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**Plant protein blends in diets for Senegalese sole affect skeletal muscle growth, flesh texture and the expression of related genes**

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**Abstract:**

Skeletal muscle growth and flesh quality of Senegalese sole fed diets containing increasing levels of plant protein blends to replace fishmeal were evaluated using muscle cellularity, texture profile and gene expression. A control fish meal-based diet (FM) was compared with three isonitrogenous (54%) and isolipidic (9%) diets with increasing levels of plant protein (PP) blends (50% PP50, 75% PP75 and 100% PP100). By the end of the experiment sole fed PP50 and PP75 had a final body length similar to the CTR (25 cm), but fish fed PP100 were significantly smaller (23 cm). Total FM replacement by PP sources resulted in significantly smaller muscle cross sectional area (CSA) mainly due to a decrease in the muscle fibre size as the total number of fibres did not vary significantly among treatments. The dietary incorporation of PP significantly reduced the expression of several key genes involved in myogenesis and muscle growth (*mrf4*, *fgf6*, *myhc* and *mylc2*). Fillet texture analysed instrumentally was affected by the total substitution of FM. Fish fed PP100 diet had a significantly higher modulus of elasticity, i.e. lower flesh stiffness, compared with the other groups. Muscle fibre size was moderately related (r=-0.573) to the modulus of elasticity and positively correlated with the expression of lysyl oxidase (r=0.495). The observed changes in muscle cellularity could not be associated with the expression of texture-related genes (*capn2*, *ctsb*, *ctsd*), since no significant differences were be observed among diets. The present results point towards a modulation of the expression of several muscle growth related

genes by increasing levels of PP sources that alter muscle cellularity and textural properties of Senegalese sole when total FM is replaced by PP.

**Statement of relevance**

The biological basis through which sustainable and practical plant protein diets (PP) affect flesh texture determinants is extremely scarce so the present results will be valuable to the aquafeed industry, fish producers and final consumers. This study clearly shows that PP diets reduced expression of several key genes involved in myogenesis and muscle growth and can hence affect fish growth potential at long term. This study identifies useful markers that correlated well with muscle cellularity and muscle growth and can be further used to select the most appropriate diets for a fish species.

**Abbreviations:**

FM Fish meal, PP Plant protein, CSA Cross sectional area

**Keywords**: flatfish nutrition; Solea senegalensis; muscle cellularity; texture-related

genes; flesh stiffness

## 1. Introduction

Global fish consumption has increased over the past four decades, rising from 9.0 kg per capita in 1961 to an estimated 19 kg in 2012 (FAO, 2015) as a result of different promotion campaigns and consumer concern regarding the importance of a healthy balanced diet (Chowdhury et al., 2012; Pieniak et al., 2008; Wu et al., 2015). The image of fish quality among consumers is more often related to nutritional value and sensory properties of the flesh than other characteristics (Grigorakis, 2007). In most markets, consumers show a preference for a firm flesh texture (Haard, 1992), which is strongly influenced by a set of intrinsic traits such as muscle chemical composition, number and size distribution of muscle fibres and associated connective tissue (Hurling et al., 1996; Valente et al., 2011a). Moreover, environmental responsible practices of aquaculture also became important concerns for both the producer and the consumer (Frewer et al., 2005).

The replacement of fish meal (FM) by sustainable and eco-friendly plant protein (PP) sources is a major trend in aquaculture feeds and it has been accomplished in different species without affecting growth performance (Cabral et al., 2013; Johnsen et al., 2011; Kaushik et al., 2004; Pratoomyot et al., 2010). Most studies evaluated the impact of such diets on the nutritional value of the fillet, but few focused on organoleptic attributes and even less on textural properties (de Francesco et al., 2004; Johnsen et al., 2011; Matos et al., 2012). Flesh texture is a function of muscle fibres, connective tissue and fat cells (Johnston et al., 2000). The biological basis through which sustainable PP diets affect flesh texture determinants is scarce and the genetic network underpinning those processes involves a complex response which remains largely unknown. Myofibrillar proteins are the largest skeletal muscle proteins

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responsible for the contractile, functional and textural properties being major regulators of muscle growth (Goll et al., 2008). Moreover, endogenous proteolytic enzymes, which mainly include the cytosolic calpains, the lysossomal cathepsins and the ubiquitin-proteasome pathway play an important role in protein degradation (Jackman and Kandarian, 2004) with consequences in flesh texture (Salmerón et al., 2013). Several genetic pathways regulating muscle fibre hypertrophy and/or nuclear accretion have recently been identified in fish, and related to its nutritional status (Alami-Durante et al., 2010a; 2010b; Bower and Johnston, 2010; Valente et al., 2012; 2013). The dietary inclusion of PP sources in rainbow trout was linked to changes in the dynamics of white muscle growth and correlated with changes in expression of myogenic regulatory factors *(MyoD)*, structural genes (*fast-MHC*) (Alami-Durante et al., 2010b) and genes involved in muscle lysosomal proteolysis (cathepsin D) (Alami-Durante et al., 2010a).

Senegalese sole (*Solea senegalensis*) is a high market value flatfish considered as candidate to diversify the Southern Europe aquaculture (Morais et al., 2014). Recent studies demonstrated that sole can effectively use diets with high levels of PP sources, without impairing growth performance or nutrient utilization (Cabral et al., 2011; Silva et al., 2009; Valente et al., 2011b), and still preserving high nutritional value for human consumption (Cabral et al., 2013). However, the impact of vegetable sources on the dynamics of white muscle growth and sole flesh textural properties has never been evaluated. In the present study, the flesh quality of Senegalese sole fed diets containing increasing levels of PP blends (50%, 75% and 100%) to replace FM will be evaluated using texture profile analyses, muscle cellularity and gene expression. Relative expression of key genes involved in myogenesis (*myod1*, *myod2*, *myf5*, *mrf4*, *myog*), muscle growth (*igf-I*, *igf-II*, *igf1r, insr, fst, fgf6, myhc and mylc2*), protein turnover

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(*calpain2*, *cathepsinB*, *cathepsinD*) and stabilization of collagen fibrils (*lox*) will be related to textural and cellular properties.

## 2. Material and methods

The experiment was directed by trained scientists (following category C FELASA recommendations) and conducted according to the European guidelines on protection of animals used for scientific purposes (directive 2010/63/EU of European Parliament and of the Council of European Union).

### 2.1 Experimental diets

The trial comprised four extruded isonitrogenous (54% dry mater, DM), isolipidic (9% DM) and isoenergetic (21 KJ g$^{-1}$ DM) dietary treatments. A control fish meal–based diet (FM) was compared with three test diets containing increasing levels of a plant protein blend (soybean meal, peas, corn gluten, and wheat): 50% (PP50), 75% (PP75), and 100% (PP100). Details on diets formulation and proximate composition are presented in Table 1. All diets were formulated with practical ingredients. PP-based diets were supplemented with crystalline lysine (Lys) and methionine (Met) to simulate those levels existing in the FM based diet, whereas fish oil (FO) levels were adjusted accordingly to keep the diets isolipidic. All ingredients were finely grounded (hammer mill, 0.8 mm sieve), mixed and then extruded (twin screw extruder, 3.0 mm pellet size, SPAROS, Lda., Portugal). Diets were finally dried at 60 ºC for 3 h and stored at 4ºC until use.

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## 2.2 Fish, rearing conditions and sampling

The growth trial was conducted at the experimental facilities of CIIMAR, Porto, Portugal. Senegalese sole juveniles were supplied by a commercial fish farm (Stolt Sea Farm, Spain). After arrival at the experimental unit, fish were kept under quarantine conditions for 4 weeks and fed with a commercial diet (Skretting: 61% protein and 19% fat). Once they were acclimated to the new rearing facilities, triplicate groups of 26 homogeneous fish (106±15 g) per dietary treatment were distributed among 12 fibreglass tanks (0.7 m x 0.7 m, initial fish density of 6 kgm$^{-2}$) in a closed recirculation system. Each tank was supplied with filtered and heated (19±1°C) seawater (30 ± 1‰ NaCl) at a flow rate of 2 L min$^{-1}$. Oxygen level was kept above saturation (>90%) and nitrogenous compounds and pH level were daily monitored during the entire trial to ensure levels within the recommended limits for marine fish species. An artificial photoperiod of 12 h light:12 h dark was established. Fish were fed until visual satiety using automatic feeders that distributed 4 meals day$^{-1}$ throughout 140 days. The ration offered was daily adjusted based on the presence/absence of uneaten feed in each tank as previously described by Cabral et al. (2013).

At the end of the growth trial and following a 30 h starvation period, all fish were individually weighed (g) and measured (total length, cm). The condition factor (K) was calculated as final body weight (g)/[final body length (cm)]$^{3}$. Six fish were sampled per dietary treatment and sacrificed by a sharp blow on the head for muscle tissue evaluation. After removing the scales, one cross-sectional body slab (2-3 mm thickness) was taken from the anterior region to dorsal fin, fixed in Bouin solution (Panreac, Barcelona, Spain) during 48 h and stored in ethanol (70%) until morphometric analysis. Additionally, a piece of white muscle (circa 2g) was dissected from each fish,

immediately frozen in liquid nitrogen and stored at -80ºC for subsequent determination of gene expression. From each fish, a cross-sectioned slices of 3-4 cm wide was also taken from the head side and kept in ice for further instrumental colour and texture analysis.

*2.3 Histological analysis*

Fixed muscle samples were decalcified, dehydrated in a graded ethanol series, cleared in xylol and embedded in paraffin and analysed according to Valente et al. (2011a). Transversal sections (10 μm-thickness) were cut in each block and stained with haematoxylin-eosin before being coversliped for morphometric analysis. Measurements were obtained using an interactive image analysis system (Olympus Cell Family), capture by CCD-video camera (ColorView Soft Imaging System, Olympus) and a light microscope (BX51, Olympus, Japan). The dorsal area occupied by white muscle (muscle CSA, mm$^2$) was computed through Multiple Image Alignment (MIA) using Cell*Family software, after circumscribing the physical limit of the muscle section only. White muscle fibres density ($N_A$) was calculated as: $\Sigma N * \Sigma A^{-1}$, where $\Sigma N$ is the total number of fibres counted over all sampled fields in a section (>6) and $\Sigma A$ is the total area of the fibre counting fields (mm$^2$). Total number (N) of white muscle fibres was calculated as: $N_A$ * muscle CSA, where $N_A$ is the number of white muscle fibres per unit area (fibres mm$^{-2}$) and muscle CSA is the total white muscle area (mm$^2$). Mean individual fibre area ($\bar{a}$, μm$^2$) was determined by circumscribing the physical limits of >900 white muscle fibre sections and the corresponding white muscle fibre diameter (μm) was computed as follows: D (muscle) = 2 $\bar{a}^{0.5} \pi^{-0.5}$. All measurements concerning white fibres used 10x magnification objectives.

*2.4 Instrumental analyses*

Fillet texture was analysed 24h *post mortem* using a Texture Analyser Model Instron 4301 (Instron Engineering, Canton, MA, USA), equipped with a load cell of 1 kN and a 4.0 mm diameter spherical probe. Texture profile analyses were obtained by double compression (constant speed and penetration depth of 1 mm s$^{-1}$ and 3.0 mm, respectively) on maximum thickness part of each raw fillet. Penetration depth was selected according to the maximum distance that did not induce fibres breaking and, therefore, muscle structure affectation.

*2.5 Gene cloning and sequencing*

BLAST similarity searches against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed for the remaining target genes (*capn2*, *ctsb*, *ctsd*, *lox*) in order to identify their orthologues in other teleost species. After CLUSTALW alignments (http://align.genome.jp/), degenerate primers were designed against the most conserved regions of the sequences. Their melting temperatures and potential presence of dimers and hairpins were predicted by *Netprimer* software (http://www.premierbiosoft.com/netprimer/). Products of interest obtained after polymerase chain reaction (PCR) amplification were analysed by agarose gel electrophoresis, excised from the gel with QIAquick kit (Qiagen) and cloned onto 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). They

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were sent for sequencing at the University Hospital of North Norway (UNN, Tromsø, Norway). DNA sequences were analysed with CodonCode Aligner v.2.0.6 (CodonCode Corporation, Dedham, MA, USA) and their identities determined by BLASTN similarity searching against the NCBI database.

*2.6 RNA isolation and gene expression analysis*

Sole muscle samples (approximately 100 mg) were placed into Lysing Matrix D tubes (QBiogene/Medinor, Oslo, Norway) containing QIAzol (Qiagen, Nydalen, Sweden) and homogenised for 40s 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 wipe out buffer supplied (5 min) by QuantiTect reverse transcription kit (Qiagen) to remove any trace of genomic DNA contamination. Assessment of RNA quality was performed by electrophoresis technique 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) higher than 1.9 indicated high purity of extracted RNA. Total RNA (1 µg) from each sample was reverse transcribed using the QuantiTect transcription kit (Qiagen) for 30 min at 42ºC.

Specific primers were designed based on published Senegalese sole *igf-I* and *igf-II* (Funes et al., 2006) and *myf5, mrf4, myog, myod1, myod2, fst, fgf6, myhc, mylc2, igf1r* and *insr* (Campos et al., 2010) gene sequences (Table 2). Gene expression was quantified by quantitative real time PCR (qPCR) with SYBR Green chemistry (Qiagen) on a LightCycler® 480 instrument (Roche) as detailed in Fernandes et al. (2008).

Reactions (total volume of 10µl) were prepared in 96-well plates including 4µl of 50x diluted cDNA template, 1µl of each primer pair (5µmol l$^{-1}$) and 5µl of QuantiTect SYBR Green containing ROX as reference dye (Qiagen). After sealing the plates, samples were denatured for 15 min at 95ºC and then subjected to 45 cycles of amplification following the next thermocycling profile: denaturation for 15s at 94ºC, annealing for 20s at 60ºC and extension for 20s at 72ºC. Specificity of the qPCR reaction and presence of primer dimers were checked in the melting curves generated under dissociation protocol from 65ºC to 97ºC. Standard curves corresponding to 5-fold dilution series (1:1-1:16) of pooled RNA from all samples allowed to calculate the PCR efficiency. 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 (Ct) values were determined by the fit-point method using the LightCycler® 480 software and arbitrarily set to 0.3 fluorescence threshold. The potential suitability of reference gene (*ubq*, *rps4*, *eef1a1*) (Table 3) was assessed according to GeNorm analysis (Vandesompele et al., 2002). Normalisation factors were calculated as the geometric average of the two most stable and suitable reference gene (*Ubq*, *rpS4*). After Ct values transformation into quantities from standard curves, normalised gene expression levels were obtained applying the appropriate normalisation factors to raw data.

*2.7 Statistical analysis*

Statistical analyses followed methods outlined by Zar (1999). All data were checked for normality (Shapiro-Wilk test) and ln-transformed when required. Homogeneity of variances was confirmed by Levene´s test and significant differences

determined by one-way ANOVA using the STATISTICA 11 (StatSoft Inc., Tulsa, USA) software. Individual means were compared using a Tukey's HSD (honest significant difference) test. When data did not meet the ANOVA assumptions, the Kruskal-Wallis non-parametric test was chosen instead. Relationships between all parameters were evaluated by non parametric Spearman Rank order correlations. Significant differences were considered when P<0.05.

## 3. Results

### 3.1. Growth performance

After feeding all fish for 140 days, full substitution of dietary FM by PP (PP100) resulted in a significantly lower final body weight and size compared to other dietary treatments (Table 4). Fish fed PP50 and PP75 showed a similar growth performance to the FM fed fish. The condition factor (K), a measure of the condition of fish, was similar among treatments (1.4-1.5).

### 3.2. Muscle cellularity and instrumental texture analysis

Dorsal muscle CSA was significantly affected by the dietary conditions (P< 0.05). Fish fed PP50 and PP75 had similar CSA areas to the FM fed fish, but PP100 had the lowest CSA section. White fibre diameter was not significantly affected by the dietary inclusion of PP sources up to 75 %, but fish fed PP100 had the smallest white fibres. The total number of white fibres and the percentage of small-sized fibres (<30µm) was similar among dietary treatments (P≥0.05), resulting in similar fibre

distribution among dietary treatments. PDF distribution showed that the PP50 group skewed to the right–hand tail of the distribution, although not significantly (Fig. 1). The dietary inclusion of 100% PP caused significant differences on fillet stiffness determined by the modulus of elasticity (Table 4). Fish fed PP100 diet exhibited a significantly higher modulus of elasticity (0.5 GPa) i.e. lower fillet stiffness compared to other dietary treatments (0.4 GPa). Moreover, this textural parameter was negatively correlated with fish length (-0.600), and moderately correlated with white muscle fibre size (-0.573) (Table 5).

3.3. *Muscle gene expression*

The dietary incorporation of PP significantly affected the relative transcript levels of several muscle-related genes (Fig. 2). The relative gene expression of *myod1* decreased concomitantly with increasing PP levels (Fig. 2 A) with PP100 showing a significantly lower expression than the FM fed fish, whereas *myod2* showed no clear trend (data not shown). Moreover, myogenic regulatory factor *mrf4* transcript levels decreased significantly with the inclusion of PP sources (Fig. 2 B). Myogenin expression also tended to decrease with PP diets, but without statistical significance, whereas myf5 showed no clear trend (Fig. 2 C, D). Gene expression of *igf1r, insr* and *fst* in skeletal muscle revealed a common expression pattern with soles fed PP100 diet significantly reducing their levels of transcripts approximately 3.0 fold in comparison to groups fed FM diet (Fig. 2 G, H and I). Other genes also related with overall growth – *igf1* and *igf2* – did not modify their expression as a function of dietary treatment (Fig. 2 E, F). The relative expression of fibroblast growth factor 6 (*fgf6*) and myosin superfamily elements (*myhc* and *mylc2)* was significantly reduced (>2 fold) by the

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dietary inclusion of PP sources, with soles fed 100PP diet showing the lowest values (Fig. 2 J, K, L). These genes were highly and positively correlated between each other (Table 5).

Texture-related genes (*ctsb, ctsd, capn2* and *lox*) tended to decrease their transcript levels when PP meal was incorporated in sole diets (Fig. 2 M, N, O, P), but could not be related with the modulus of elasticity ($P \geq 0.05$). The expression of *lox* showed a 3-fold reduction in fish fed PP100 compared to those fed FM. Moreover, the expression of *lox* was positively and significantly correlated with white muscle fibre diameter (0.495) and negatively correlated with fibre density (-0.602) (Table 5).

## 4. Discussion

Senegalese sole seems to have great capacity of using high levels of dietary PP protein sources as previously reported in several studies with juveniles (Cabral et al., 2011; Silva et al., 2009). Cabral et al (2013) has recently showed that large-sized sole could also effectively use practical diets containing up to 75% of PP sources, without affecting feed intake, growth performance or nutrient utilization whilst preserving the nutritional fillet value. However, total FM substitution impaired growth. The present study shows that the inclusion of PP sources in Senegalese sole diets significantly affects muscle growth dynamics, confirming earlier observations in several fish species (Alami-Durante et al., 2010a; Matos et al., 2012). In seabream, a 90% PP diet significantly decreased white fibre diameter with a concomitant increase of fibre density, although final body size remained similar to the control (Matos et al., 2012). In rainbow trout, total FM substitution also induced a significant decrease of the white muscle fibres diameter, but that resulted in smaller-sized fish (Alami-Durante et al., 2010a). Similarly, in Senegalese sole, PP100 diet impaired growth and resulted in significantly smaller muscle cross sectional area (CSA). This muscle CSA reduction was mainly due to a decrease in the muscle fibre size as the total number of fibres did not vary significantly among treatments. These results suggest a prevalence of hyperplastic growth by input of new fibres in fish fed 100PP, rather than by hypertrophy of the existing ones.

In Senegalese sole diets, FM replacement by PP sources in diets significantly affected the expression of several growth-related genes. Among the myogenic regulatory factors (MRFs), a significant down-regulation of *myod1* expression (one of the earliest markers of myogenic commitment) was observed in fish fed PP100 diet,

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without affecting the percentage of small-sized fibres or the total number of white fibres. In rainbow trout, soybean-meal rich diets also resulted in a significant decrease of *myoD1* expression, but increased the percentage of small white muscle fibres (diameter below 20 µm) (Alami-Durante et al., 2010b). The two *myod* paralogs evaluated in Senegalese sole responded differently to the treatments, as *myod2* was not significantly affected by PP diets. Likewise, *myogenin* expression was not significantly affected by increasing levels of PP diets, confirming previous results in rainbow trout (Alami-Durante et al., 2010a; 2010b). The present results showed that the expression of *mrf4,* another MRF required for both cell specification and differentiation (Chen and Tsai, 2008), decreased significantly with the dietary inclusion of PP, irrespectively of its inclusion level. Reduced expression of muscle *mrf4*, but not *myf5*, was previously associated with lower growth in Senegalese sole fed high dietary lipid levels (Campos et al., 2010), pointing towards a nutritional modulation of muscle development. Moreover, higher expression of *mrf4* was associated with fibre area increase in sole early stages (Campos et al., 2013). In the present study growth impairment and muscle fibre size reduction could be observed in fish fed with the highest PP level (PP100). Moreover, the expression of *mrf4* was highly and positively correlated (r=0.9; Table 5) with the expression of *myosins* (*myhc* and *mylc2*), as both gradually decreased with the increasing FM replacement by PP sources in diets, ultimately resulting in a significant decrease of fibres diameter and final body size in the PP100 diet. Similarly, Campos et al. (2010) have previously reported a positive correlation between *mrf4* and *mylc2* expression and protein gain in Senegalese sole fed different lipid levels, suggesting their expression could be useful markers for this species growth. The effects of dietary PP sources on the myosins expression are controversial. In rainbow trout, the expression of *myhc* in white muscle was not modified by diets with increasing levels of a PP mixture

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(Alami-Durante et al., 2010a), but it was upregulated in fish fed a soybean rich diet (Alami-Durante et al., 2010b). These results suggest that the mRNA levels of MHC may depend on the dietary amino-acid profile.

The insulin-like growth factor (IGF) signalling pathway consists of multiple IGF ligands, IGF receptors, and IGF-binding proteins (IGFBPs) that play a central role in the regulation of growth in all vertebrates (Duan, 1998). Although this system is known to be highly regulated by the nutritional status of fish (Chauvigné et al., 2003; Hevrøy et al., 2007; Montserrat et al., 2007; Valente et al., 2012; 2013) very few studies evaluated the effects of dietary PP sources on fish *igf´s* expression in muscle. In the present study, the *igf-I* and *igf-II* mRNA transcripts remained unaffected by dietary treatments, but the skeletal muscle expression of *igf1r* and *insr* receptors decreased significantly in response to total substitution of FM by PP sources (PP100 diet). IGF´s might have kept their mitogenic capability resulting in similar total number of cells among treatments, but the lowest cell size observed in fish fed PP100 suggests that their hypertrophic role could be negatively affected by a lower receptor density. Lie at al. (2011) reported reduced expression of IGF-IIR in the liver of Atlantic cod *Gadus morhua* fed diets with 75 and 100% PP, resulting in a significant decrease of growth. This is consistent with previous data in gilthead sea bream showing a decrease in circulating IGF-I levels in fish fed PP-based diets (Gomez-Requeni et al., 2004). In the present study IGF's circulating levels were not evaluated and its transcription remained unaltered. Nevertheless, the expression of its receptors was strongly affected by high dietary PP levels probably mediating several metabolic functions that resulted in growth impairment.

Members of the Fibroblast Growth Factor family (i.e. FGF6) are potent moderators of critical phases of muscle development. Prolonged expression of FGF6 is

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involved in the continuous proliferation of myogenic cells during fish growth (Rescan, 1998). Campos et al. (2010) reported that *fgf6* expression in *S. senegalensis* fast muscle showed a positively correlation with protein efficiency ratio and negatively with feed conversion ratio. In our study, *fgf6* expression in Senegalese sole white muscle showed a significant decrease in all PP groups, displaying a positive correlation with *igf* receptors (*igf1r*, r=0.761; *insr*, r=0.858). These results suggest an important role of *fgf6* in the mechanisms involved in growth regulation in white muscle. Follistatin (FST), an antagonistic member of the transforming growth factor-β (e.g. myostatin), is an activin-binding protein known to be involved in muscle growth and development (Lee and McPherron, 2001). In rainbow trout, overexpression of follistatin was shown to enhance or stimulate muscle growth (Medeiros et al., 2009). In the present study, *fst* gene expression was downregulated with increasing inclusion of PP sources in the diet. The lowest value of *fst* expression was detected in PP100 group, as well as final body weight and fibre diameter. However, statistical analysis indicated no correlation between *fst* expression and final body weight or fibre diameter. Similarly, Campos et al. (2010) found no correlation between growth and *fst* expression in Senegalese sole fed with different dietary lipids levels. Still, there was a strong positive correlation between *igfs* receptors and *fst* expression (*igf1r*, r=0.727; *insr*, r=0.783). Present results point towards a possible modulation of *fst* and *igfs* receptors expression and muscle growth by PP dietary levels in Senegalese sole.

Flavor and textural properties are important attributes of seafood. Among texture attributes, firmness also termed hardness is closely associated with the human visible acceptability of fish products. Instrumental methods provide an overall understanding on the mechanical properties of fish that closely reflect sensory perception of muscle texture (Coppes et al., 2002). Recent work on human mastication

highlights the importance of two mechanical properties- toughness and modulus of elasticity (i.e, stiffness) for food breakdown during mastication (Williams et al., 2005). In the present study fish fed PP100 diet exhibited a significantly higher modulus of elasticity, i.e. lower fillet stiffness, compared to other dietary treatments. Valente et al (2011a) have previously reported a negative correlation between flesh firmness and white fibre diameter in gilthead seabream from distinct production systems. But the present results show that the modulus of elasticity was only moderately related with fibre density (r=0.510) and muscle fibre size (r=-0.573). Hagen et al. (2007) has previously suggested that muscle texture is more influenced by collagen crosslinking and collagen content than fibre density. It has been suggested an important role of lysyl oxidase (LOX) in cross-linking of collagen contributing for flesh firmness (Consuegra and Johnston, 2006; Johnston et al., 2006). In the present study, the expression of *lox* (protein-lysine 6-oxidase) could not be related with flesh stiffness (P≥0.05), but was positively correlated with white muscle fibre size (0.495), and negatively correlated with fibre density (-0.602). Nevertheless LOX protein levels were not analysed being difficult to clearly deduce a possible collagen crosslinking through evaluation of *lox* expression *per se* and associate it with flesh firmness. According to Salmerón et al (2013) calpain expression could be modulated by nutritional status and diet composition of gilthead seabream and correlated with muscle texture, but that could not be confirmed in the present study. The observed phenotypic changes at the muscle cellular level were not associated with the expression of texture-related genes evaluated (*capn2*, *ctsb*, *ctsd*), since no significant differences were verified in the expression of these genes among fish fed increasing PP levels confirming previous data on rainbow trout (Alami-Durante et al., 2010a).

In conclusion, Senegalese sole can use diets with PP sources up to 75% of fish meal replacement, but total substitution of dietary FM (PP100) impairs growth. The smallest muscle CSA and fibre diameter observed in fish fed PP100 was associated with reduced expression of several key genes involved in myogenesis and muscle growth (*mrf4*, *fgf6*, *myhc* and *mylc2*). Decreased stiffness (higher modulus of elasticity) in PP100 fillets was probably associated with cell size reduction suggesting a prevalence of hyperplastic growth in this fish. The expression of *lox* was positively correlated with white muscle fibre size (0.495), and negatively correlated with fibre density (-0.602), and could be a useful indicator for predicting flesh textural properties. The present results point towards a modulation of the expression of several muscle growth related genes by increasing levels of PP sources that alter muscle cellularity and textural properties of Senegalese sole when the level of substitution was total (PP100).

Table 1. Feed ingredients and proximate composition of the dietary treatments.

| | Dietary treatments | | | |
|---|---|---|---|---|
| | FM | PP50 | PP75 | PP100 |
| *Feed ingredients (%)* | | | | |
| Fishmeal 70 LT[a] | 24.5 | 8.0 | 5.5 | 0.0 |
| Fishmeal 60[b] | 27.0 | 13.0 | 0.0 | 0.0 |
| CPSP90[c] | 5.0 | 5.0 | 5.0 | 0.0 |
| Squid meal | 5.0 | 5.0 | 5.0 | 0.0 |
| Pea | 0.0 | 11.5 | 14.0 | 17.0 |
| Soycomil PC[d] | 0.0 | 4.0 | 6.0 | 12.3 |
| Soybean meal[e] | 12.5 | 9.8 | 9.0 | 8.0 |
| Potato concentrate | 0.0 | 2.5 | 6.0 | 6.0 |
| Wheat gluten | 0.0 | 3.5 | 7.0 | 13.4 |
| Corn gluten | 0.0 | 7.5 | 9.0 | 12.0 |
| Aquatex G2000[f] | 11.0 | 9.4 | 11.5 | 9.0 |
| Wheat meal | 11.0 | 12.0 | 9.6 | 7.3 |
| Fish oil | 2.0 | 4.5 | 5.7 | 6.8 |
| Vit[g] & Min Premix[h] | 1.0 | 1.0 | 1.0 | 1.0 |
| Di-calcium phosphate | 0.0 | 1.6 | 4.0 | 5.0 |
| L-Lysine | 0.0 | 0.5 | 0.5 | 0.9 |
| DL-Methionine | 0.0 | 0.2 | 0.2 | 0.3 |
| Binder[i] | 1.0 | 1.0 | 1.0 | 1.0 |
| | | | | |
| *Proximate composition* | | | | |
| Dry matter (DM, %) | 91.1 | 93.7 | 93.4 | 93.5 |
| Ash (% DM) | 13.5 | 9.5 | 8.8 | 8.7 |
| Crude protein (% DM) | 54.2 | 54.0 | 54.7 | 55.1 |
| Crude fat (% DM) | 8.8 | 9.2 | 9.4 | 9.8 |
| Total phosphorus (%DM) | 1.7 | 1.4 | 1.5 | 1.4 |
| Gross energy (kJ/g DM) | 20.2 | 20.7 | 20.8 | 20.9 |
| Digestible protein (DP,% DM) | 39.9 | 38.2 | 41.4 | 43.7 |
| Digestible energy (DE, kJ/g DM) | 14.0 | 12.5 | 13.0 | 14.6 |
| DP/DE mg/kJ | 28.4 | 30.6 | 32.0 | 30.0 |

[a]Peruvian fishmeal LT (71% crude protein, 11% crude fat, EXALMAR, Peru)
[b]Fair Average Quality (FAQ) fishmeal (62% crude protein, 12% crude fat, COFACO, Portugal)
[c]Soluble fish protein hydrolysate (87% crude protein, 6.5% crude fat, Sopropêche, France)
[d]Soycomil-P (soy protein concentrate, 65% crude protein, 0.7% crude fat, ADM, The Netherlands)
[e]Dehulled solvent extracted soybean meal (micronized)
[f]Aquatex G2000 (Dehulled, grinded pea grits, 24% crude protein, 0.4% crude fat, SOTEXPRO, France)
[g]Vitamins (mg, mcg or IU/kg diet): Vitamin A (retinyl acetate), 20.000 UI; vitamin D3 (DL-cholecalciferol), 2000 UI; vitamin E (Lutavit E50), 100 mg; vitamin K3 (menadione sodium bisulfitete), 25 mg; vitamin B1(thiamine hydrochloride), 30 mg; vitamin B2 (riboflavin), 30 mg; calcium pantothenate, 100 mg; nicotinic acid, 200 mg; vitamin B6 (pyridoxine hydrochloride), 20 mg; vitamin B9 (folic acid), 15 mg; vitamin B12 (cyanocobalamin), 100 mcg; vitamin H (biotin), 3000 mcg; vitamin C (Lutavit C35), 1000 mg; inositol, 500 mg; colin chloride, 1000 mg; betaine (Betafin S1), 500 mg.
[h]Minerals (mg or %/kg diet): Co (cobalt carbonate), 0.65 mg; Cu (cupric sulphate), 9 mg; Fe (iron sulphate), 6 mg; I (potassium iodide), 0.5 mg; Mn (manganese oxyde), 9.6 mg; Se (sodium selenite), 0.01 mg; Zn (zinc sulphate) 7.5 mg; Ca (calcium carbonate), 18.6%; KCl, 2.41%; NaCl, 4.0 %.
[i]Diatomaceous earth: Kielseguhr: LIGRANA GmbH, Germany.

Table 2. Nucleotides sequences of the PCR primers used to evaluate mRNA abundance by qPCR.

| Gene | Fwd sequence (5'→3') | Rev sequence (5'→3') | Acession | Size(bp) | *E(%)* |
|---|---|---|---|---|---|
| *igf-I* | CAGGCTATGGCTACAACACAC | CACAGTACATCTCCAGGCG | AB248825 | 93 | 105 |
| *igf-II* | GCAGAATGAAGGTCAAGAAGATG | CGAGACCACTTCCACAGC | AB248826 | 89 | 104 |
| *igf 1r* | GCTGTTAAAATAGGAGATTTCGG | GGAGCAAACCCTTACCACC | FJ515914 | 82 | 98 |
| *insr* | CGTGGTTCGTCTTTTGGG | CTCTTCAGGTCACCGTGAGTC | FJ515913 | 84 | 91 |
| *myf5* | AAGAACGAGAGTTTGGGTGGTA | TGGTCGTGTCTCTGTGAGCG | FJ515910 | 125 | 110 |
| *mrf4* | GAGAGGAGGAGGCTCAAGAAG | CAGGTCCTGTAATCTCTCAATG | EU934042 | 137 | 101 |
| *myog* | CCCTGGTGTCTTCCCTCA | GACGACGACACTCTGGGC | EU934044 | 94 | 96 |
| *myod1* | CTCCTCCTCCCCGTCATC | TTGGTGGTCTTCCGCTTG | FJ009109 | 144 | 90 |
| *myod2* | ACAGCCACCAGCCCAAAC | GTGAAATCCATCATGCCATC | FJ009108 | 194 | 102 |
| *fst* | CATCAAAGCTAAGTCGTGTGAG | CACCGCCTCCTCTGTCTTG | EU934045 | 133 | 96 |
| *fgf6* | AAGGTTATACGGAACGACAGTC | CGCAATGTAGAAGCCTCTGTAG | FJ009110 | 122 | 112 |
| *myhc* | GAAAAATCTGACAGAGGAAATGG | CCTTGGTGAGAGTGTTGACTTTG | FJ515911 | 143 | 104 |
| *mylc2* | GTACAAGGAGGCGTTCACAATC | CCAGCACGTCCCTAAGGTC | FJ515912 | 77 | 106 |
| *capn2* | GCCTATGCCAAAGTCAATGG | ATAGTTCTCAGCGATGCCTCC | JN990079 | 96 | 88 |
| *ctsb* | CTGCCTGGGAGTTCTGGAC | AGGGCGGCAACCTATGTG | KC237277 | 74 | 107 |
| *ctsd* | GCATGATCGGAGACCTGGC | CCCCGTCCACAGATATGCG | KC237276 | 132 | 93 |
| *lox* | GGACCTGTAGGCTGAGCTTG | CGTCCCCGATCCATACTATGT | KC237278 | 112 | 111 |

For each gene, its GenBank accession number, amplicon size (bp) and amplification efficiency (E). The annealing temperature of all primer pairs is 60ºC. *igf-I* and *igf-II*, insulin-like growth factor I and II; *igf 1r* and *insr*, insulin-like growth factor receptor I and insulin receptor; *myf5*, myogenic factor 5; *mrf4*, muscle-specific regulatory factor 4; *myog*, myogenin; *myod1*, myoblast determination protein 1; *myod2*, myoblast determination protein 2; *fst*, follistatin; *fgf6*, fibroblast growth factor 6; *myhc*, myosin heavy chain; *mylc2*, myosin light chain; *capn2*, calpain 2; *ctsb* ,cathepsin b; *ctsd*, cathepsin d; *lox*, protein-lysine 6-oxidase.

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

| Gene | Fwd sequence (5'→3') | Rev sequence (5'→3') | Acession | Size(bp) | E(%) | $R^2$ |
|---|---|---|---|---|---|---|
| *Ubq* | TCTGCGTGGTGGTCTCATC | TGACCACACTTCTTCTTGCG | AB291588 | 135 | 99 | 0.979 |
| *rpS4* | CTGCTGGATTCATGGATGTG | GGCAGTGATGCGGTGGAC | AB291557 | 100 | 92 | 0.995 |
| *Eef1a1* | ATTGGCGGCATTGGAACA | CATCTCCACAGACTTGACCTC | AB326302 | 116 | 117 | 0.986 |

For each gene, its GenBank accession number, amplicon size, amplification efficiency (E) and correlation coefficient (R2) of the calibration curve are indicated. Ubq, ubiquitin; rpS4, ribosomal protein S4; Eef1a1, elongation factor 1 alpha isoform 1.

Table 4. Growth performance, muscle cellularity and texture of white muscle in Senegalese sole fed the experimental diets for 140 days

| | Dietary treatments | | | |
|---|---|---|---|---|
| | FM | PP50 | PP75 | PP100 |
| *Growth performance* | | | | |
| Final body weight (g) | $242.3 \pm 29.7^a$ | $238.4 \pm 26.1^a$ | $215.8 \pm 19.9^a$ | $172.7 \pm 15.5^b$ |
| Final body length (cm) | $25.9 \pm 0.9^a$ | $25.00 \pm 1.1^a$ | $24.8 \pm 1.1^a$ | $23.25 \pm 0.5^b$ |
| K | $1.4 \pm 0.1$ | $1.5 \pm 0.1$ | $1.4 \pm 0.2$ | $1.4 \pm 0.2$ |
| *Muscle Cellularity* | | | | |
| Muscle CSA ($mm^2$) | $255.2 \pm 26.1^a$ | $274.2 \pm 32.6^a$ | $265.9 \pm 11.0^a$ | $210.00 \pm 42.0^b$ |
| Fibre diameter ($\mu m$) | $65.7 \pm 7.6^{ab}$ | $72.1 \pm 5.3^a$ | $62.8 \pm 2.7^{ab}$ | $60.0 \pm 6.3^b$ |
| Total number of fibres x $10^3$ (N) | $63.7 \pm 17.3$ | $58.8 \pm 12.5$ | $72.4 \pm 6.2$ | $62.3 \pm 14.6$ |
| Fibre density ($N/mm^2$) | $250.1 \pm 64.95^{ab}$ | $214.4 \pm 39.02^b$ | $272.7 \pm 27.2^{ab}$ | $297.9 \pm 50.03^a$ |
| % Fibre <30$\mu m$ | $1.9 \pm 1.8$ | $0.5 \pm 0.5$ | $1.1 \pm 1.0$ | $1.1 \pm 0.4$ |
| *Texture* | | | | |
| Modulus of Elasticity (GPa) | $0.40^b \pm 0.07$ | $0.40^b \pm 0.03$ | $0.38^b \pm 0.06$ | $0.50^a \pm 0.09$ |

Data represent mean values ± SD. Different letters indicate significant differences ($P<0.05$) between diets.

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Table 5. Spearman´s Rank order Correlation coefficient and significance level between muscle cellularity, texture and the expression of related genes (N=21).

|  | Modulus of elasticity | *lox* | *myhc* | *mylc2* | *Igf1r* | *Insr* |
|---|---|---|---|---|---|---|
| **Body length** | -0.600* | 0.464* | 0.456* | 0.562** | 0.308 | 0.406 |
| **Fibre diameter** | -0.573 | 0.495* | 0.244 | 0.314 | 0.121 | 0.151 |
| **Fibre density** | 0.510 | - | -0.354 | -0.384 | -0.196 | -0.288 |
| *Myod1* | -0.476 | 0.814** | 0.852** | 0.839** | 0.725** | 0.786** |
| *Myog* | -0.140 | 0.856** | 0.832** | 0.634** | 0.631** | 0.682** |
| *mrf4* | -0.175 | 0.758* | 0.908* | 0.852* | 0.727* | 0.853* |
| *fgf6* | -0.168 | 0.542* | 0.766* | 0.791* | 0.761* | 0.858* |
| *fst* | 0.301 | 0.771* | 0.858* | 0.836* | 0.727* | 0.783* |
| *lox* | 0.364 | - | 0.835* | 0.784* | 0.571* | 0.722* |

*P<0.05; **P<0.01

**Figure caption**

Fig.1 Probability density functions (PDFs) of muscle fibre diameter for white muscle in Senegalese sole fed the experimental diets for 140 days (n=1000 per dietary treatment). Dashed lines represent the average PDFs for each dietary group and the solid central line corresponds to the average PDF for combined groups. The shaded area shows 1000 bootstrap estimates from combined populations of fibre diameter.

Fig.2 Expression of Myogenic Regulatory Factors (*myod1*, *myod2*, *myf5*, *myog* and *mrf4*), Insulin-like Growth Factors (*igf-I*, *igf-II* and *igf1r*), Insulin Family (*insr*), *fst*, *fgf6*, Myosin Superfamily (*myhc* and *mylc2*), *lox* and Proteases family (*capn2*, *ctsb*, *ctsd*) in the skeletal white muscle from sole fed the experimental diets. Data represent mean values ± SE. Different letters indicate significant differences (p<0.05) between diets.
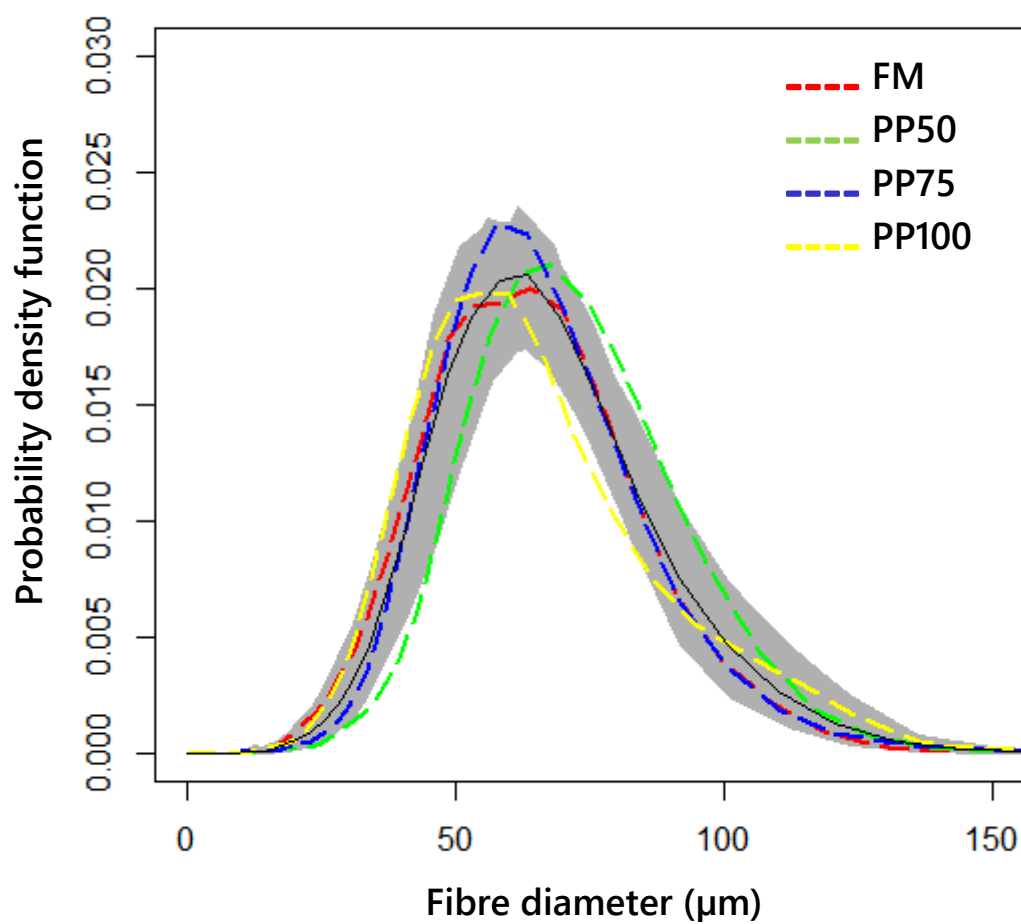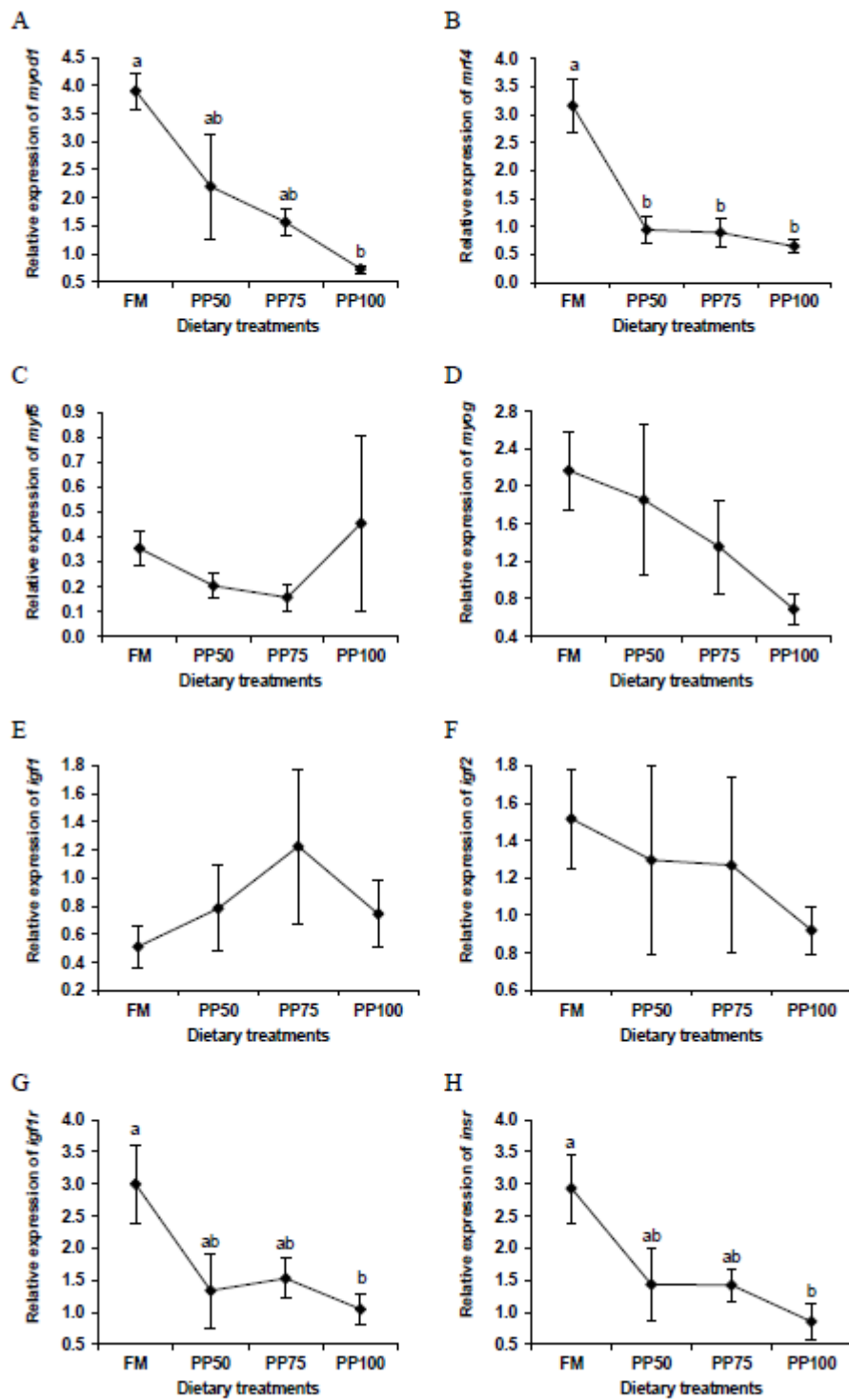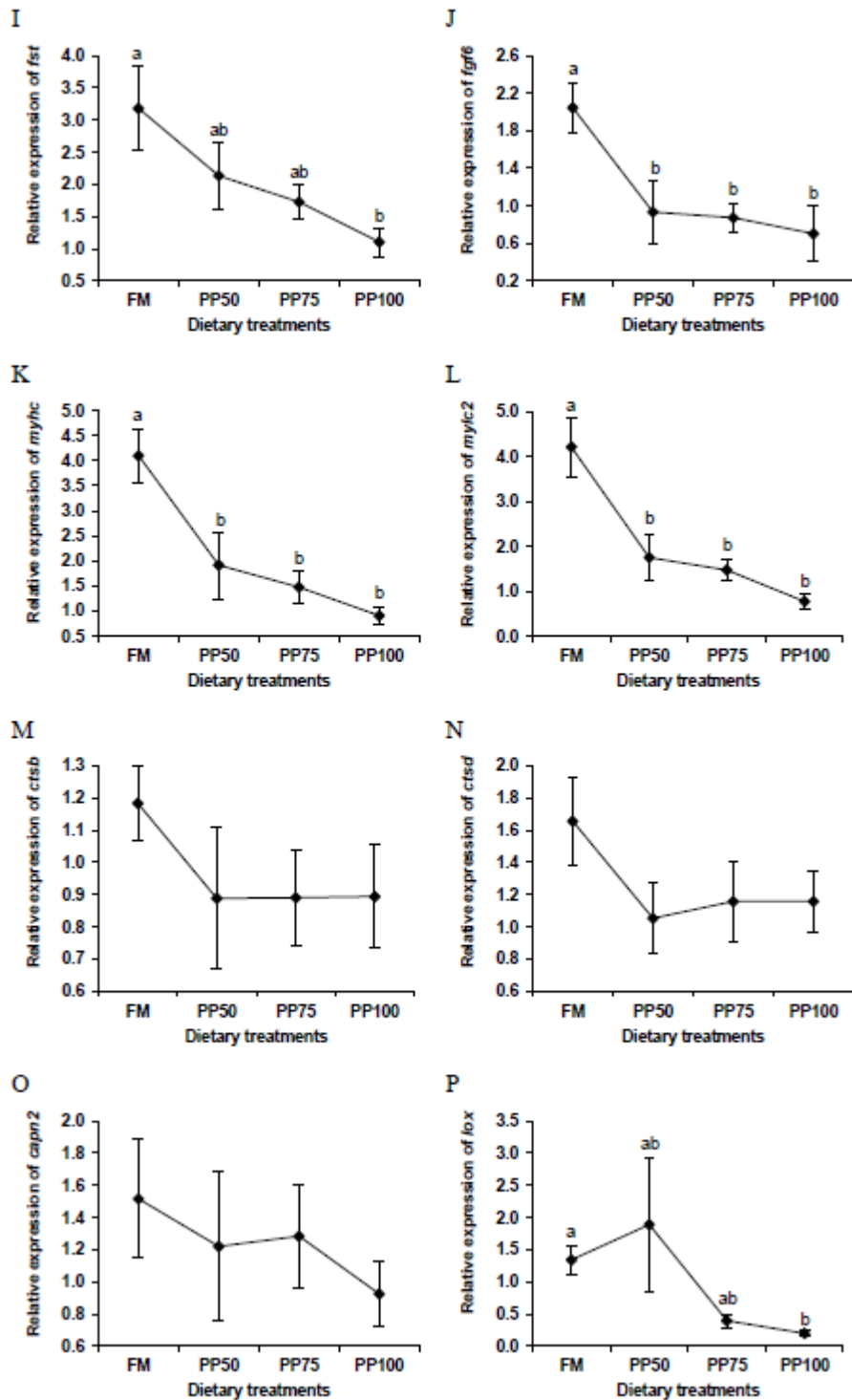
**Figure 1**

Figure 2

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**Highlights**

1. Senegalese sole can effectively use diets with PP sources up to 75% of fish meal replacement

2. Total FM substitution by PP (PP100) decreased final body size and muscle cross sectional area (CSA)

3. Smaller CSA in fish fed PP100 were associated with smaller fibre size

4. PP diets reduced expression of several key genes involved in myogenesis and muscle growth

5. Decreased stiffness (higher modulus of elasticity) in PP100 fillets was partially related with cell size reduction