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Effects of variable protein and lipid proportion in gilthead sea bream (*Sparus aurata*) diets on fillet structure and quality

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Abstract

Juvenile gilthead sea bream (*Sparus aurata*) were fed with seven experimental diets containing different proportions of protein and lipid (P/L): 38/29, 41/26, 44/25, 47/23, 50/22, 54/21 and 57/20 per cent of dry matter, respectively. After 12 weeks, fish fed on diet 38/29 (P/L) showed the lowest food efficiency and final weight. A correlation between the hepatosomatic index and fat content in the diets was observed, together with a decrease in muscle glycogen in animals fed with a 38/29 (P/L) diet. Whilst no statistical differences were found in flesh texture, a clear relationship was observed between elasticity and fibre density. Fillet Chroma was altered in the animals fed with the 38/29 (P/L) diet. The same group showed a decrease in hepatic growth factor receptor (c-met) and proliferating cell nuclear antigen (PCNA) expression in white muscle. Protein content in the diets correlated with mammalian target of rapamycin (mTOR) phosphorylation in muscle. In conclusion, we have shown that the substitution of protein by lipid in sea bream diets can be performed up to a certain percentage without affecting fish growth, and we have demonstrated that several molecules such as MyoD, mTOR, PCNA and c-met are affected by the dietary ratio of protein to lipid and could be potential flesh quality markers.

KEY WORDS: c-met, fat, fibres, mammalian target of rapamycin, muscle, myoblast determination factor, proliferating cell nuclear antigen, texture

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Introduction

The dietary protein fraction is the most important factor affecting fish growth and represents the largest proportion of feed production costs (Tacon & Metian 2008). It is commonly accepted that an increase in the protein fraction in diets improves fish growth, especially in carnivorous fish (Watanabe 2002). However, the high price of fish meal, the unreliability of the supplies (FAO 2008) and ammonia pollution (Shyong *et al.* 1998) constitute major problems for high-protein diets.

Many studies have focused on determining the minimum percentage of dietary protein necessary to obtain good growth rates and thus reduce feed production costs (Santinha *et al.* 1996; Shiau & Lan 1996; Santinha *et al.* 1999; Moon 2001; Lee *et al.* 2002; Espinós *et al.* 2003; Skalli *et al.* 2004). The efficiency of protein utilization for growth can be improved by replacing dietary protein with non-protein energy sources, such as lipids and/or carbohydrates, to produce a protein-sparing effect (Daniels & Robinson 1986; Boujard & Médale 1994; Pérez *et al.* 1997; Company *et al.* 1999; Benedito-Palos *et al.* 2008; Li *et al.* 2011). This approach allows the protein content of the diets to be reduced without seriously affecting the growth rates.

Different studies have addressed the ideal relationship between protein and lipid fractions to meet these objectives mentioned such as salmonids (Helland & Grisdale-Helland 1998) and marine fish (Santinha *et al.* 1996; Santinha *et al.* 1999; Vergara *et al.* 1999).

Gilthead sea bream (*Sparus aurata*) are an important species for aquaculture of the southern European coasts, and production increases annually (Department of Marketing & Institute of Aquaculture University of Stirling 2004; FAO 2008). Some studies have concentrated on determining the protein level necessary for good growth in this species. This

was first determined as being around 400g kg^{-1} of dietary dry matter (Sabaut & Luquet 1973) and later revised by Santinha *et al*. (1996), who suggested a minimum of 450g kg^{-1} protein for adequate growth rates.

In terms of lipids, some authors have proposed that 90g kg^{-1} fat can be formulated with 440g kg^{-1} protein in gilthead sea bream diets (Marais & Kissil 1979), whilst other studies have suggested 220g kg^{-1} lipids can be incorporated into the diet without affecting the quality of the fillet (Vergara *et al*. 1999).

Commercial diets for sparids like gilthead sea bream and dentex (*Dentex dentex*) routinely include 200g kg^{-1} lipids combined with 480g kg^{-1} protein for adult fish, although these proportions can vary during the lifecycle (Tibaldi *et al*. 1996; Espinós *et al*. 2003; Skalli *et al*. 2004; Benedito-Palos *et al*. 2008). Meanwhile, in salmonids, it is common to include until a 300g kg^{-1} lipids in the diet, which causes no negative effects (Rosenlund *et al*. 2001).

The effect of protein optimization on the final quality of the product for future commercialization must also be considered, but to date, this has been insufficiently studied. The quality of the final product is comprised of a complex network of organoleptic parameters, plus fillet composition, colour, texture and appearance, among others (reviewed for gilthead sea bream in Grigorakis 2007). An increase in dietary lipid content can affect consumer acceptance of the final product, because of the common belief that farmed fish tend to be fatter than wild fish, which generates a degree of rejection in the market (Haard 1992; Grigorakis & Alexis 2005). Because the final objective of the aquaculture is the commercialization of the fish, it is important to evaluate how an increase in the lipid content on fish diets affects quality.

The studies that analyse how lipid fraction affects quality focus on fillet composition but do not consider colour and texture. Such an approach ignores the fact that colour and texture are two of the most important parameters for consumers when buying fish. Both these factors are complex components of the quality, and there is no agreement as to the most important factors that determine them, but lipid content and muscle histology seem to be crucial (reviewed in Johnston 2006; and for a gilthead sea bream in Grigorakis 2007). Because the muscle structure is important for quality, it is essential to understand the processes that determine fibre formation and growth. Satellite cells play a central role in fish muscle growth and formation (reviewed in Johnston 2006). During muscle growth, these satellite cells contribute to the formation of new fibres (hyperplasia) or to an increase in the size of existent ones (hypertrophy). For this reason, many studies have tried to determine the molecules, pathways and stimuli that control satellite cell activation and progress (reviewed in Johnston 2006).

Molecules involved in muscle growth include proliferating cell nuclear antigen (PCNA), myogenic differentiation factor (MyoD) and hepatic growth factor receptor (c-met) among others. PCNA has been described as an essential molecule in cell proliferation (Johnson & Allen 1993). MyoD is a member of the myogenic regulatory factors family that regulates myogenic programme initiation, which, together with PCNA, are involved in muscle growth as a result of exercise and nutritional effects (Brodeur *et al*. 2002; Martin & Johnston 2005). In contrast, c-met is generally considered a satellite cell marker, essential during embryogenesis (Dietrich *et al*. 1999). *In vitro* studies have also shown c-met to be involved in myoblast proliferation (Barani *et al*. 2003; García de la Serrana *et al*. 2007).

The PI3K/Akt/mTOR system is also important, and it is related to muscle hypertrophy and protein synthesis (Glass 2005; Periago *et al*. 2005). *In vitro* analysis of fish myoblast cultures has demonstrated that the PI3K/Akt pathway is sensitive to the action of insulin-like growth factors and it is involved in muscle differentiation (Castillo *et al*. 2006; Montserrat *et al*. 2007; Codina *et al*. 2008), and mTOR has been related to the dietary amino acids in trout (Seiliez *et al*. 2008).

In this study, we have analysed the influence of the dietary protein/lipid fraction on the final quality of the fillet of gilthead sea bream. Also, we have studied the histological structure of the muscle and myoblast activity, with the aim of establishing possible markers to relate growth, muscle composition, cell activity, fibre structure and flesh quality.

Materials and methods

Experimental diets

Seven experimental diets were formulated to contain gradual increase in protein/lipid ratios (P/L): diet A (38/29 P/L), B (41/26 P/L), C (44/25 P/L), D (47/23 P/L), E (50/22 P/L), F (54/21 P/L) and G (57/20 P/L), as Table 1 summarized. They were formulated as isoenergetic diets (23 MJ kg^{-1}) by Skretting Aquaculture, Norway.

Animal conditions and sampling

During the experimental trial, gilthead sea bream (*S. aurata*) were maintained in Institut de Recerca i Tecnologia

Table 1 Formulation and chemical composition of the experimental diets fed to gilthead sea bream. Skretting Aquaculture designed the experimental diets and also performed nutrient analysis

Diets (g kg^{-1}protein/g kg^{-1} lipid)	A (38/29)	B (41/26)	C (44/25)	D (47/23)	E (50/22)	F (54/21)	G (57/20)
Raw material (g kg^{-1})							
Wheat	367.6	325.2	291.9	258.7	225.4	192.1	158.1
Wheat gluten	60.0	106.0	117.5	128.1	138.6	149.2	159.9
Fish meal	268.8	267.2	293.7	320.2	346.6	373.1	399.7
Soybean concentrate	90.0	106.9	117.5	128.1	138.6	149.2	159.9
Fish oil	210.0	190.2	175.8	161.5	147.1	132.8	118.9
Vitamin premix	2.6	2.6	2.6	2.6	2.6	2.6	2.6
Total	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0
Nutrient analysis (g kg^{-1})							
Dry matter	921.8	921.4	922.0	922.6	923.3	923.9	924.8
Crude protein	379.7	412.3	444.6	476.8	509.0	541.2	573.1
Crude fat	290.7	262.3	250.0	237.7	225.5	213.2	201.4
Amino acid analysis (g kg^{-1} dry matter)							
Asparagine	30.3	31.7	30.6	34.9	41.2	44.9	49.8
Serine	17.5	18.5	18.4	20.9	24.0	26.4	29.5
Glutamine	77.2	84.6	87.7	99.1	112.3	121.7	130.7
Prolactin	23.4	25.4	26.4	30.1	33.6	35.9	38.7
Glycine	21.1	34.0	18.6	21.2	24.5	26.4	29.4
Alanine	18.7	19.2	18.4	21.3	25.6	26.5	29.7
Cysteine	2.4	2.7	2.6	3.1	3.7	4.0	4.4
Valine	11.2	11.8	11.5	13.3	17.1	16.5	17.8
Threonine	12.2	12.6	12.5	14.3	16.7	18.7	20.8
Methionine	8.7	8.8	10.2	11.9	13.8	15.2	17.1
Isoleucine	10.0	10.6	10.5	12.1	13.9	14.9	16.2
Leucine	25.1	26.5	26.2	30.1	34.0	36.8	40.6
Tyrosine	9.1	9.3	9.1	10.7	12.2	13.5	14.5
Phenylalanine	15.3	16.3	16.7	18.6	21.4	22.6	24.8
Lysine	22.4	24.5	23.5	26.8	30.7	33.2	36.7
Histidine	7.1	7.5	7.4	8.7	9.4	10.1	11.2
Arginine	18.4	19.2	18.7	21.5	24.8	27.1	30.1
Total	330.1	363.1	349.1	398.6	458.9	494.4	542.0
EAA	139.4	147.1	146.4	167.9	194.0	208.6	229.8
NEAA	190.7	216.0	202.7	230.6	264.9	285.7	312.2
EAA/NEAA	0.73	0.68	0.72	0.73	0.73	0.73	0.74

EAA, essential amino acids; NEAA, non-essential amino acids.

Agroalimentària (IRTA) installations at Sant Carles de la Ràpita, Tarragona, Spain. Fish, weighing around 70 g, were adapted to the installation conditions over 2 weeks using a standard commercial fish feed. Following the adaptation period, the fish were randomly distributed into three identical fibre glass tanks of 400 L (30 fish stocked per tank) for each experimental condition (21 tanks in total). Animals were fed until satiation twice a day, 5 days a week for 3 months (May–July) with the different experimental diets. Fish were fed with 4 mm extruded pellet. During this period, water temperature, oxygen level, salinity, photoperiod and temperature were maintained under natural conditions. To determine feed consumption, fish were fed until satiety and then not consumed; pellets were collected and weighted. Total biomass from all triplicated tanks was obtained from Skretting to evaluate the weight gain during the experimental period and used to calculate feed efficiency.

To perform the different measurements once the experimental period had finished, the fish were fasted for 8 or 24 h before sampling. Twelve fish (four per tank) for each time point were captured at random from each feeding group and anaesthetized with 2-phenoxyethanol (100 mg kg^{-1}) diluted in seawater for muscle sampling (n = 24). For muscle composition, texture, colour and histology, only animals fasted 24 h were used. In all cases, fish were extracted rapidly, to avoid possible differences in protein phosphorylation between animals in the same treatment. Also, last meal was administrated within 30 min between groups to have enough time for sampling and avoid differences in phosphorylation between treatments.

The fish were killed by cutting the spinal column and were eviscerated to measure mesenteric fat and liver weight. White muscle from the middle/front region was

extracted and frozen immediately in liquid nitrogen before storing at −80 °C until analysis.

For texture, 2 × 2 cm pieces of fillet were extracted from the lateral anterior region of the fish after carefully removing skin and red muscle. Portions were kept in a plastic bag and stored on ice until texture analysis 24 h later.

Muscle for histological analysis was extracted from the middle region of the fish and frozen in 1 mL of cold 2-methylbutane. The samples were then maintained in liquid nitrogen until analysis.

Muscle composition

Prior to analysis, the muscle from nine fish fasted 24 h (three per tank) per condition was ground in a mortar with liquid nitrogen. Muscle pieces were used for composition determination (lipid, protein, glycogen and water) and Western blot analysis.

Lipid extraction was based on the chloroform/methanol method (Folch *et al.* 1957): 700 mg of muscle was mixed with 8 mL of methanol/chloroform 2 : 1 mix per duplicate. Chloroform in the lipid extraction was completely evaporated under a hood. Lipid content was evaluated by a gravimetric procedure, and the results were expressed as mg lipid per 100 mg wet weight.

For glycogen evaluation, an extraction based on ethanol precipitation was used: 600 mg of muscle was digested with 30% KOH and mixed with 2 mL of absolute ethanol for glycogen precipitation (Good *et al.* 1933). After the ethanol precipitation, the pellet was diluted and used for analysis. Glycogen was evaluated by colour using an anthrone (SIGMA, Madrid, Spain) and sulphuric acid solution. The reaction generates a green solution detectable at 625 nm (Fraga 1956) and can, therefore, be counted using 96-well plates in a Tecan Infinite M2000 spectrophotometer (Tecan Group Lt, Männedorf, Switzerland). The results are expressed as mg glycogen per 100 mg wet weight.

Muscle water content was determined by gravimetric procedures, using 500–1000 mg of white muscle dried at 105 °C for 24 h. The results are expressed as mg water per 100 mg wet weight.

Ten to twenty milligram of muscle was used to estimate the soluble protein fraction. Samples were homogenized in 1 : 5 volumes of Tris–HCl. Protein concentration was determined using the Bradford method (Bradford 1976), and the results are expressed as mg protein per 100 mg wet weight.

Protein homogenates and Western blot analysis

For Western blot analysis, 20–40 mg of protein was homogenized in ice with 500 μL of RIPA buffer, and protease and phosphatase inhibitors (protease inhibitor cocktail P8340, 50 mmol L^{-1} NaF and 0.4 mmol L^{-1} Na_2VO_4; SIGMA). Homogenates were incubated in continuous movement at 4 °C to extract all protein and centrifuged at 13 800 ***g*** in a bench centrifuge at 4 °C for 15 min. The supernatant was stored at −80 °C until Western blot analysis.

Forty microgram of protein was resolved by SDS-PAGE for 90 min at 100 V (mTOR was separated overnight at 35 V and 4 °C). Proteins were transferred to a polyvinylidene fluoride membrane using precold transfer buffer (25 mmol L^{-1} Tris–HCl, 192 mmol L^{-1} glycine and 20% methanol) for 2 h at 100 V (in the case of mTOR, for 4 h at 40 V and 4 °C).

Next, the membranes were blocked with 5% non-fat milk buffer (50 mmol L^{-1} Tris–HCl, 150 mmol L^{-1} NaCl, 0.1% Tween 20, 5% non-fat milk) for 2 h at room temperature. The membranes were incubated overnight at 4 °C with the appropriate antibody against the target protein in wash or block buffer with 0.01% sodium azide, all primary antibodies used were diluted 1/1000 (v/v). After the primary antibody, the membranes were incubated for 1 h at room temperature with an appropriate horseradish peroxidase (HRP)-linked secondary antibody (Santa Cruz Biotechnologies, Helderberg, Germany) (1/10 000). Immunoreactive bands were developed by enhanced chemiluminescence and quantified using an image analyzer (TotalLab v1.00; Nonlinear Dynamics Ltd, 2000, Newcastle, UK). One sample was loaded onto all gels as a loading control and for the comparison of the different westerns. Total Akt and mTOR were used to normalize their respective phosphoprotein values. Values are expressed as percentage in relation with group A set as 100%. C-met and PCNA were normalized by β-actin. In this study, Akt-p (9271; Cell Signaling, Barcelona, Spain), total Akt (9272; Cell Signaling), mTOR-p (2971; Cell Signaling), total mTOR (2949; SIGMA), PCNA (P10 clone; sc-56; Santa Cruz Biotechnologies) and c-met (sp-260 clone, sc-162; Santa Cruz Biotechnologies) antibodies were used for detection.

Immunohistological analysis

Fish muscle, stored in liquid nitrogen with 2-methylbutane, was cut in a Leica cryomicrotome (Meyer Instruments, Houston, TX, USA) at −20 °C. Pieces approximately 6-μm

thick were plated onto Poly-Lysine precoated slides (P0425; SIGMA) and stored at −80 °C until analysis.

The samples were fixed and treated, as previously described for salmon (*Salmo salar*) (Johnston *et al.* 1999). For MyoD detection, we used an anti-MyoD antibody provided by Professor Ian A. Johnston (Scottish Oceans Institute, Saint Andrews, UK). Sections were co-stained with Mayer's haematoxylin to facilitate the identification of positive nuclei.

Five different regions of each sample were captured using a 10× objective with an Olympus camera (CC12 model, Olympus, Barcelona, Spain) coupled to a microscope (BX40 model, Olympus). Positive cells were counted using image analysis software (analySYS®; Soft Imaging System, Münster, Germany).

Histological fibre analysis

Six muscle samples per feeding group were cut using a Leica cryomicrotome at −20 °C. Sections 20 μm wide were obtained and plated onto 1% gelatin-gold pretreated slides. These were stored at −80°C until histological analysis.

The slides were fixed in 5% paraformaldehyde for 5 min at room temperature and stained for 10 min using filtered Mayer's haematoxylin at room temperature. Colour was developed under tap water for 10 min, dehydrated with increasing levels of ethanol and given a final xylene wash prior to mounting with DPX (SIGMA).

Pictures from five different regions per muscle were captured using an Olympus camera coupled to a microscope. All pictures were acquired using the 10× objective. Approximately 900–1000 fibres per sample were evaluated using image analysis software (analySYS®; Soft Imaging System).

Colour measurements

Muscle colour was measured at the time of sampling per duplicate using a portable CR400 Chroma Meter (Konica Minolta, Madrid, Spain). Measurements were acquired from the proximal zone of the fish. The machine was calibrated using a white standard provided by the company.

The colour system L^*, a^* and b^* was used for colour analysis. The L^* variable represents lightness ($L^* = 0$ for black, $L^* = 100$ for white), the a^* scale indicates red/green ($+a^*$ intensity in red and $-a^*$ intensity in green), and the b^* scale represents yellow/blue ($+b^*$ intensity in yellow and $-b^*$ intensity in blue) (Hunter & Harold 1987). Chroma (colour intensity) and Hue angle (composed colour) were calculated from the data obtained with the colorimeter (Chroma $= \sqrt{(a^{*2} + b^{*2})}$; Hue $= \tan^{-1} b^*/a^*$).

Texture measurements

Texture analysis was performed in the Departament d' Agrotecnologia of the Universitat Politècnica de Catalunya using a TA.XT2i Texture Analyzer and Exponent 4.0.9 software (Stable Micro Systems Ltd., Godalming, UK). The texturometer was coupled to a Mini Kramer HDP/MKO5 (Stable Micro Systems), a compression test using a load cell of 30 kg with a test speed and postspeed of 1 mm s^{-1}. The blade was at a 90° angle and perpendicular to muscle fibres.

Total work, maximal strength and elasticity were measured in all samples 24 h after sampling to reduce the *rigor mortis* effect on muscle texture analysis. The size of the muscle pieces (2 × 2 cm) was verified immediately prior to analysis and adjusted where necessary to ensure all the samples were the same size. To work at a constant temperature with all the samples, the muscle pieces were conserved on ice until texture analysis was carried out. At the same time, the depth of all the pieces of muscle was evaluated.

Maximal strength is defined as the maximal force applied during a complete texture analysis. Elasticity is the capacity of the muscle to recover its original aspect following the application of force. Elasticity coincides with the linear portion of the texture curve before break point. Total work is the parameter used to define the force required in order to cut the sample completely. Values were normalized to a depth of 1 cm.

Statistical analysis

All parameters were tested for normalization using the Kolmogorov–Smirnov non-parametric test for normal distribution.

Data were plotted using SPSS 13 for Windows (IBM, San Jose, CA, USA) and represented as mean ± standard error. Statistical differences between treatments were analysed by nested one-way analysis of variance (ANOVA) with tank as random factor to test possible tank effect. When tank effect was not found, an ANOVA followed by Tukey's *post hoc* test was performed when possible. Statistical differences were considered significant when P-values were <0.05.

Linear correlations between parameters were accepted as significant when the P-value was <0.05. The Pearson correlation index was calculated for all significant correlations

found; it is indicated in all cases when significant correlations were found.

Results

Animal growth and body parameters

Final body weight was significantly lower in animals from treatment A compared with the other groups. Length was lower in animals' fed diet A than diet B and significantly lower in the other groups in which the percentage of protein in the diet was higher and lipid lower. Weight values were over 200 g when the protein component was higher than 410g kg^{-1} and fat lower than 230g kg^{-1} (Table 2). Fish fed diet A showed the lowest food intake and a significant lower feed efficiency when compared with diets D–G.

Groups A and B showed hepatosomatic index (HSI) values significantly higher than groups E–G. The HSI decreased progressively in the other groups, in inverse relation to the protein/lipid ratio. No differences were observed for the mesenteric fat index between groups.

The specific growth rate (SGR) of group A was the lowest with respect to other groups, showing the group F the highest SGR (Table 2). We also found a positive but not significant correlation between dietary protein content and SGR and an inverse relationship with dietary fat content (Fig. 1a,b).

Regarding muscle composition, no significant differences were found in white muscle protein and water between groups. Group A showed significantly lower glycogen levels than groups D–G. Group A also showed a non-significant increase in the concentration of fat in muscle (Fig. 2).

Muscle texture analysis

Maximal strength, elasticity and total work were analysed in white muscle pieces of fish from the different experimental groups (Table 3). No significant differences were found in any of the parameters analysed in fish fed with different diets, despite elasticity showed a trend to decrease in fish fed diets with higher protein/lipid ratio.

Histological fibre analysis

The histological parameters of white muscle fibres (area, density and immunohistochemistry analysis of MyoD) are shown in Table 3.

Animals from group A presented significantly lower fibre area values than all the other groups. Group G showed the highest mean fibres ($P < 0.05$ compared with B, C and E and $P < 0.01$ compared to A), and the other groups appeared with values between these two extremes (Table 3).

Similar results were found for fibre density analysis, and group A showed significantly the highest fibre density, with minimal density in group G (Table 3).

Myogenic differentiation factor-positive cells in white muscle showed an increase in cells per mm^2 with protein

Table 2 Body parameters and index in gilthead sea bream fed experimental diets (A–G). Animals were fasted overnight before sampling

Diets (g kg^{-1}protein/ g kg^{-1}lipid)	A (380/290)	B (410/260)	C (440/250)	D (470/230)	E (500/220)	F (540/210)	G (570/200)
Final weight (g)	170.3 ± 4.5^{a}	204.1 ± 6.3^{bc}	196.1 ± 4.9^{b}	201.1 ± 0.8^{b}	210.1 ± 1.0^{bc}	220.6 ± 4.1^{c}	208.9 ± 3.1^{bc}
Length (cm)	21.6 ± 0.2^{a}	22.2 ± 0.2^{ab}	22.4 ± 0.2^{bc}	22.5 ± 0.1^{bcd}	23.0 ± 0.0^{cd}	23.2 ± 0.2^{d}	23.0 ± 0.1^{bcd}
Liver weight (g)	2.9 ± 0.2	3.4 ± 0.1	3.0 ± 0.1	3.0 ± 0.03	2.8 ± 0.0	3.1 ± 0.1	2.8 ± 0.1
Mesenteric fat (g)	2.0 ± 0.2	2.3 ± 0.3	2.5 ± 0.3	2.8 ± 0.1	2.5 ± 0.0	2.9 ± 0.3	2.0 ± 0.2
Total food intake (g of DM)	4792 ± 63^{a}	5604 ± 68^{bc}	5452 ± 82^{b}	5849 ± 53^{cd}	5743 ± 48^{bcd}	6071 ± 96^{d}	5975 ± 110^{d}
HSI (%)[1]	1.7 ± 0.1^{a}	1.7 ± 0.1^{a}	1.5 ± 0.1^{ab}	1.5 ± 0.0^{ab}	1.3 ± 0.0^{b}	1.4 ± 0.1^{b}	1.3 ± 0.1^{b}
MFI (%)[2]	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.4 ± 0.0	1.2 ± 0.0	1.3 ± 0.1	0.9 ± 0.1
CF (%)[3]	1.7 ± 0.03^{a}	1.9 ± 0.06^{b}	1.7 ± 0.02^{ab}	1.8 ± 0.01^{ab}	1.7 ± 0.01^{a}	1.8 ± 0.02^{ab}	1.7 ± 0.02^{a}
SGR (%)[4]	0.9 ± 0.03^{a}	1.2 ± 0.03^{bc}	1.1 ± 0.03^{b}	1.2 ± 0.01^{bc}	1.2 ± 0.01^{bc}	1.3 ± 0.02^{c}	1.2 ± 0.02^{bc}
FE[5]	0.72 ± 0.00^{a}	0.80 ± 0.01^{abc}	0.79 ± 0.01^{ab}	0.86 ± 0.00^{bc}	0.86 ± 0.01^{bc}	0.87 ± 0.01^{bc}	0.89 ± 0.00^{c}

DM, dry matter.

Values are expressed as Mean ± SEM ($n = 24$; $n = 3$ for food intake and FE). Letters indicate significant differences ($P < 0.05$).

[1] Hepatosomatic Index (HSI) = (liver weight/animal weight) × 100.

[2] Mesenteric Fat Index (MFI) = (fat weight/animal weight) × 100.

[3] Condition Factor (CF) = (weight/$size^3$) × 100.

[4] Specific Growth Rate (SGR) = (ln initial weight−ln final weight)*$days^{-1}$*100.

[5] Feed efficiency (FE) = Weight gain/Feed intake as dry matter.

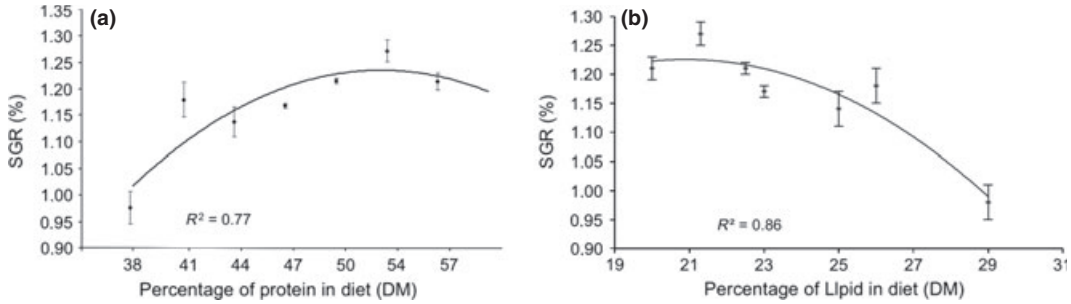

Figure 1 (a) Specific growth rate (SGR) and dietary protein percentage correlation. (b) SGR and dietary lipid percentage correlation. Pearson correlations of a binomial trend line are shown as square *r*-value.

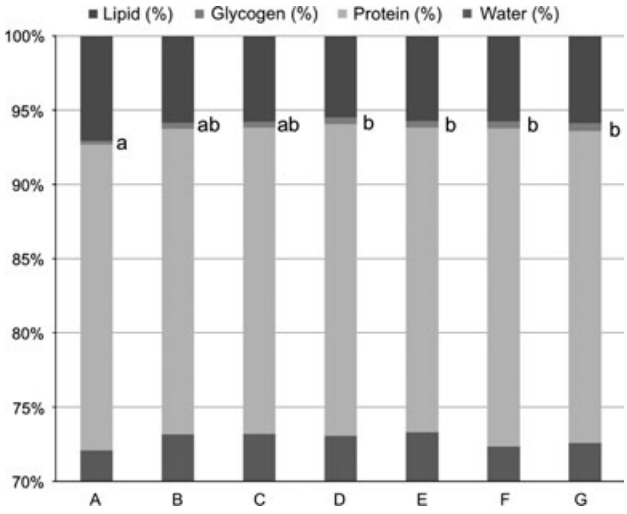

Figure 2 White muscle composition in sea bream fed experimental diets (A–G). Values are expressed as percentage on the column ($n = 9$). All animals were fasted for 24 h before collection of the samples for analysis. Letters indicate significant differences between groups ($P < 0.05$).

inclusion in the diet. Animals from group A showed significant lower values than those from E–G groups. The number of cells expressing MyoD increased across the experimental diets, with maximal values in E–G ($P < 0.05$ compared with group A) (Table 3). A positive correlation between MyoD-positive cells and body weight was found ($r = 0.35$; $P = 0.05$, Fig. 4c).

Analysing fibre distribution (Fig. 3), we observed that animals from group A appeared to have a high percentage (35%) of small fibres (<2000 μm^2) in comparison with groups with maximal protein levels, in diets F and G (23% and 22%, respectively; $P < 0.01$). Considering the 2000–4000 μm^2 fibre range, group D showed significantly lower values (19%) than group E (26%) ($P < 0.05$). When the fibre range increased to 6000–8000 μm^2, group G presented a higher percentage (14.5%) than A (9.5%). Again, in the fibre range between 14 000 and 16 000 μm^2, group G showed significantly higher values than A–C groups ($P < 0.05$). A similar result was found in the 16 000–18 000 μm^2 ranges, with highest values in group G (1.7%) compared to A (0.24%) ($P < 0.05$) (Fig. 3).

A positive correlation was found between fibre area and animal weight ($r = 0.60$; $P = 0.01$, Fig. 4a). Furthermore, a direct correlation between fibre density and elasticity was also found ($r = 0.61$; $P < 0.01$, Fig. 4b).

Colour analysis

Skin *L**, *a**, *b**, Chroma and Hue angle values did not show any significant differences between the animals fed with the different diets (data not shown).

Muscle *L** and *a** values were similar between groups. Animals from group A showed the highest *b** values, while statistically minimal values appeared in groups E and G when compared with A ($P < 0.05$) (Table 4). The Chroma parameter was also affected in group A, with the lowest values compared to groups E and G ($P < 0.05$).

Akt and mTOR phosphorylation in different groups

Figure 5 shows phosphorylation of Akt and mTOR in animals 8 and 24 h postfeeding.

At 8 h postfeeding, levels of phosphorylated Akt (Akt-p) in animals fed diet C presented the highest values and were significantly different from those of group F ($P < 0.05$). At 24 h postfeeding, Akt-p showed significantly higher values in groups A, D, E and G than in group F. The phosphorylation of Akt varied considerably between groups and did not seem to be related to dietary composition.

Table 3 Texture analysis and fibre structure in gilthead sea bream fed experimental diets

Diets (g kg^{-1}protein/ g kg^{-1} lipid)	A (380/290)	B (410/260)	C (440/250)	D (470/230)	E (500/220)	F (540/210)	G (570/200)
Muscle texture							
Maximal strength (N cm^{-1})	77.4 ± 3.7	70.4 ± 2.4	72.0 ± 2.5	68.18 ± 0.3	71.75 ± 1.4	68.1 ± 0.9	71.6 ± 2.8
Elasticity (N cm s^{-1})	14.20 ± 1.0	12.1 ± 0.5	11.8 ± 0.5	11.6 ± 0.1	12.99 ± 0.2	11.02 ± 0.6	10.9 ± 0.4
Total work (N)	529.5 ± 29.9	505.3 ± 20.8	521.2 ± 15.7	491.24 ± 3.0	492.32 ± 11.9	630.9 ± 52.6	541.6 ± 20.8
Fibre structure							
Area (μm^2)	4280 ± 63.2^a	4788 ± 73.3^b	4821 ± 64.8^b	5115 ± 73.6^{bc}	4612 ± 87.9^b	5162 ± 92.4^{bc}	5827 ± 124^c
Density (fibres cm^{-2})	176.3 ± 3.5^a	147.6 ± 1.9^b	143.0 ± 4.6^{bc}	138.4 ± 0.4^{bc}	153.8 ± 2.9^c	145.8 ± 5.2^b	126.6 ± 1.7^c
MyoD$^+$ cells mm^{-2}	42.2 ± 1.6^a	47.0 ± 1.6^{ab}	49.1 ± 1.6^{ab}	46.0 ± 2.2^{ab}	51.3 ± 2.2^b	50.9 ± 2.1^b	50.8 ± 2.2^b

MyoD, myogenic differentiation factor.
Values are expressed as a Mean ± SEM expressed in Newtons. Letters indicate significant differences between groups ($P < 0.05$) ($n = 9$).

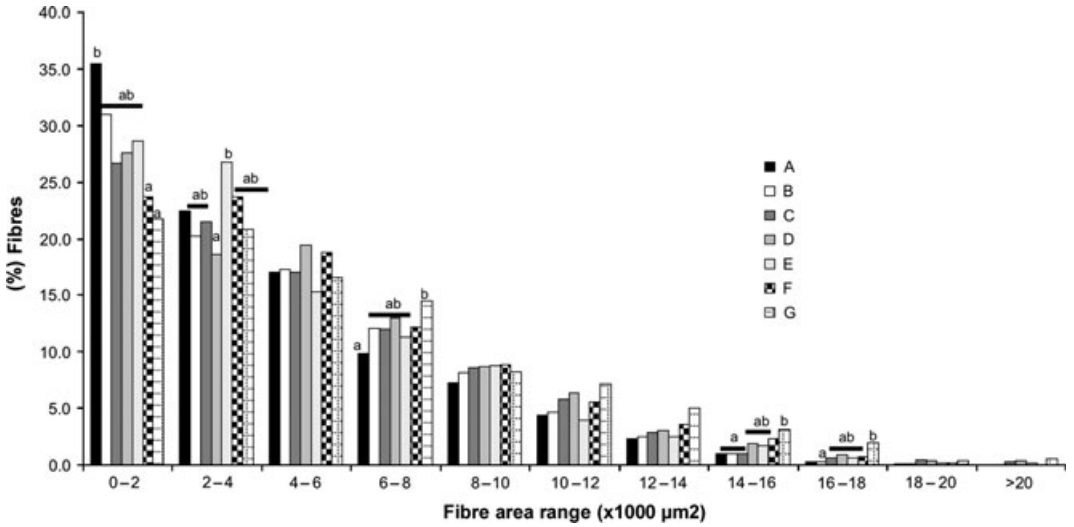

Figure 3 White muscle fibre area distribution in sea bream fed experimental diets (A–G). Fibres were analysed in five different regions of white muscle section from five animals per condition. Fibres are distributed in 2000 μm^2 intervals to reduce complexity. Values are expressed as percentage for each range of fibre sizes. Letters indicate significant differences between groups ($P < 0.05$).

At both 8 and 24 h postfeeding, the animals from experimental group A showed the lowest values of mTOR activation. Levels of phosphorylated mTOR (mTOR-p) increased across the other groups with maximal values in groups E–G.

PCNA and c-met protein expression

The indicators of proliferation, PCNA and c-met showed very similar profiles of expression in fish fed with the different diets (Fig. 6). The lowest values of PCNA expression were found in group A and increased across the other experimental diets, reaching maximal expression in fish fed the D diet ($P < 0.01$ compared to A).

C-met also showed the lowest values of expression in animals from group A, and this difference was significant when compared with groups C and D.

Discussion

The addition of non-protein components to fish diets determines that fish use fewer amino acids as an energy source, thus saving them for growth (Kaushik & Médale 1994). The addition of these non-protein elements covers the energy demands of the fish and reduces protein requirement in the diets (Kaushik & Médale 1994; Company *et al*. 1999; Lee *et al*. 2002). Lipids are normally used as an energy-dense nutrient in fish diets to obtain a protein-sparing effect (Li

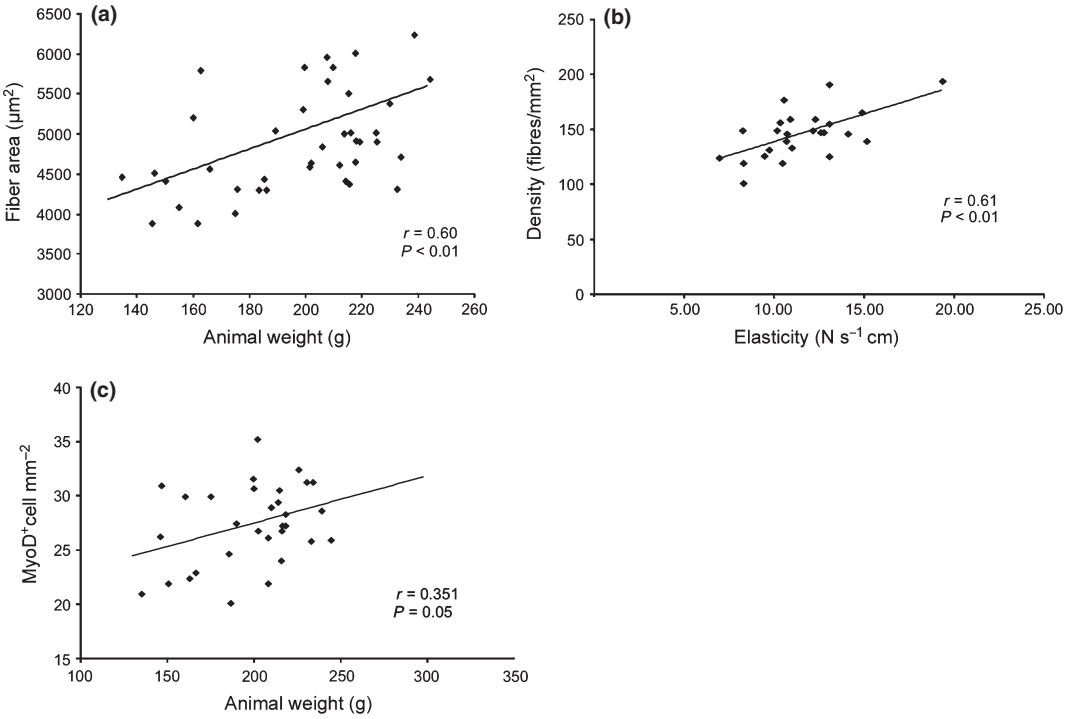

Figure 4 Correlations between: (a) fibre area and animal weight and (b) fibre density and fillet elasticity and (c) $MyoD^+$ per mm^2 and animal weight. The linear correlation was calculated after demonstrating the normality of all the values used. The Pearson correlation is shown as the *r*-value. The *P*-value represents the unilateral significance.

Table 4 White muscle colour analysis in sea bream fed experimental diets

Diets (g kg^{-1}protein/g kg^{-1} lipid)	A (380/290)	B (410/260)	C (440/250)	D (470/230)	E (500/220)	F (540/210)	G (570/200)
Muscle							
*L**	52.3 ± 0.9	54.2 ± 1.3	52.2 ± 0.9	51.6 ± 0.7	50.7 ± 0.8	49.3 ± 2.3	51.7 ± 0.8
*a**	−1.4 ± 0.1	−1.6 ± 0.0	−1.5 ± 0.1	−1.7 ± 0.0	−1.7 ± 0.0	−1.6 ± 0.0	−1.7 ± 0.1
*b**	−0.2 ± 0.3	−0.6 ± 0.0	−0.7 ± 0.1	−0.9 ± 0.2	−1.5 ± 0.2	−0.6 ± 0.2	−1.3 ± 0.2
Chroma[1]	1.7 ± 0.1[a]	1.9 ± 0.1[ab]	1.9 ± 0.3[ab]	2.5 ± 0.3[ab]	3.0 ± 0.3[b]	2.6 ± 0.1[ab]	3.2 ± 0.4[b]
Hue[2]	−3.7 ± 2.1	0.8 ± 0.8	0.8 ± 0.5	1.3 ± 0.9	0.3 ± 0.5	1.7 ± 0.5	0.8 ± 0.7

Values are expressed as Mean ± SEM. Letters indicate significant differences between groups ($P < 0.05$) ($n = 9$).
[1] Chroma = $\sqrt{(a^*2+b^*2)}$.
[2] Hue = $(\tan^{-1}(b^*/a^*))$.

et al. 2011) and have been used in sparids and sea bass (*Dicentrarchus labrax*) with positive results (Company *et al*. 1999). Lipids are preferred to carbohydrates because the protein-sparing action of the latter is less marked than that of dietary lipids among certain species (Adron *et al*. 1976). Traditionally, fish were considered to have a limited ability to use carbohydrates for energy (Takeuchi *et al*. 1978). Literature on carbohydrate utilization by fish is contradictory, and the inclusion of carbohydrates in fish diets has been questioned (reviewed in Lupatsch *et al*. 2001). Therefore, in this study, we used lipids as an energy source to study their effect on fish quality and muscle growth.

The digestibility of the diets was not measured because of the limited quantity of faeces collected. However, feed efficiency suggests that diets B–G had similar digestibility. Previous studies have demonstrated that a high inclusion of wheat in fish diets, like in this study, does not have a negative effect on digestibility and fish growth (Venou *et al*. 2003).

The lower weight and length values of the animals from group A are remarkable. It is well known that diets with low protein levels tend to provoke a decrease on somatic parameters (Sabaut & Luquet 1973; Santinha *et al*. 1996). These results are in agreement with the low food intake, SGR and feed efficiency of diet A in comparison with diets

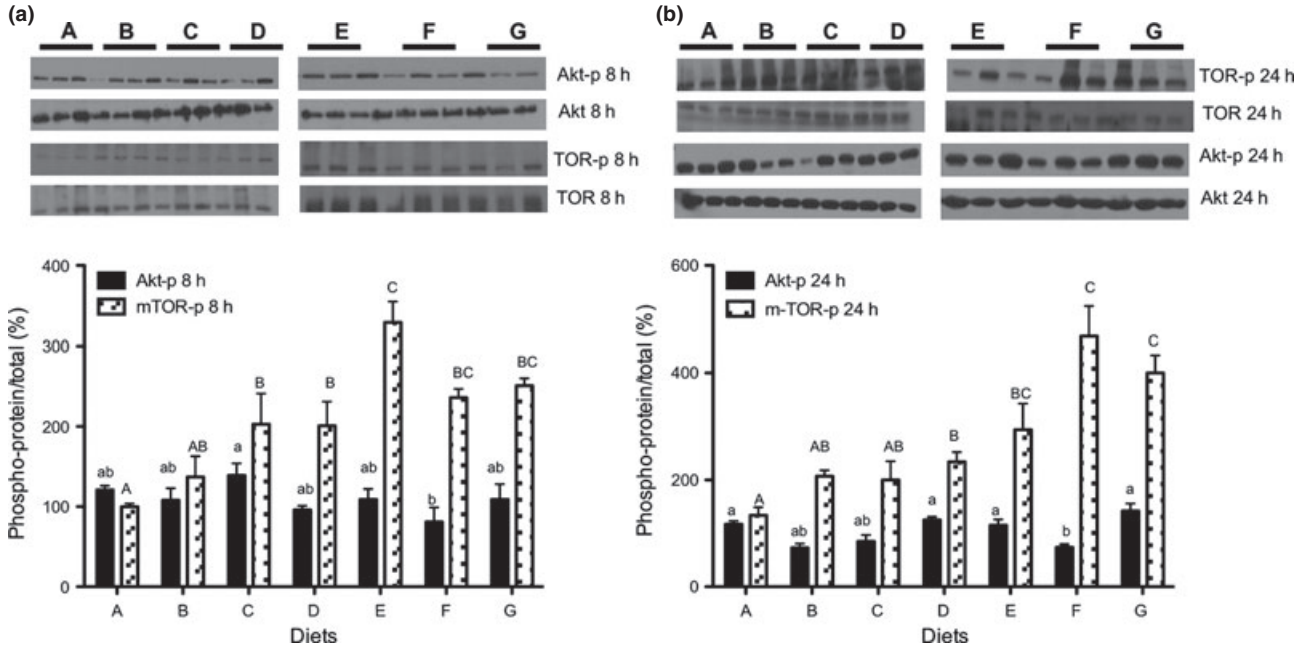

Figure 5 Western blot results from sea bream experimental groups (A–G) fasted for 8 and 24 h. Graph (a): Akt-p and mTOR-p 8 h post-feeding. Capital letters mean mTOR-p significant differences and small letters are significant differences in Akt-p. Graph (b): Akt-p and mTOR-p 24 h hours postfeeding. Capital letters mean mTOR-p significant differences and small letters are significant differences in Akt-p. Values were obtained with Akt-p and mTOR-p densitometries normalized with total Akt and mTOR, respectively, and expressed as percentage relative to diet A (set to 100%).

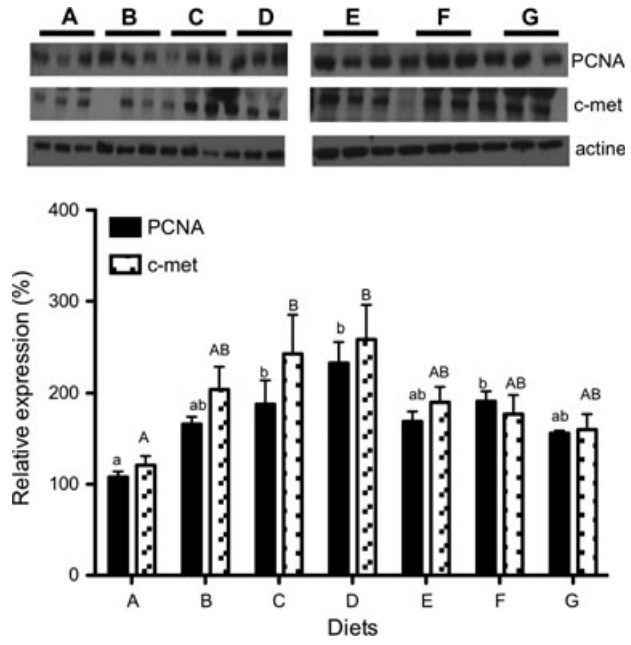

Figure 6 Western blot results from sea bream fed experimental diets (A–G) for c-met and proliferating cell nuclear antigen (PCNA). Capital letters mean c-met significant differences and small letters are significant differences in PCNA. Values were obtained with c-met and PCNA normalized with β-actin and expressed as percentage relative to diet A (set to 100%).

D–G. The negative effect of high lipid diets on growth has been reported previously in gilthead sea bream (Company *et al*. 1999) and other species like dentex (Espinós *et al*. 2003), sea bass (Péres & Oliva-Teles 1999), cobia (*Rachycentron canadum*) (Chou *et al*. 2001) and meagre (*Argyrosomus regius*) (Chatzifotis *et al*. 2010). Some studies suggested that the reduction in fish growth when fed with high lipid diets is because of the excessive energy that reduces food consumption (Kaushik & Médale 1994; Moon 2001). As diets used in this study were designed to be isoenergetic, the excess of energy does not seem to be the cause of the reduction in food intake observed in group A. Another possibility is the negative effect of the reduction in essential amino acids on fish growth in feeding group A, but further studies in digestibility are necessary to establish the actual origin of growth, food intake and feed efficiency reductions.

The experimental groups with lower ratio in P/L content in the diet (A and B) showed a tendency to increase HSI. In this study, liver composition was not analysed and the increase in the HSI because of the fast deposition is not discernable. Previous studies have suggested that amino acid composition of the diet modulates fat deposition in liver. Espe *et al*. (2010) have demonstrated that diets with suboptimal levels of essential amino acids, such as methionine, stimulate

triacylglycerides deposition in the Atlantic salmon (*S. salar*) liver. Table 1 shows that essential amino acids decreased with the replacement of protein by lipid. The lowest values in diets A and B, where HSI levels were higher, suggest that fat deposition can be originated by this deficiency.

Looking at quality parameters such as flesh texture, we found that the different diets in our study had little effect. However, we did observe a clear correlation between muscle structure and elasticity, indicating an influence of the muscle fibre distribution on final texture. A similar relationship has been found in studies with other species such as sea bass and salmon (Johnston *et al.* 2000; Johnston *et al.* 2004; Park *et al.* 2005).

Previous studies have reported a tendency for sea bream muscle to whiten as the fat component increases (Grigorakis *et al.* 2003). The changes in *b** values in animals fed diet A suggested that sea bream muscle tends to be whiter in this group, which could be attributed to the increased muscle fat content in these animals.

There is a relation between the molecules that control satellite cell activity and muscle development with fish weight. It is well known that MyoD is an important transcription factor that controls activation of the myoblast and it is essential for myogenesis (reviewed in Johnston 2006). The MyoD-positive cell reduction found in animals from group A may be related to the lower final weight observed. C-met and PCNA also showed a decrease in expression in animals fed with diet A, which supports the lowest MyoD-positive cells. Both molecules, c-met and PCNA are involved in proliferation (Brodeur *et al.* 2002; Barani *et al.* 2003; García de la Serrana *et al.* 2007) and their decrease is very well correlated with growth delay in group A.

These findings, which suggest a reduction in muscle growth in diet A group, are also supported by the mTOR activation pattern. mTOR is related to growth-promoting protein synthesis and hypertrophic growth in muscle (Ohanna *et al.* 2005; Periago *et al.* 2005; Drummond *et al.* 2008). In fish, it has been demonstrated that mTOR is sensitive to food intake and amino acids present in the diet (Seiliez *et al.* 2008).

The present data show that animals fed with diet A also had lower mTOR activation. The fact that phosphorylation of Akt was very variable would suggest that in this case, the activation of mTOR is more influenced by amino acids availability. So, the lower mTOR activation in group A could be related to the relatively low level of proteins in diet A joined to the lower feed intake. This suggests a lower protein synthesis rate in this group that could be the origin of the lower final weight and the relatively smaller muscle fibres because the extent of protein synthesis seems to be closely related to the size of the meal (Houlihan *et al.* 1995). Although more studies at different sampling times and correlation with insulin and IGF-I circulating levels are necessary, the mTOR pathway seems to be a good marker of the dietary effects on muscle growth. Correlation between mTOR phosphorylation and protein synthesis was tested by Western blot detection of S6K1 and EBP1, two molecules downstream mTOR and more related with protein synthesis (Drummond *et al.* 2008). Antibodies previously used by Seiliez *et al.* (2008) were used in gilthead sea bream with no band detection (data not shown). Although mTOR phosphorylation and mTOR activity can be slightly different we believe that at this time, the assumption of mTOR phosphorylation as an estimation of mTOR activity provides valuable information about protein synthesis.

Our results suggest a model summarized in Fig. 7. We hypothesize that when the dietary lipid fraction increases, juvenile sea bream start to increase fat deposition in the liver, increasing HSI, but not in other body compartments. When fish diet has over a 250g kg^{-1} of lipid content (diet A), animals store fat in muscle in exchange of glycogen. This increase in fat probably is the origin of changes in muscle colour, but do not have significant effects on texture.

Diet A composition and consumption is the probable origin of a protein synthesis reduction indicated by lower mTOR activation. This lower protein synthesis together with a reduction in myoblast activity (MyoD-positive cells and PCNA reduction) would explain the lower final weight of this group.

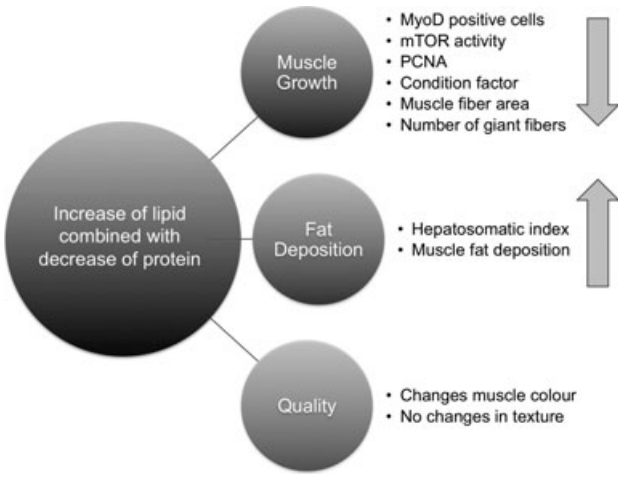

Figure 7 Summary of changes observed in sea bream juveniles when lipid proportion on the diets is increased.

Conclusions

In conclusion, this study demonstrates that it is possible to reduce protein content in diets with lipids addition, without seriously affecting fillet composition, texture or colour. We have also demonstrated a direct correlation between muscle fibre and elasticity. Furthermore, a positive correlation between final weight and expression of satellite cell markers such as MyoD, PCNA and c-met was found. Finally, mTOR phosphorylation was affected by diet composition, probably by protein fraction.

The results of this study indicate that parameters such as MyoD, PCNA, c-met and mTOR activation could be used to measure muscle status with a view of determining growth potential and fillet quality.

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Author contributions

Daniel Garcia de la serrana participated in all the samplings, performed all the protocols reported in the manuscript, prepared the data and performed the statistical analysis. Ramón Fontanillas and Wolfgang Koppe designed the experimental diets and controlled the animal food intake during the experimental trial. Dr Jaume Fernàndez, Dr Josefina Blasco and Miguel Martín were involved in all the sampling procedures. Dr Isabel Navarro provided technical support for the experimental analysis. Dr Joaquim Gutiérrez was the coordinator of the research.

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