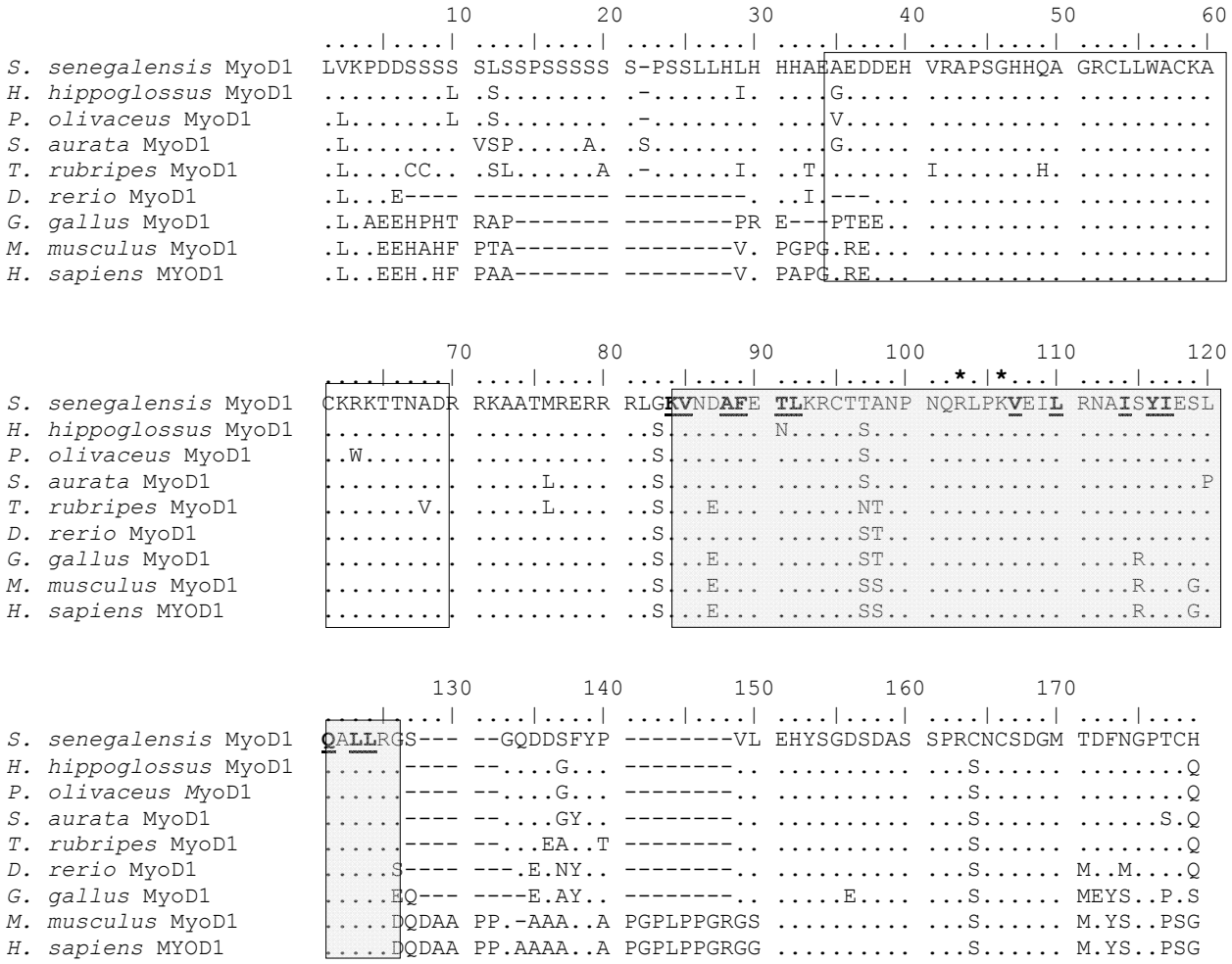
Supplementary Figure S4



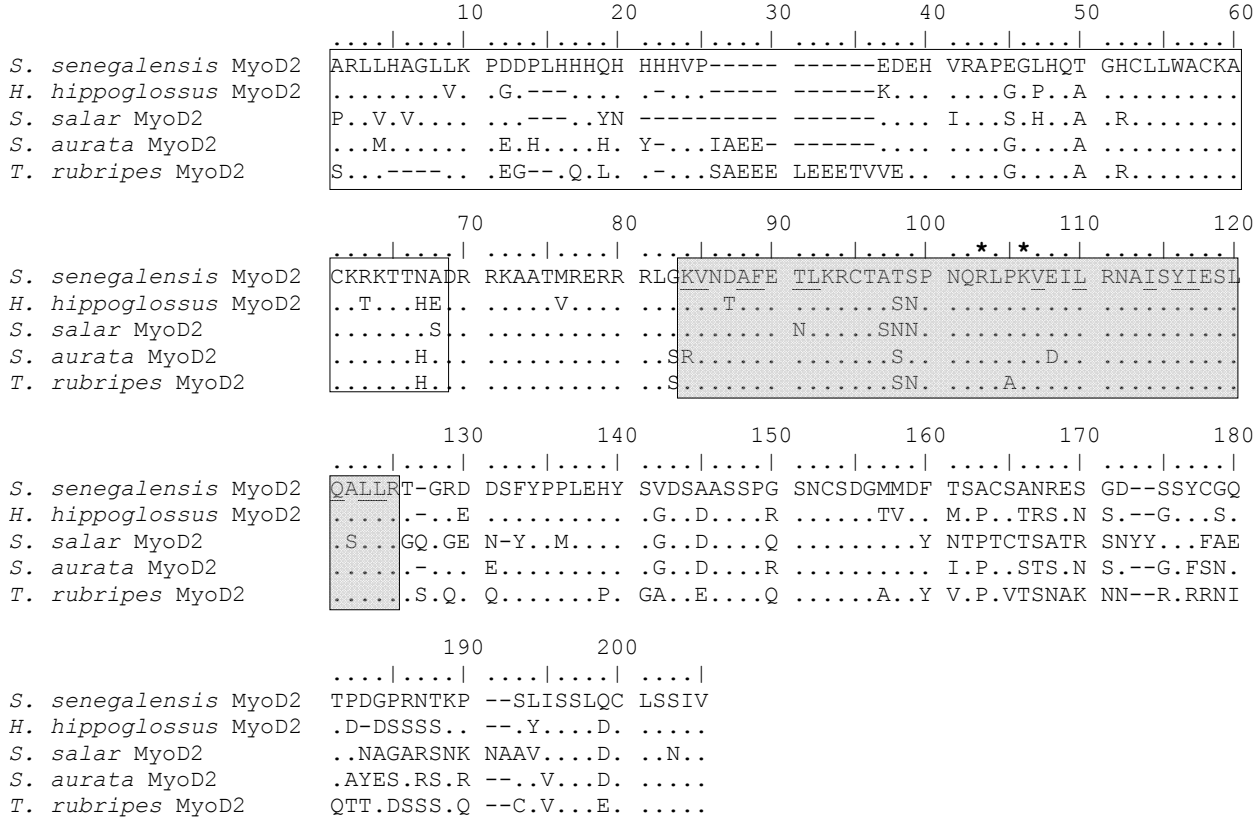
Supplementary Figure S5



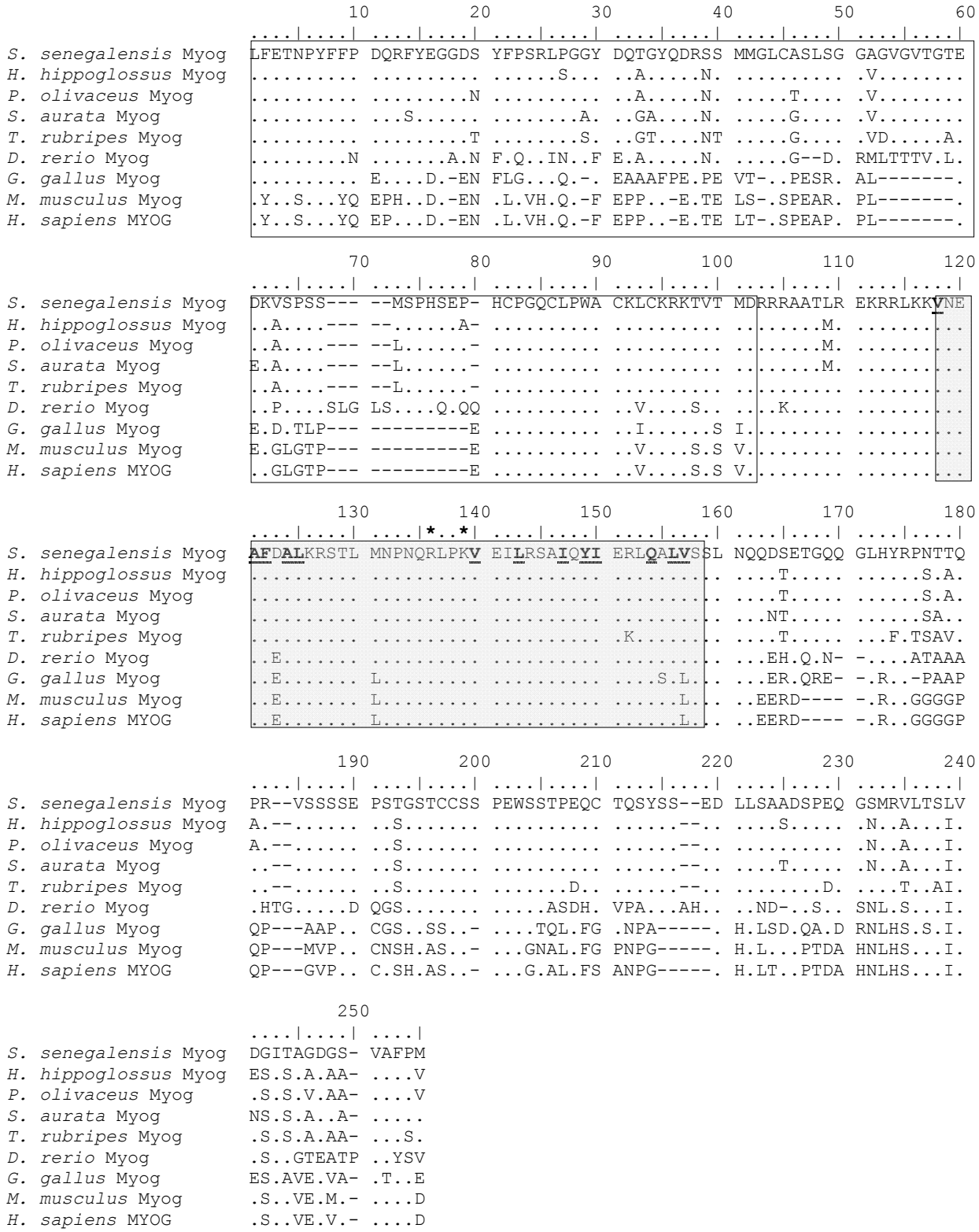
Supplementary Figure S6



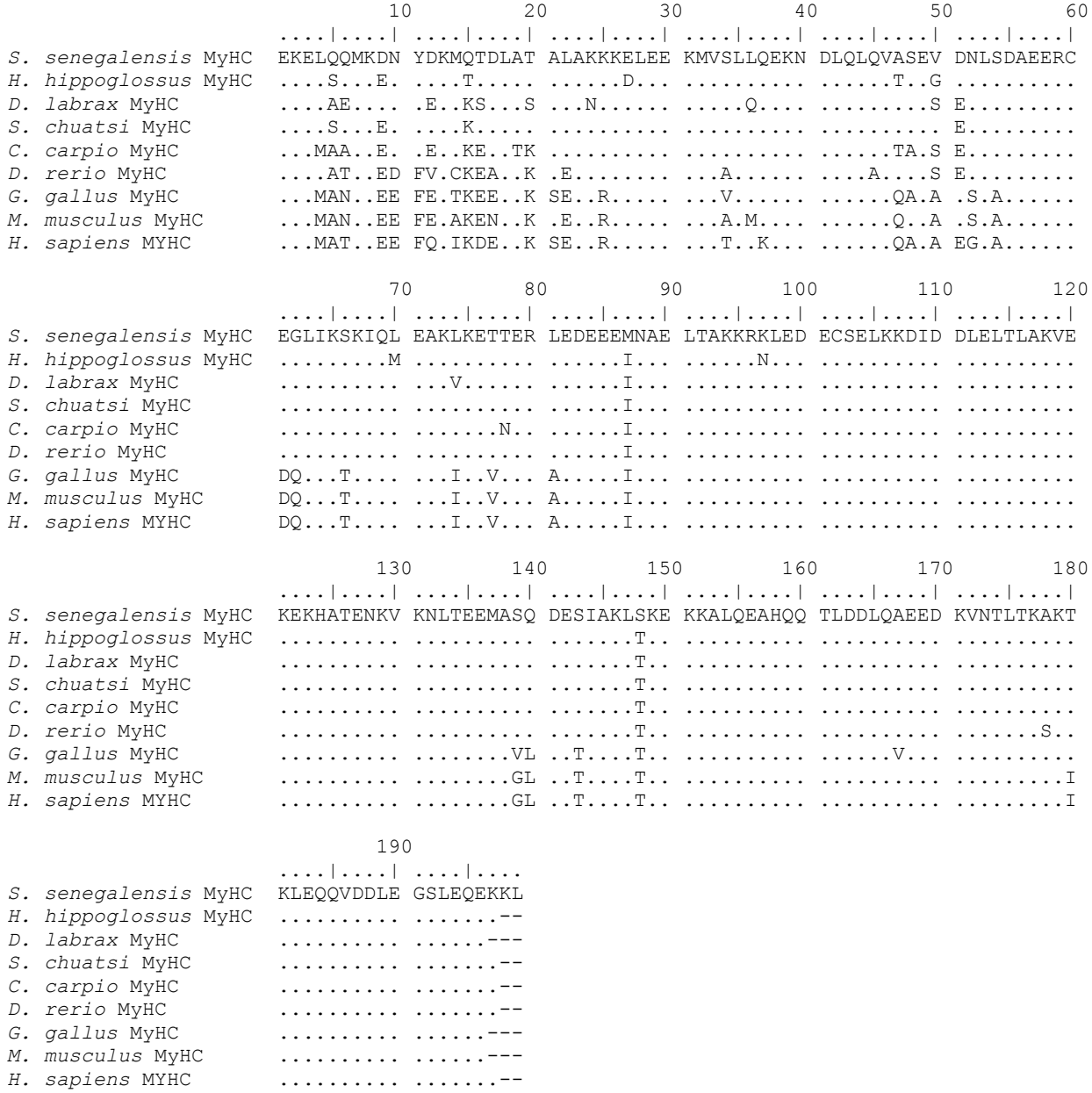
Supplementary Figure S7



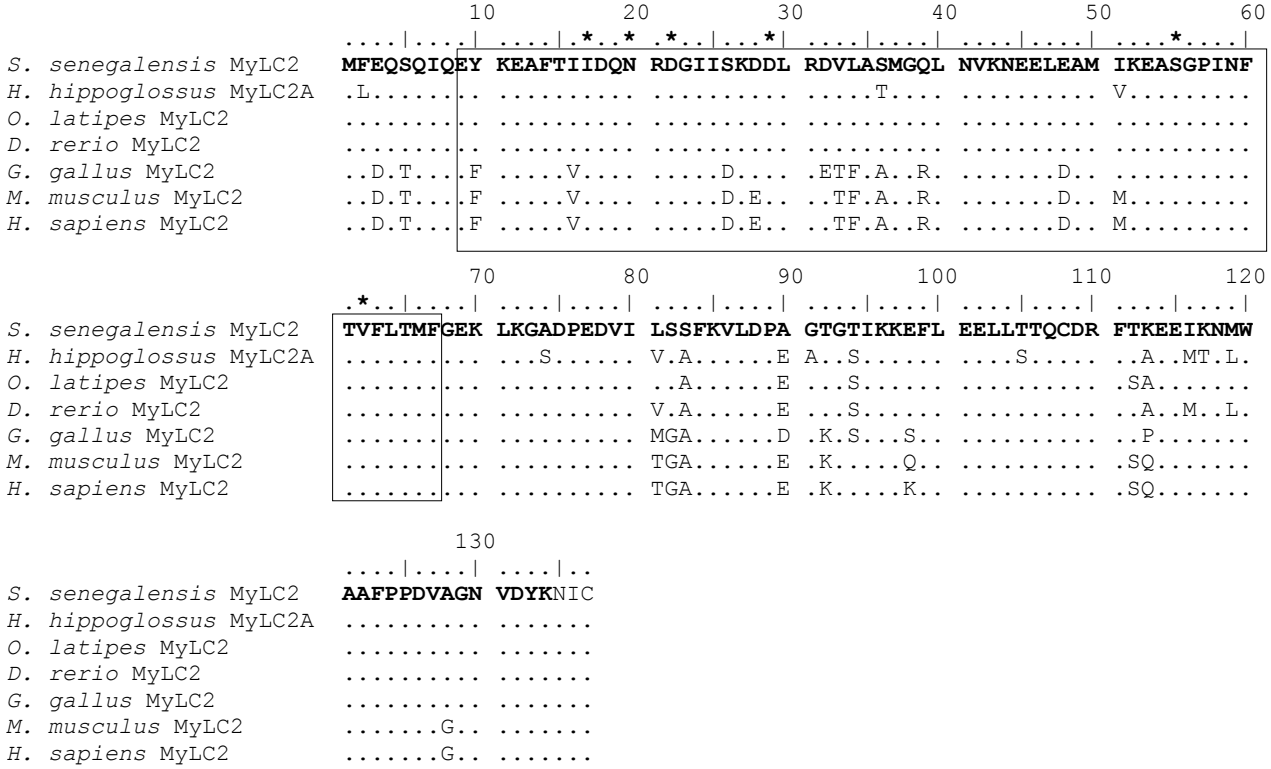
Supplementary Figure S8



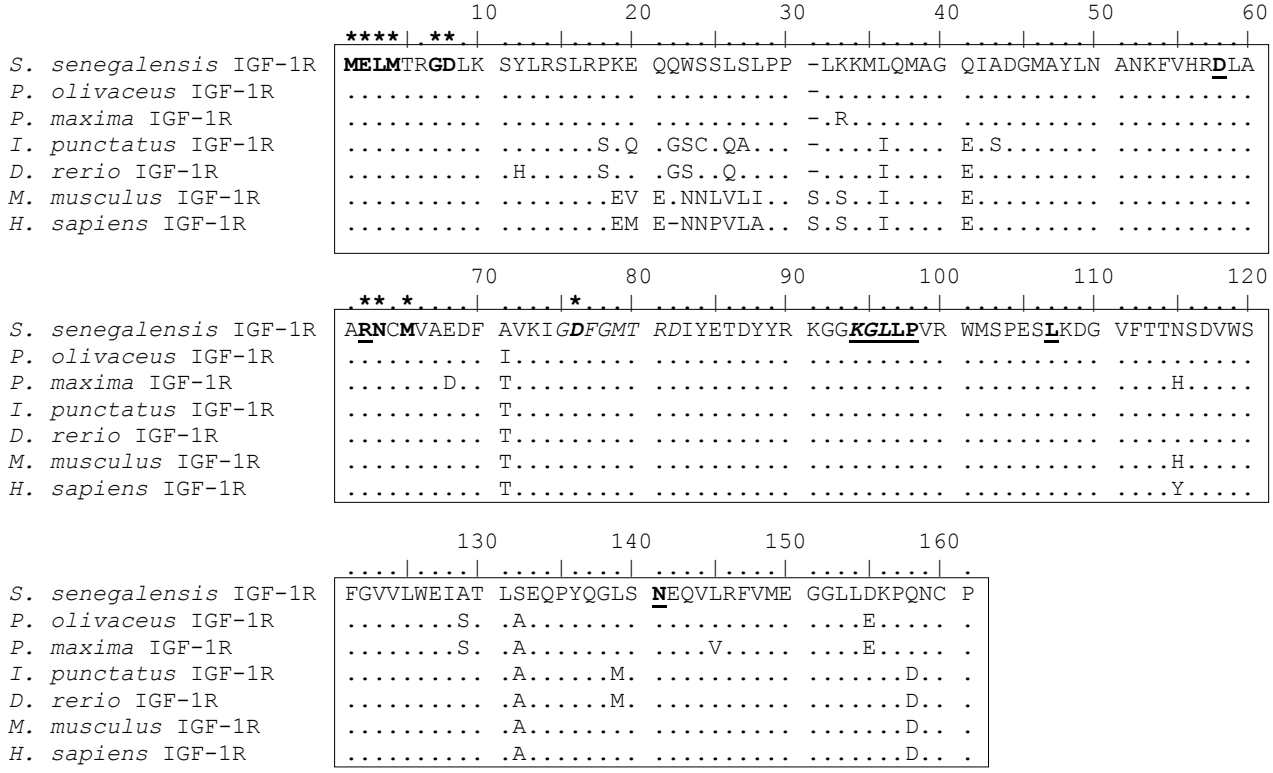
Supplementary Figure S9



Supplementary Figure S10



Supplementary Figure S11



Supplementary Figure S12

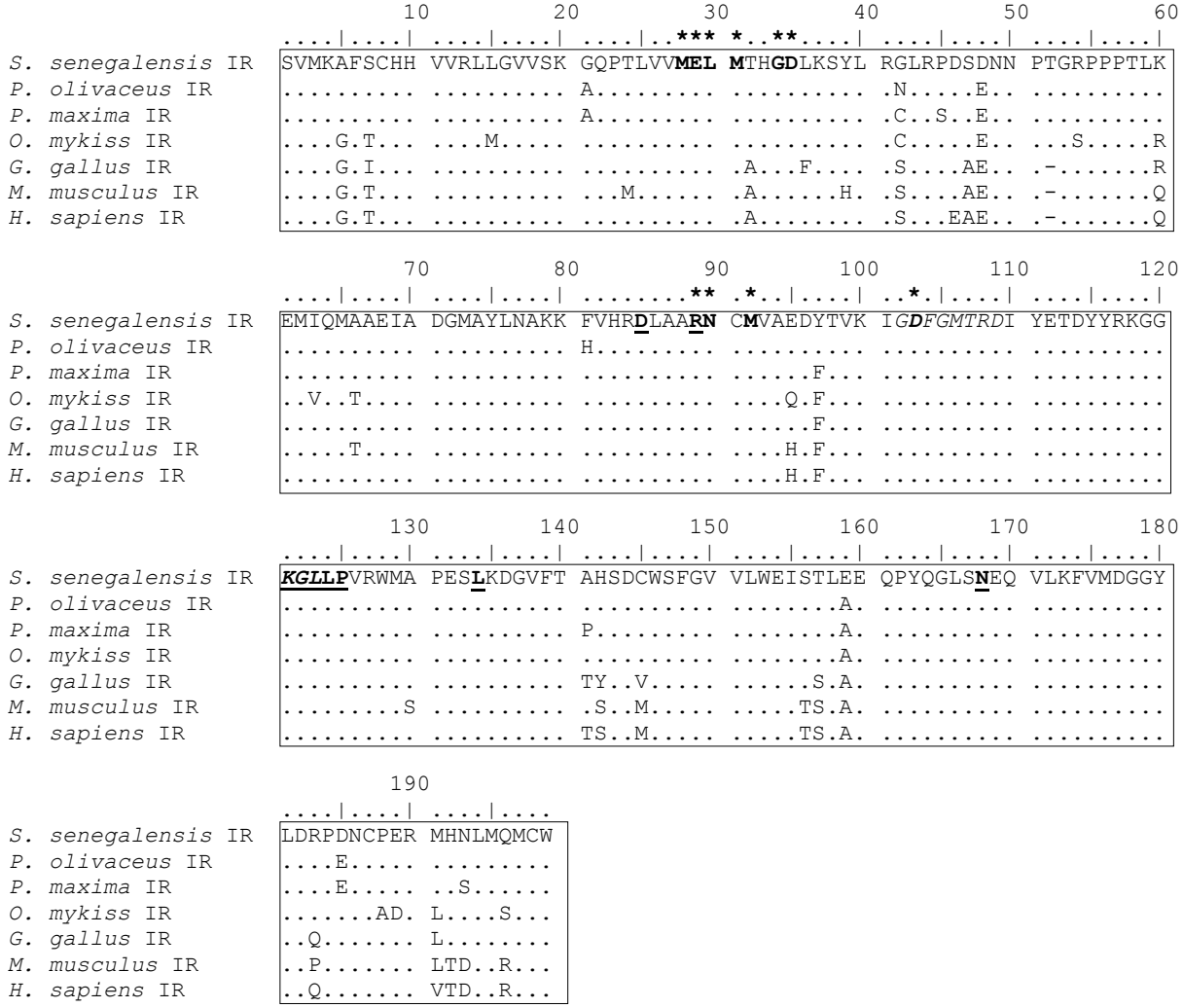


Table S1. Details of the primers used to quantify expression of the target genes by real-time PCR

Gene	Forward sequence (5′→3′)	Reverse sequence (5′→3′)	Size (bp)	*E* (%)	R^2
myf5	GAGCAGGTGGAGAACTACTACG	CCAACCATGCCGTCAGAG	89	102	0.986
mrf4	GAGAGGAGGAGGCTCAAGAAG	CAGGTCCTGTAATCTCTCAATG	137	90	0.978
myog	GTCACAGGAACAGAGGACAAAG	TGGTCACTGTCTTCCTTTTGC	118	89	0.985
myod1	CTCCTCCTCCCCGTCATC	TTGGTGGTCTTCCGCTTG	144	99	0.997
myod2	AGACTGGACACTGCCTGCTG	GTTCACTTTGCCAAGCCG	113	96	0.950
mstn1	GGGAGATGACAACAGGGATG	TGGATCCGGTTCAGTGGC	91	98	0.982
fst	CATCAAAGCTAAGTCGTGTGAG	CACCGCCTCCTCTGTCTTG	133	105	0.993
fgf6	CGGTGGAGAGAGGAGTCG	AAGACTGTCGTTCCGTATAACC	94	91	0.996
myHC	GAAAAATCTGACAGAGGAAATGG	CCTTGGTGAGAGTGTTGACTTTG	143	94	0.994
mylc2	GTACAAGGAGGCGTTCACAATC	CCAGCACGTCCCTAAGGTC	77	98	0.999
igf-I	AGCGATGTGCTGTATCTCCTG	AGCCTCTCTCCCCGCACA	148	91	0.990
igf-II	GCAGAATGAAGGTCAAGAAGATG	CGAGACCACTTCCACAGC	89	104	0.968
Igf1r	GCTGTTAAAATAGGAGATTTCGG	GGAGCAAACCCTTACCACC	82	106	0.944
insr	CGTGGTTCGTCTTTTGGG	CTCTTCAGGTCACCGTGAGTC	84	98	0.937

Amplicon size and efficiency of the reaction and correlation coefficients of the calibration curves are shown.

Table S2. Comparison of the partial amino acid sequence of Senegalese sole key growth genes with their orthologues from other fish and vertebrate species

·	*H. hippoglossus*	*P. olivaceus*	*D. labrax*	*S. salar*	*S. aurata*	*O. mykiss*	*T. rubripes*	*D. rerio*	*G. gallus*	*M. musculus*	*H. sapiens*
Myf5	–	87%	–	75%	–	78%	85%	76%	56%	58%	57%
Mrf4	–	–	–	84%	–	85%	94%	85%	77%	75%	75%
MyoD1	93%	93%	–	–	90%	–	87%	75%	67%	63%	63%
MyoD2	78%	–	–	65%	79%	–	66%	–	–	–	–
Myog	91%	91%	–	78%	90%	77%	87%	68%	53%	51%	52%
Mstn1	–	88%	89%	81%	85%	81%	87%	78%	63%	61%	63%
Fst	–	96%	–	87%	97%	86%	95%	89%	78%	74%	74%
Fgf6	–	–	92%	–	–	85%	–	79%	–	66%	66%
MyHC	94%	–	92%	–	–	–	–	89%	82%	82%	81%
MyLC2	95%	–	–	91%	92%	90%	–	95%	86%	85%	85%
Igf1R	–	98%	–	–	–	–	–	93%	–	89%	89%
IR	–	97%	–	–	–	92%	–	–	91%	88%	89%