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ORIGINAL PAPER

# Dietary protein content influences both growth and size distribution of anterior and posterior muscle fibres in juveniles of *Pagellus bogaraveo* (Brunnich)

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**Abstract** Muscle cellularity was studied in *Pagellus bogaraveo* juveniles fed on diets with different protein contents. Measured in transversal body sections, at both post-opercular and post-anal locations, the morphometric variables estimated were: total muscle area ($A$), total number of fibres ($N$), number of fibres per unit area of muscle ($N_A$) and cross-sectional fibre area ($\bar{a}$), of the two main muscle fibre types. At the end of the experiment fish fed on diets having more than 40% of protein displayed significantly higher body weight. Fish fed on protein-rich diets exhibited greater $\bar{a}$ and $N$. For fish fed on 30 and 50% protein diets the morphometric parameters measured grew linearly with the fish weight. High-protein diets favoured muscle hyperplasia. When comparing rostral and caudal locations, a greater $N$ and a smaller $\bar{a}$ of posterior red fibres were the consistent differences found—a fact, to our knowledge, so far unreported for fish.

**Keywords** Fish muscle · Growth · Histology · Hyperplasia · Hypertrophy · Morphometry · Protein

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## Introduction

The myotomal muscle of teleosts contains two main fibre types, grouped in two layers: (1) subdermal red muscle, with oxidative metabolism and slow contraction, and (2) deep white muscle, with glycolytic metabolism and fast contraction (Johnston et al. 1975, 1977; Bone 1978). Between those layers, an intermediate zone is found, with fibres characterised by being essentially fast contracting, with both intermediate resistances to fatigue and shortening speed (Johnston et al. 1977).

Post-larval muscle growth in fishes involves both hyperplasia and hypertrophy mechanisms, thus differing from mammals where muscle hyperplasia is largely restricted to the pre- and perinatal periods (Goldspink 1972; Stickland 1981). In many fishes, a long-term hyperplastic growth is the likely cause of the so-called "mosaic appearance" of the white muscle, which can be either restricted to particular seasonal periods or permanent, depending on the species. Indeed, the smallest fibres are thought to be the newly generated ones (Carpenè and Veggetti 1981; Rowlerson et al. 1985). The rates of muscle fibre hypertrophy and hyperplasia for reaching a given girth vary among species and different strains of a species (Weatherley et al. 1979) and can be influenced by controlled rearing conditions, such as diet (Kiessling et al. 1991; Fauconneau et al. 1997; Johnston et al. 2002), exercise training (Johnston and Moon 1980) and temperature (Ayala et al. 2000, 2001; López-Albors et al. 2003).

Protein, the most expensive component in fish diets, plays an important role in the growth of fish. The use of diet protein is related to both protein level and availability of non-protein energy sources. A number of studies have been conducted to determine the optimal diet protein level for some sparids fishes (Santinha et al. 1996; Vergara et al.

1996; Tibaldi et al. 1996; Sá et al. 2006) and have estimated 40–50% as the optimal diet protein level in terms of growth performance. Being the muscle the final product in fish farming it is surprising that there is very little work specifically linking diet protein level to muscle growth dynamics in juveniles. Johnston et al. (2002) investigated the effect of two diet protein levels (high and low) on muscle cellularity in two strains of Atlantic salmon (*Salmo salar*, Linnaeus); in one strain they found no differences either in fibre size distribution or in fibre number, whereas in the other strain the high-protein diet favoured growth by fibre recruitment.

We are evaluating the aquaculture potential of the blackspot seabream, *Pagellus bogaraveo* (Brunnich), and therefore the knowledge of its muscle growth dynamics is rather important. A first nutritional assay with *P. bogaraveo* juveniles revealed, as found in other sparids, a high-protein dependence (>40%) in order to achieve maximal growth (Silva et al. 2006). A parallel morphometric study was simultaneously conducted in that assay in order to evaluate the effect of diet protein level (D20–D60) on the muscle growth after 12 weeks. Moreover, intermediate samplings (4 and 8 weeks) were also conducted for two of the diets (D30 and D50) to follow the muscle growth kinetics. Another question that we wanted to address concerned the hypothesis that, all the way along a "rostro-caudal" gradient, diverse diet protein levels may influence the muscle growth in a different way. This is particularly relevant as a fundamental question, because most studies on fish muscle growth dynamics were made in samples taken from the caudal zones, the cellularity of which may notably differ from that found in the cranial ones.

## Materials and methods

### Fish and treatments

Five isolipidic (crude fat: 12% dry matter, DM) diets were formulated to contain graded protein levels (20–60%; Table 1). All ingredients were finely ground, mixed and dry pelleted through a 2.4 mm die at 50°C (CPM model 3000, San Francisco, CA, USA). Diets were subsequently stored at room temperature and light-protected during use.

The trial was conducted on wild-caught *P. bogaraveo* at the Mariculture Center of Calheta, in Madeira Island, Portugal. After acclimatisation, homogeneous groups of 120 juveniles (initial body weight, IBW = 23 g) were randomly distributed among 10 PVC tanks (500 l) in a flow-through seawater system (salinity: 38 g $l^{-1}$), at a temperature of 21–24°C (Min.–Max.). Fish were exposed to a 12-hour light/12-hour dark photoperiod (dawn at 0700 and dusk at 1900 hours). The five diets were randomly allotted to the duplicates tanks, and fish were hand fed to apparent satiety, twice a day, for over 12 weeks.

**Table 1** Formulation and proximate composition of the experimental diets

| | Diet treatments | | | | |
|---|---|---|---|---|---|
| | D20 | D30 | D40 | D50 | D60 |
| *Ingredients* (%) | | | | | |
| Extruded peas meal[a] | 10.0 | 10.0 | 10.0 | 5.0 | 9.0 |
| Wheat meal | 55.7 | 37.5 | 22.4 | 11.2 | 5.0 |
| LT fishmeal[b] | 5.0 | 17.7 | 33.2 | 50.7 | 50.0 |
| Wheat gluten | 2.0 | 2.0 | 2.0 | 2.0 | 16.3 |
| Defatted soybean meal (48% crude protein) | 13.7 | 20.0 | 20.0 | 20.0 | 0.0 |
| Fish protein concentrate[c] | 1.0 | 1.0 | 2.0 | 2.0 | 13.2 |
| Mineral mix[d] | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Vitamin mix[e] | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Binder | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| Fish oil | 9.9 | 9.1 | 7.7 | 6.4 | 3.9 |
| *Proximate composition* | | | | | |
| Dry matter (DM, %) | 91.0 | 91.3 | 92.1 | 92.5 | 93.5 |
| Crude protein (% DM) | 24.1 | 35.7 | 45.3 | 54.4 | 63.7 |
| Crude fat (% DM) | 12.7 | 12.7 | 12.4 | 12.4 | 11.6 |
| Ash (% DM) | 5.7 | 7.9 | 10.1 | 12.2 | 11.8 |
| NFE (% DM)[f] | 48.6 | 34.9 | 24.3 | 13.4 | 6.4 |
| Gross energy (kJ $g^{-1}$ DM) | 18.8 | 19.3 | 19.5 | 19.7 | 20.4 |
| Digestible protein (DP, % DM) | 17.4 | 29.7 | 39.4 | 48.8 | 58.3 |
| Digestible energy (DE) (kJ $g^{-1}$ DM) | 9.2 | 11.7 | 13.8 | 15.0 | 17.3 |
| DP/DE ratio (mg $kJ^{-1}$) | 18.8 | 25.5 | 28.5 | 32.6 | 33.7 |

[a] Aquatex (20.5 Crude protein (C.P.); Sotexpro, France)

[b] Norse LTay

[c] Soluble fish protein hydrolizate (75.26 crude protein; Sopropêche, France)

[d] Mineral mixture (g or mg $kg^{-1}$ diet): calcium carbonate (40% Ca), 2.15 g; magnesium oxide (60% Mg), 1.24 g; ferric citrate, 0.2 g; potassium iodide (75% I), 0.4 mg; zinc sulphate (36% Zn), 0.4 g; copper sulphate (25% Cu), 0.3 g; manganese sulphate (33% Mn), 0.3 g; dibasic calcium phosphate (20% Ca, 18% P), 5 g; cobalt sulphate, 2 mg; sodium selenite (30% Se), 3 mg; potassium chloride, 0.9 g; sodium chloride, 0.4 g

[e] Vitamin mixture (IU or mg $kg^{-1}$ diet): DL-alpha tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15,000 IU; DL-cholecalciferol, 3,000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; cyanocobalamin, 0.05 mg; nicotinic acid, 175 mg; folic acid, 5 mg; ascorbic acid, 500 mg; inositol, 1,000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2,000 mg

[f] Nitrogen-free extract—estimated by difference

Diets were analysed for dry matter, ash by combustion in a muffle furnace (550°C for 12 h); crude protein (Micro-Kjeldahl; $N \times 6.25$) after acid digestion, lipids by petroleum ether extraction (at Soxhlet 40–60°C), and gross energy, by direct combustion in an adiabatic bomb calorimeter (model PARR 1261, Parr, Molin, IL, USA).

## Fish sampling

Taking into account the wet weight gain data from the nutritional assay (Silva et al. 2006), the morphometrical analysis performed for this study was designed using six specimens per each diet from the initial and final sampling and using the same number of fish per diets containing 30 and 50% protein from intermediate samplings (4 and 8 weeks).

At the beginning of the growth trial, and every 4 weeks thereafter, randomly net sampled fish were killed through deep anaesthesia by immersion in a bath of isoeugenol (2-methoxy-4-propenylphenol; 5–10 ml 1,000 $l^{-1}$), immediately dried up with filter paper, individually weighed to the nearest ± 0.1 g and finally measured (fork length) to the nearest ± 0.1 cm. Then, the fins were cut, the scales gently removed, and two cross-sectional body slabs (about 3 mm thick) removed; one from the region immediately before the first dorsal fin and the other from the region immediately following the anal opening. The samples ($n = 6$/experimental group) were fixed in Bouin's (Panreac, Barcelona, Spain) fluid for 72 h, with sporadic shaking. After fixation, and under a stereomicroscope (Leica Zoom 2000, Germany), each slab was longitudinally cut in two (right and left) equal-sized (mirror) halves. The pieces were then routinely dehydrated in a graded ethanol series, cleared in xylol, and finally embedded in paraffin. One lottery chosen block (right or left half of the fish) was used per fish and per sampling level (i.e. cranial and caudal). A perfect 10 μm-thick section was cut per block, and then stained with haematoxylin-eosin before being coversliped for morphometric analysis.

## Morphometrical analysis

The study was made using an interactive image analysis system (CAST-Grid, Olympus Danmark A/S, Version 1.6), working with a live-image captured by a CCD-video camera (Sony, Japan). The light microscope (BX50, Olympus, Japan) used was equipped with a fully motorized stage (Prior Scientific, USA), thus allowing meander sampling with an (X-Y axis) accuracy of 1 μm. For practical purposes, the muscle tissue was operationally divided in two morphologically well-differentiated zones: a main (and innermost) white fibres area and a thinner (and outermost) red fibre rich area. Just for comparative purposes, the subcutaneous adipose tissue was also studied. Relative and absolute morphometric parameters of the fish body and components of interest were estimated as follows.

### *Fish cross-sectional area*

The cross-sectional (half) body area ($A$), in $mm^2$, was precisely computed by the software after the operator (P. Silva) finished circumscribing the physical limits of interest in the section. Such delimitation was made using the mouse pointer over a 160× magnified image seen in the monitor, captured under a 4× objective lens. An unbiased estimate of the total area [$A$ (*body*)] was made by doubling the computed value. Here and below, the procedures were alike among studied fish groups.

### *Relative area of body components*

\The relative area occupied by a particular tissue component [$A_A$ (*tissue*, *body*)], either muscle (white and red) or adipose, was estimated by manual point-counting, using a 1:9 two lattice point-system grid (with 16 repeating units) overlaid on the live video-image displayed on a 17′ monitor (Fig. 1). For both adipose tissue and white muscle, the 1:1 point ratio was used. The tighter 1:9 point ratio was used for the red muscle. The final estimates are unbiased by the technical nature of the procedure and were computed as follows (Howard and Reed 1998):

$$A_A(\text{tissue}, \text{body}) = \sum P(\text{tissue}) \div \left[R \cdot \sum P(\text{body})\right]$$

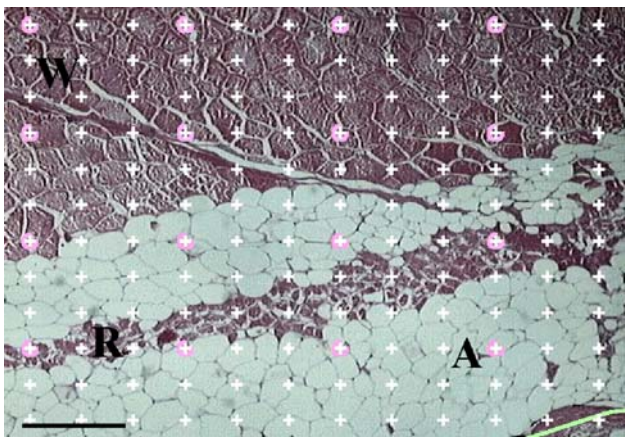


**Fig. 1** Test grid used for light microscopical analysis containing 16 point-counting units, each consisting of a point double lattice, with either 1 or 9 points, each defined by one *cross*. All *crosses* are used to estimate the area of red muscle whereas the *encircled crosses* are only used to estimate the reference area, the white muscle and the adipose tissue. In this example, 11 points hit red muscle, 9 (*encircled*) hit the white muscle, and 5 (*encircled*) hit the adipose tissue, respectively. In addition, 16 (*encircled*) points hit the reference space. So, in this field of view, the area fraction of red muscle per total area can be estimated as $13/(9 \times 16) = 0.09$, because 9 times as many *crosses* are used to estimate red muscle area in relation to the total area. In addition, the white muscle fractional area can be estimated as $9/16 = 0.56$, as the lattice grid used (1:1 point ratio) is the same for both compartments. Symbols: white muscle (*W*); red muscle (*R*) and subcutaneous adipose tissue (*A*) (stained with haematoxylin-eosin). *Scale bar* 250 μm

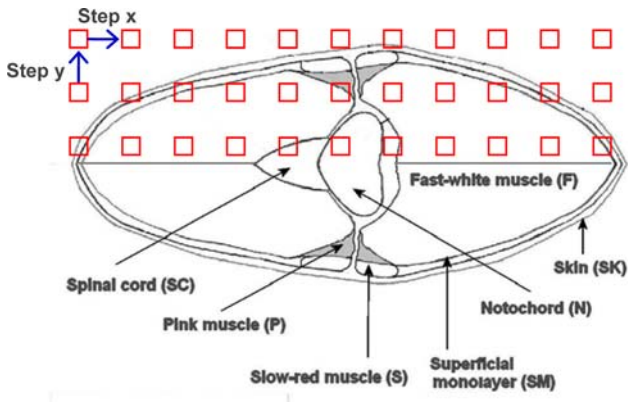


**Fig. 2** Diagram illustrating the sampling track taken over a single section, at the fish caudal portion. Point-counting was made in systematically sampled fields (with the first field being randomly positioned with the software) and working with the ×4 objective lens and a final screen magnification of ×160. The systematic sampling track of fields was carried out by software-controlled stepwise movements of the precise motorized stage, in the *x*- and *y*-directions ($x = y = 1{,}250$ μm)

where $\sum P$(tissue) and $\sum P$(body) correspond to the total number of points in a section hitting the tissue of interest or the fish body (reference space), respectively, and $R$ is the inverse of the point ratio used for each particular $A_A$ estimation (i.e. 1 or 9).

Point-counting was made in systematically sampled fields and working with the 4× objective lens and a final screen magnification of 160×. The sampling was assured by the software-controlled motorized stage, being defined a stepwise *x*–*y* movement of 1,250 μm as distance between fields (Fig. 2).

Each $A_A$ (*tissue*, *body*) was estimated to be used solely as an intermediate value in further computations of other morphometric parameters, as detailed below.

*Total area of body components*

The total area of each body component [$A$ (*tissue*)], namely white muscle, red muscle and adipose tissue, expressed in $mm^2$, and was unbiasedly estimated as follows:

$$A(\text{tissue}) = A_A(\text{tissue}, \text{body}) \cdot A(\text{body})$$

*Relative number (density) of muscle fibres*

The numbers of white and of red muscle fibres per unit area ($mm^2$), within the respective muscle zones [$N_A$ (*fibres*, *muscle*)], were unbiasedly estimated as follows (Howard and Reed 1998):

$$N_A(\text{fibres}, \text{muscle}) = \sum N(\text{fibres}) \div \left[a \cdot \sum P(\text{muscle})\right]$$

where $\sum N$(fibres) is the total number of (white or red) fibres counted over all sampled fields in a section, $a$ is the area of the counting frame used (8,141 $\mu m^2$) when counting white and red fibres, and $\sum P$(muscle) is the sum of the (unique) frame associated point hitting the reference space (i.e. white muscle or red muscle) across sampled fields. For avoiding bias due to edge effects, an unbiased counting frame bearing forbidden lines was used (Gundersen 1977); in accordance, fibres were counted only when they were within the counting frame or touching the inclusion lines but did not touch the forbidden lines or their extensions (Fig. 3).

Cell counting was made in systematically sampled fields and working with the 20× objective lens and a final magnification of 799×. After pilot trials, the stepwise stage *x*–*y* movement was defined according to the fish size so to provide a minimum of 100 sampled fields per section. For the heavier fishes (providing bigger section sizes), a less tight sampling scheme was used. A smaller *x*–*y* step was defined for sampling red fibres. The adopted steps varied

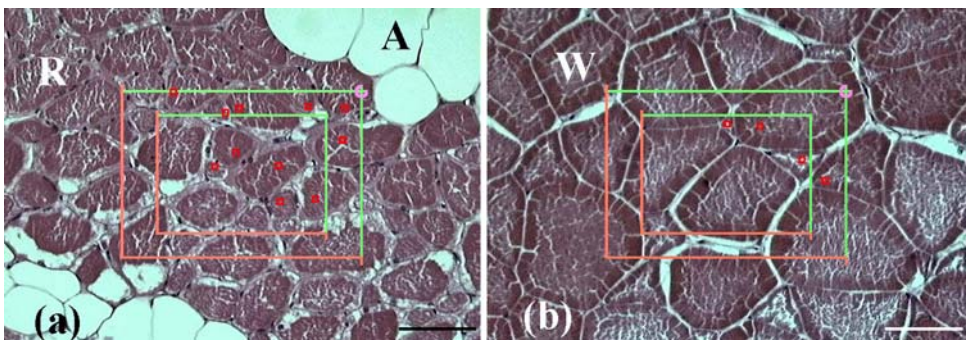


**Fig. 3** Light micrograph of a 10-μm-thick section. **a** Red muscle fibres (*R*); **b** white muscle fibres (*W*) (stained with haematoxylin-eosin). Two sampling/counting, with the exclusion (*red*) and inclusion lines (*green*) are represented. The larger grid was used for sampling cells for applying the 2D-nucleator technique and the smaller one was used for cell counting. In the pictures it is given an example of number counting, per 8,141 $\mu m^2$ (smaller grid area): **a** 11 red muscle fibres counted; **b** 4 white muscle fibres counted. Fibres are unbiasedly counted if they touch the inclusion line or if they are partially our totally inside the grid area, as far as they do not touch the forbidden lines. *A*, adipose tissue. *Scale bar* 50 μm

from 750 to 1,250 μm, for sampling white fibres, and from 175 to 400 μm, for red fibres.

*Total number of muscle fibres*

The total number of (red or white) muscle fibres per fish cross section ($N$) was unbiasedly estimated after general principles for handling ratios and absolute values (Howard and Reed 1998), namely by combining three previously estimated parameters, as follows:

$$N(\text{fibres}) = N_{\text{A}}(\text{fibres}, \text{muscle}) \cdot A_{\text{A}}(\text{muscle}, \text{body}) \cdot A(\text{body})$$

*Mean cross-sectional fibre area*

The mean individual muscle fibre area [$\bar{a}$ (*muscle*, *fibre*)] was estimated by the 2D-nucleator technique. The latter unbiasedly estimates the area of any object profile irrespective of its size, shape, and orientation by measuring the distance between a "central point" within the object and the intersections between the object boundary and radiating test lines (four in our case) (Larsen et al. 1998; Savnik et al. 2002). The formula reads as follows:

$$\bar{a}(\text{muscle fibre}) = \pi \times \bar{I}^2$$

where $I$ is the distance from the "central point" to the object boundary measured in a pre-determined number of random directions. For our case, the "central point" was chosen by the operator (P. Silva) as the virtual most approximate centre of a cross-sectioned fibre. All the procedure and measurement were assisted by the Grid software. Muscle fibres were sampled by using the same rules used for the cell counting (see details above), using an unbiased sampling frame with 16,281 $\mu m^2$, but the fibres were measured only when simultaneously captured by the sampling frame and the observer judging them as being cross-sectioned. All measurements were made when working with the 20× objective lens and with a final screen image magnification of 799×.

Statistical analysis

Data were analysed using the software Statistica (version 6, StatSoft). For all statistical tests, the significance level was set at $\alpha = 0.05$. For each group of animals data are presented as means, followed by the respective coefficients of variation (CV = standard deviation ÷ mean), thus providing instant information on interindividual variability. All variables were checked for normality and homogeneity of variance, by using the Kolmogorov–Smirnoff and the Levene tests, respectively.

The significance of the difference when comparing a particular experimental group to the fish at the start of the assay (time zero) was evaluated by the Student $t$-test for independent samples. The effects of diet on body weight and body length were tested using the one-way analysis of variance (ANOVA). For each morphometric parameter, analysed at a particular sampling time, data were submitted to an analysis of covariance (ANCOVA), in which diet protein content was set as the independent variable and body weight as a covariate. After a significant ANOVA or ANCOVA, pairs of means were compared by the Duncan's multiple range test.

For a particular animal group, and for each parameter, the significance of the differences between rostral and caudal values was compared by the Student $t$-test for dependent samples, after checking the test assumptions as above. Pairwise $t$-tests were also used to compare slopes and elevations of regression lines (Zar 1996), as obtained by regression analysis between fish weight and morphometric data from muscle.

A first analysis of the results revealed that the average individual cross-sectional white fibre area was superior to 2,000 $\mu m^2$ in most trial groups. It was decided to use, therefore, the percentage of fibres with an area inferior to that value as an indication of the contribution of hyperplasia, since the increase in the number of smaller fibres unquestionable denotes hyperplastic growth, i.e. recruitment of new fibres. The significance of the differences between the percentage of smaller fibres (area $< 2{,}000$ $\mu m^2$) seen in the trial groups was tested calculating the $z$-ratio and associated two-tail probabilities for the difference between two independent proportions, as applied in VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html).

## Results

Biometric parameters

The mean body weight and length increase of the fish are presented in Table 2. After 12 weeks all groups increased their body weight significantly. Moreover, the body weight was higher in fish fed on diets with a protein content greater than 40%. The fork length also increased with diet protein levels up to 40%, with no further improvements being observed with higher protein levels (Table 2).

Adipose tissue

The total cross-sectional area occupied by the subcutaneous adipose tissue was greater (expect for one case) at the end of the trial (Table 2). The values tended to increase with body weight, but, considering weight as covariate, no

**Table 2** Body weight (BW), fork length (FL) and subcutaneous adipose tissue total area [*A* (*adipose tissue*)] in anterior and posterior parts of *Pagellus bogaraveo* fed the different diet treatments (protein varying from 20 to 60%) for over 12 weeks

| | Initial | Diets | | | | |
|---|---|---|---|---|---|---|
| | | D20 | D30 | D40 | D50 | D60 |
| BW (g) | 24.32(0.13)* | 35.97(0.19)$^{**,a}$ | 48.75(0.18)$^{**,b}$ | 62.72(0.19)$^{**,c}$ | 66.38(0.19)$^{**,c}$ | 68.9(0.13)$^{**,c}$ |
| FL (cm) | 10.47(0.04)* | 11.90(0.05)$^{**,a}$ | 13.20(0.04)$^{**,b}$ | 13.90(0.05)$^{**,b,c}$ | 14.20(0.05)$^{**,c}$ | 14.50(0.03)$^{**,c}$ |
| *A* (*adipose tissue*) (mm$^2$) | | | | | | |
| Anterior | 32.45(0.03)$^{*,\#}$ | 54.51(0.13)$^{**,\#}$ | 81.28(0.23)$^{**,\#}$ | 82.62(0.21)$^{**,\#}$ | 99.58(0.33)$^{**,\#}$ | 91.62(0.20)$^{**,\#}$ |
| Posterior | 21.49(0.17)* | 27.84(0.24)* | 39.39(0.27)** | 56.12(0.21)** | 49.30(0.26)** | 50.46(0.17)** |

Values are presented as means (CV), $n = 6$/group. Within a row, means with the superscript symbol (**) differ significantly from the initial mean and means without a common superscript letter differ significantly ($P < 0.05$). Within a column, anterior mean with the superscript symbol ($^{\#}$) differ significantly ($P < 0.05$) from the posterior mean. Absence of superscript indicates no significant difference between treatments

significant gains were observed among protein levels. Marked differences existed between the anterior and posterior parts of the fish, with the former consistently presenting greater amounts of fat (typically, twice more).

## Muscle growth

The distinction between white and red muscle was largely evident in all fish (Fig. 1), due to the distinct positioning and also separating myosepta. Overall, growth from 0 to 12 weeks mainly resulted from an increase in fibres size ($\bar{a}$), which induced a larger *A* (*body*); an increase in fibre number was, however, only observed in the posterior part of the fish fed on diets with more than 50% protein. All the results related with size and numbers are summarized in Tables 3 and 4.

## White muscle

At 12 weeks, and although no significant protein effect was found among treatments, the white *A* (*white muscle*) showed increasing trends with increasing body weight. The enlarged body size during the experiment was explained by an increase of the white *A* (*white muscle*), both at cranial and caudal locations (Table 3). There was a positive regression ($P < 0.05$) between the white *A* (*white muscle*) and the body weight (Table 5), but no major differences between the slopes of the regression lines of the two diet groups at the post-opercular level of the fish were found; although at the post-anal level the slope of the D30 group line was higher (Table 5). At the beginning of the assay, the *A* (*white muscle*) at the cranial part was larger than at the caudal level (Table 3), and although the mean values

**Table 3** Total muscle area [*A* (*white muscle*)], total number of fibres [*N* (*white fibres*)], number of fibres per unit area [$N_A$ (*white fibres*, *muscle*)] and cross-sectional fibre area [$\bar{a}$ (*white fibres*)] measured in white muscle, in anterior and posterior part of *Pagellus bogaraveo* fed the different diet treatments (protein varying from 20 to 60%) for over 12 weeks

| | Initial | Diets | | | | |
|---|---|---|---|---|---|---|
| | | D20 | D30 | D40 | D50 | D60 |
| *A* (*white muscle*) (mm$^2$) | | | | | | |
| Anterior | 1.3E+02(0.10)$^{*,\#}$ | 1.4E+02(0.19)* | 1.9E+02(0.17)** | 2.2E+02(0.11)** | 2.5E+02(0.21)$^{**,\#}$ | 2.5E+02(0.21)** |
| Posterior | 1.1E+02(0.09)* | 1.1E+02(0.22)* | 1.8E+02(0.15)** | 2.1E+02 (0.17)** | 2.1E+02(0.14)** | 2.3E+02(0.16)** |
| $N_A$ (*white fibres*, *muscle*) (N/mm$^2$) | | | | | | |
| Anterior | 3.0E+02(0.10)* | 2.3E+02(0.12)** | 2.0E+02(0.26)** | 1.8E+02(0.11)** | 1.7E+02(0.13)$^{**,\#}$ | 1.9E+02(0.06)** |
| Posterior | 3.1E+02(0.05)* | 2.3E+02 (0.16)** | 2.3E+02(0.20)** | 1.9E+02(0.14)** | 2.1E+02(0.04)** | 2.2E+02(0.20)** |
| *N* (*white fibres*) | | | | | | |
| Anterior | 3.9E+04(0.09) | 3.1+04(0.24) | 3.7E+04(0.11) | 4.0E+04(0.10) | 4,3E+04(0.14) | 4.6 E + 04(0.23) |
| Posterior | 3.5E+04(0.09)* | 2.5E+04(0.26)$^{**,a}$ | 4.0E+04(0.13)$^{*,b}$ | 3.9E+04(0.17)$^{*,b}$ | 4.4E+04(0.11)$^{**,b,c}$ | 4.9E+04(0.18)$^{**,c}$ |
| $\bar{a}$ (*white fibres*) (μm$^2$) | | | | | | |
| Anterior | 1.9E+03(0.15)* | 2.1E+03(0.16)* | 2.4E+03(0.14)** | 2.8E+03(0.14)** | 2.9E+03(0.09)** | 2.7E+03(0.10)** |
| Posterior | 2.0E+03(0.11)* | 2.3E+03(0.21)* | 2.4E+03(0.17)* | 2.6E+03(0.19)** | 2.8E+03(0.09)** | 2.6E+03(0.12)** |

Values are presented as means (CV), $n = 6$/group. Within a row, means with the superscript symbol (**) differ significantly from the initial mean and means without a common superscript letter differ significantly ($P < 0.05$). Within a column, anterior mean with the superscript symbol ($^{\#}$) differ significantly ($P < 0.05$) from the posterior mean. Absence of superscript indicates no significant difference between treatments

**Table 4** Total muscle area [*A* (*red muscle*)], total number of muscle fibres [*N* (*red fibres*)], number of muscle fibres per unit area [$N_A$ (*red fibres*, *muscle*)] and cross-sectional fibre area [$\bar{a}$ (*red fibres*)] measured in red muscle, in anterior and posterior part of *Pagellus bogaraveo* fed the different diet treatments (protein varying from 20 to 60%) for over 12 weeks

| Red muscle | | | | | | |
|---|---|---|---|---|---|---|
| | Initial | Diets | | | | |
| | | D20 | D30 | D40 | D50 | D60 |
| *A* (*red muscle*) ($mm^2$) | | | | | | |
| Anterior | 4.8E+00(0.40)* | 6.4E+00(0.27)* | 9.0E+00(0.34)$^{**,\#}$ | 1.1E+01(0.46)** | 9.9E+00(0.34)** | 9.4E+00(0.16)$^{**,\#}$ |
| Posterior | 5.8E+00(0.38)* | 7.9E+00(0.35)* | 1.2E+01(0.24)** | 1.3E+01(0.31)** | 1.3E+01(0.26)** | 1.5E+01(0.34)** |
| $N_A$ (*red fibres*, *muscle*) ($N/mm^2$) | | | | | | |
| Anterior | 5.2E+02(0.14)* | 5.7E+02(0.28)* | 3.5E+02(0.35)**$^{,\#}$ | 4.0E+02(0.25)** | 3.8E+02(0.13)$^{**,\#}$ | 3.7E+02(0.18)$^{**,\#}$ |
| Posterior | 7.0E+02(0.21)* | 7.1E+02(0.18)* | 6.9E+02(0.20)* | 4.6E+02(0.29)** | 5.7E+02(0.19)* | 6.0E+02(0.19)* |
| *N* (*red fibres*) | | | | | | |
| Anterior | 2.6E+03(0.48) | 3.5E+03(0.25)$^{\#}$ | 3.2E+03(0.62)$^{\#}$ | 4.1E+03(0.35) | 3.7E+03(0.39)$^{\#}$ | 3.5E+03(0.27)$^{\#}$ |
| Posterior | 4.1E+03(0.41)* | 5.6E+03(0.37)* | 7.8E+03(0.27)** | 5.9E+03(0.32)* | 7.3E+03(0.23)** | 8.6E+03(0.19)** |
| $\bar{a}$ (*red fibres*) ($\mu m^2$) | | | | | | |
| Anterior | 7.7E+02(0.14)$^{*,\#}$ | 6.4E+02(0.19)* | 9.6E+02(0.29)$^{*,\#}$ | 1.2E+03(0.31)$^{**,\#}$ | 1.1E+03(0.12)$^{**,\#}$ | 9.4E+02(0.16)** |
| Posterior | 6.5E+02(0.21)* | 6.5E+02(0.16)* | 7.2E+02(0.17)* | 9.3E+02(0.35)* | 8.5E+02(0.14)** | 8.5E+02(0.23)* |

Values are presented as means (CV), $n = 6$/group. Within a row, means with the superscript symbol (**) differ significantly from the initial mean and means without a common superscript letter differ significantly ($P < 0.05$). Within a column, anterior mean with the superscript symbol ($^{\#}$) differ significantly ($P < 0.05$) from the posterior mean. Absence of superscript indicates no significant difference between treatments

**Table 5** Relationships between body weight (BW) and total area [*A* (*tissue*)] ($mm^2$), number of fibres per unit area [$N_A$ (*fibres*, *muscle*)] ($N/mm^2$), total number of fibres [*N* (*white fibres*)] and cross-sectional fibre area [$\bar{a}$ (*fibres*)] ($\mu m^2$) in white muscle in anterior and posterior part of *Pagellus bogaraveo* fed the different diet treatments (D30 and D50) for over 12 weeks. $N = 24$

| White muscle | | | | |
|---|---|---|---|---|
| | Parameter | Diet | Line | $R^2$ |
| Anterior | *A* (*white muscle*) | D30 | *A* (*white muscle*) = 6.78 + 4.05 · BW | 0.97 |
| | | D50 | | |
| Posterior | *A* (*white muscle*) | D30 | *A* (*white muscle*) = 50.19 + 2.37 · BW | 0.68 |
| | | D50 | *A* (*white muscle*) = 55.98 + 2.14 · BW | 0.75 |
| Anterior | $N_A$ (*white fibres*, *muscle*) | D30 | $N_A$ (*white fibres, muscle*) = 364.25 − 3.52 · BW | 0.57 |
| | | D50 | $N_A$ (*white fibres, muscle*) = 327.50 − 2.48 · BW | 0.58 |
| Posterior | $N_A$ (*white fibres*, *muscle*) | D30 | $N_A$ (*white fibres, muscle*) = 383.43 − 2.96 · BW | 0.47 |
| | | D50 | $N_A$ (*white fibres, muscle*) = 339.69 − 2.08 · BW | 0.68 |
| Anterior | *N* (*white fibres*) | D30 | No linear correlation | 0.25 |
| | | D50 | *N* (*white fibres*) = 32,304 + 131.58 · BW | 0.17 |
| Posterior | *N* (*white fibres*) | D30 | No linear correlation | |
| | | D50 | *N* (*white fibres*) = 30,449 + 141.52 · BW | 0.19 |
| Anterior | $\bar{a}$ (*white fibres*) | D30 | $\bar{a}$ (*white fibres*) = 191.57 + 53.85 · BW | 0.93 |
| | | D50 | | |
| Posterior | $\bar{a}$ (*white fibres*) | D30 | $\bar{a}$ (*white fibres*) = 1,483.70 + 19.92 · BW | 0.44 |
| | | D50 | $\bar{a}$ (*white fibres*) = 1,681.20 + 16.63 · BW | 0.56 |

For each parameter, when the slopes and elevations did not differ from each other ($P > 0.05$) the common equation regression was computed. In all other cases, slopes differed ($P < 0.05$)

were always higher anteriorly, with time the difference turned out to be less important facing the greater interindividual variability.

As to the relative number of fibres, at 12 weeks no differences among treatments were found in the white muscle $N_A$ (*white fibres*, *muscle*). Moreover, the white

fibres $N_A$ (*white fibres*, *muscle*) declined as the body size increased, as shown in both D30 and D50 groups (Table 5). The slopes of such decline were different, being higher for the D50 group (Table 5).

Comparison of the *N* (*white fibres*) between dietary groups at the end of the experiment showed that greater numbers of fibres were present in the D50 and D60 groups at the post-anal level (Table 3). During the trial, the white muscle *N* (*white fibres*) increased at the post-anal level in fish fed on diets with more than 50% of protein, despite the fact that at the post-opercular level no statistically differences were detected among treatments (Table 3). There was also a linear and positive regression (weakly positive, but statistically significant) between the *N* (*white fibres*) and the body weight of fish fed on the D50 diet (Table 5). The size distribution fibre analysis within animals at the end of the trial revealed that the percentage of smaller-area fibres (<2,000 $\mu m^2$)—an indication of the contribution of hyperplasia—was significantly and negatively correlated with body weight in white muscle, decreasing with increasing levels of dietary protein (Fig. 4). At the end of the trial, however, still more than 1/3 of the posterior white muscle fibres in fish fed on D40, D50 and D60 diets were on this smaller sized fibres class (37, 34 and 40%, respectively).

Regarding muscle hypertrophy, as evaluated by the mean cross-sectional area ($\bar{a}$), at 12 weeks no differences among treatments were found in $\bar{a}$ (*white fibres*) (Table 3). During the trial, we observed an increase in the $\bar{a}$ (*white fibres*) of fish fed on diets with over 30% protein, at the post-opercular level, and with over 40% protein, at the post-anal level. Concerning the $\bar{a}$ (*white fibres*), it was linearly and positively correlated with the body weight at the two corporal locations (Table 5).

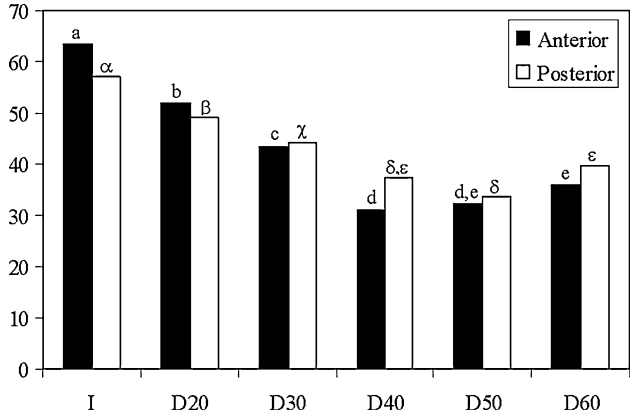


**Fig. 4** Proportion of smaller fibres (<2,000 $\mu m^2$) in white muscle in anterior and posterior part of *Pagellus bogaraveo* fed the different diet treatments (protein varying from 20 to 60%) for over 12 weeks. Within each column, means without a *common letter* differ significantly ($P < 0.05$) (*normal* and *Greek alphabet* for anterior and posterior locations, respectively). The 95% confidence intervals were between ± 0.02 or 0.03% in relation to the proportion value obtained for each diet

### Red muscle

As in the white muscle, diet treatments did not have a noticeable differential effect on the *A* (*red muscle*). After 12 weeks, the *A* (*red muscle*) was higher than at the beginning (Table 4) in fish fed on diets with a protein content superior to 30%. In short, during the trial the *A* (*red muscle*) increased by approximately 2-fold at both post-opercular and post-anal levels. As shown in Table 6, there was a strong positive linear regression between red *A* (*red muscle*) and body weight of fish fed on D30 and D50 diets, with no differences in the slopes.

As to the $N_A$ (*red fibres*, *muscle*), after 12 weeks, no differences were found among groups (Table 4). However, a few changes occurred in the $N_A$ (*red fibres*, *muscle*) during the assay, namely at the post-opercular level, where that parameter decreased in magnitude for most fish treated with diets containing over 30% of protein. For the fish fed on diet D30, no linear regressions were found between red muscle $N_A$ (*red fibres*, *muscle*) and body weight (Table 6). In most groups the red fibre density was higher at the post-anal level (Table 4).

Concerning the absolute number, at 12 weeks no differences among groups were observed. The pattern of differences in the *N* (*red fibres*) between the beginning and the end of the trial, for each treatment, was similar to those observed in the white muscle. For fish fed on D30 and D50 diets, the correlation between *N* (*red fibres*) and body weight was linear at the post-anal level (Table 6), being the D30 line slope higher. As seen for the $N_A$ (*red fibres*, *muscle*), the *N* (*red fibres*) was greater in the posterior part of the fish.

Concerning red fibre size, at 12 weeks no diet effects were found in the $\bar{a}$ (*red fibres*) at the post-anal level, and at the post-opercular level only fish fed on diets with more than 40% of protein had a bigger $\bar{a}$ (*red fibres*) when comparing to the initial values (Table 4). For D30 and D50 diet groups, the $\bar{a}$ (*red fibres*) increased linearly and positively with the body weight, both cranially and caudally (Table 6). Finally, regarding anterior vs. posterior comparisons, only in the D20 group the mean value for $\bar{a}$ (*red fibres*) was nearly equal at both locations. In the other groups, smaller fibres were found post-anally (Table 4).

## Discussion

This study was simultaneously conducted with a nutritional assay (Silva et al. 2006), which showed that the growth performance of the fish analysed herein was affected by the

**Table 6** Relationships between body weight (BW) and total area [*A* (*tissue*)] ($mm^2$), number of fibres per unit area [$N_A$ (*fibres*, *muscle*)] ($N/mm^2$), total number of fibres [*N* (*red fibres*)] and cross-sectional fibre area [$\bar{a}$ (*fibres*)] ($\mu m^2$) in red muscle in anterior and posterior part of *Pagellus bogaraveo* fed the different diet treatments (D30 and D50) for over 12 weeks. $N = 24$

| Red muscle | | | | |
|---|---|---|---|---|
| | Parameter | Diet | Line | $R^2$ |
| Anterior | *A* (*red muscle*) | D30 | *A* (*red muscle*) = 0.25 + 0.16 · BW | 0.90 |
| | | D50 | | |
| Posterior | *A* (*red muscle*) | D30 | *A* (*red muscle*) = 0.27 + 0.22 · BW | 0.92 |
| | | D50 | | |
| Anterior | $N_A$ (*red fibres*, *muscle*) | D30 | No linear correlation | |
| | | D50 | $N_A$ (*red muscle*) = 549.65–2.40 · BW | 0.17 |
| Posterior | $N_A$ (*red fibres, muscle*) | D30 | No linear correlation | |
| | | D50 | $N_A$ (*red muscle*) = 801.00–3.69 · BW | 0.22 |
| Anterior | *N* (*red fibres*) | D30 | No linear correlation | |
| | | D50 | No linear correlation | |
| Posterior | *N* (*red fibres*) | D30 | *N* (*red fibres*) = 1,979.10 + 106.89 · BW | 0.32 |
| | | D50 | *N* (*red fibres*) = 3,700.80 + 57.20 · BW | 0.20 |
| Anterior | $\bar{a}$ (*red fibres*) | D30 | $\bar{a}$ (*red fibres*) = 600.65 + 8.93 · BW | 0.18 |
| | | D50 | $\bar{a}$ (*red fibres*) = 722.27 +5.61 · BW | 0.21 |
| Posterior | $\bar{a}$ (*red fibres*) | D30 | $\bar{a}$ (*red fibres*) = 64.67 + 17.47 · BW | 0.92 |
| | | D50 | | |

For each parameter, when the slopes and elevations did not differ from each other ($P > 0.05$) the common equation regression was computed. In all other cases, slopes differed ($P < 0.05$)

dietary protein content. Increasing diet protein contents improved the biometric records, with fish fed on 40, 50 and 60% protein diets having had a similar final (average) body weight. The cited nutritional assay also revealed that the higher protein levels induced a similar daily growth index and that feed conversion ratio gradually improved with the increment of diet protein up to 40%, when growth was maximal. Silva et al. (2006) also observed that diet protein levels below 40% resulted in lower growth as well as lower protein and energy use. In the present study, a strong relationship between body weight and the morphometric variables (total muscle area, number and size of fibres) was found, and this fact underlines the importance of including body weight as a covariate when studying changes in muscle fibres.

Considering that white muscle is the main accumulating tissue in fishes (Houlihan and Laurent 1987), it was somewhat surprising that the effect of the increase of the diet protein content was not more expressively reflected in the inner-out expanding growth of muscle mass. A feasible explanation could be that differences in growth of *P. bogaraveo* induced by the protein feed level affected also lipid deposition rather than protein build up only (Silva et al. 2006). During this study, however, when the body weight was a covariate, no parallelism was seen between the increasing body weight and the mounting of adipose tissue area in body sections, which supports the conclusion that the excess of dietary protein was used to build up muscle tissue.

In accordance with the nutritional assay, one of the present findings was that *P. bogaraveo* fed with different protein levels had different growth rates, reflected in dissimilar mean cross-sectional muscle fibre areas. Overall, these differences only became apparent in fish fed on protein-rich diets (>40%). Increases in the cross-sectional area of white muscle fibres during growth were also observed in other fish species (Weatherley et al. 1980; Kiessling et al. 1991; Koumans et al. 1993; Fauconneau et al. 1997; Suresh and Sheehan 1998)—here we further proved that such increases are maximal only above a threshold of diet protein intake.

Comparisons among the different somatic growth rates linked to the diet protein content showed, that the white fibre number was significantly higher in the posterior part of the fish fed on the highest protein diets (D50–D60). This result indicates a higher hyperplastic muscle growth of this fish during the experiment. Moreover, the higher percentage of smaller fibres (<2,000 $\mu m^2$) observed in the groups fed high-protein diets (>40%) suggest a greater potential for hypertrophic growth thereafter. Previous studies reported substantial differences in the relative contribution of both hypertrophy and hyperplasia of muscle fibres between rapid and slow growing strains of the same species (Weatherley et al. 1979; Higgins and Thorpe 1990; Alami-Durante et al.

1997; Valente et al. 1999; Johnston et al. 2000) in such a manner that rapid somatic growth is commonly associated to a higher rate of hyperplasia. These changes in fibre numbers were described in fish exhibiting different body sizes, and so, if size is taken into account these differences could be minimal (Kiessling et al. 1991). However, in the present study, this does not seem to be the case; when the body size (covariate) was taken into account the statistical analysis still supported that hyperplasia was influenced by the dietary protein level.

Our analyses showed that the increase in total posterior white muscle area resulted from a both fibre recruitment and hypertrophy in animals fed on high-protein diets (>40%); nonetheless, such an increase was entirely due to fibre hypertrophy in fish fed on protein-poor diets. This seems a very important result, as the fish somatic growth is mainly due to skeletal muscle development. Silva et al. (2006) proposed diets containing 40% protein as the most favourable for growth and feed conversion ratio in *P. bogaraveo*. The present results, however, showed that despite the absence of body weight differences, a higher total white fibre number was still observed in the posterior part of the fish fed on diets with high-protein content (D50 and D60). This result, coupled with the high % of smaller fibres in such diets, suggests that a protein content slightly higher than 40% may be the most adequate to further induce this species growth. In fact, as the high-protein diets mainly led to hyperplastic muscle growth in juveniles, such specimens with both more total and more numerous smaller fibres hold a greater potential to hypertrophic growth, when compared to fish with larger but fewer fibres, being able to reach the commercial size faster.

From the kinetics of muscle growth in fish fed on D30 and D50 diets, it can be concluded that, for the body size-range studied, the white muscle fibre hypertrophy increased linearly with the animal weight, which is in full agreement with a number of some other studies in fish (Weatherley et al. 1988; Kiessling et al. 1991; Valente et al. 1999). In addition, although the fibre number was only linearly correlated with total body growth for D50 fish diet group, this further stressed the importance of the hyperplasia for growth in fish fed on diets having higher protein contents.

The present work is one of the very few studying muscle cellularity of fish related to body location (caudal and cranial). As in *Dicentrarchus labrax* (Linnaeus) (Abdel et al. 2005), the present results for the average white muscle fibre area, number of fibres and fibre density did not show significant differences between the two sampling levels, despite the existing anterior-posterior difference regarding body shape. But the same constancy is not true for the red muscle, where the number of muscle fibres was higher and the size smaller at the caudal level of the fish, at least in most of all experimental groups. To the best of our knowledge, this is the first time that such scenario is reported in a fish species, despite the study conducted by Zhang et al. (1996), who, upon the examination of the distribution of red, pink and white muscle along the length of scup (*Stenotomus chrysops*, Linnaeus), reported that the total area occupied by the red muscle in body cross sections was larger in the mid and posterior body positions. Also, in a Meyer-Rochow and Ingram (1993) article there was (despite not statistically tested) a right shift in the size-diameter distribution of the aerobic red fibres towards the tail of the Southern smelt (*Retropinna retropinna*, Richardson); only graphically observable in the lacustrine form. This agreed with a reported greater total area of the red muscle, at least up to 70% of the fork length. On the contrary, another work checking for variations in the total red muscle cross-sectional area found it more anteriorly developed in thunniform swimmers (Ellerby et al. 2000). The mechanistic reason why the red muscle of *P. bogaraveo* displayed a different cellularity between the two body locations is not clear at this time, but the data strongly suggest there are different regulatory mechanisms for establishing the number and size of the fish red fibres, according to location.

In conclusion, it was shown that differences in body size between the *P. bogaraveo* fed on diets with different diet protein levels were mainly due to different muscle fibres dynamics. Our data indicated that high-protein ratio diets favoured muscle growth by fibre recruitment. The fish fed on protein-rich diets (namely the D50 and D60) showed evidences of a sustained higher recruitment of fibres endowing the fish with the potential to accomplish further growth by fibre enlargement. Finally, we proved that rostro-caudal differences exist in fibre cellularity, with a consistently higher number of smaller sized red fibres being found at caudal level when compared to the post-opercular ones.

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## References

Abdel I, López-Albors O, Ayala MD et al (2005) Muscle cellularity at cranial and caudal levels of the trunk musculature of commercial size sea bass, *Dicentrarchus labrax* (Linnaeus 1758). Anat Histol Embryol 34(5):280–285. doi:10.1111/j.1439-0264.2005.00613.x

Alami-Durante H, Fauconneau B, Rouel M et al (1997) Growth and multiplication of white skeletal muscle fibres in carp larvae in relation to somatic growth rate. J Fish Biol 50:1285–1302. doi:10.1111/j.1095-8649.1997.tb01653.x

Ayala MD, López-Albors O, Gil F et al (2000) Temperature effect on muscle growth of the axial musculature of the sea bass (*Dicentrarchus labrax* L.). Anat Histol Embryol 29(4):235–241. doi:10.1046/j.1439-0264.2000.00262.x

Ayala MD, López-Albors O, Gil F et al (2001) Temperature effects on muscle growth in two populations (Atlantic and Mediterranean) of sea bass, *Dicentrarchus labrax* L. Aquaculture 202:359–370. doi:10.1016/S0044-8486(01)00785-2

Bone Q (1978) Locomotor muscle. In: Hoar WS, Randall DJ (eds) Fish physiology, vol VII. Academic Press, New York, pp 361–424

Carpenè E, Veggetti A (1981) Increase in muscle fibers in the lateralis muscle (white portion) of Mugilidae (Pisces, Teleostei). Experientia 37:191–193. doi:10.1007/BF01963227

Ellerby DJ, Altringham JD, Williams T et al (2000) Slow muscle function of pacific bonito (*Sarda chiliensis*) during steady swimming. J Exp Biol 203:2001–2013

Fauconneau B, André S, Chmaitilly J et al (1997) Control of skeletal muscle fibers and adipose cells size in the flesh of rainbow trout. J Fish Biol 50:296–314. doi:10.1111/j.1095-8649.1997.tb01360.x

Goldspink G (1972) Postembrionic growth and differentiation of striated muscle. In: Bourne GH (ed) Structure and function of muscle, vol I. Academic Press, New York, pp 179–236

Gundersen HJG (1977) Notes on the estimation of the numerical density of arbitrary profiles: the edge effect. J Microsc 111: 21–23

Higgins PJ, Thorpe JE (1990) Hyperplasia and hypertrophy in the growth of skeletal muscle in juvenile Atlantic salmon, *Salmo salar* L. J Fish Biol 37:505–519

Houlihan DF, Laurent P (1987) Effects of exercise training on the performance, growth and protein turnover of rainbow trout. Can J Fish Aquat Sci 44:1614–1621. doi:10.1139/f87-195

Howard CV, Reed MG (1998) Unbiased stereology three-dimensional measurements in microscopy. Bios Scientific Publishers, Oxford

Johnston IA, Moon TW (1980) Exercise training in skeletal muscles of brook trout (*Salvelinus fontinalis* Mitchill). J Exp Biol 87:177–194

Johnston IA, Ward PS, Goldspink G (1975) Studies on the swimming musculature of the rainbow trout I Fiber types. J Fish Biol 7:451–458. doi:10.1111/j.1095-8649.1975.tb04620.x

Johnston IA, Davison W, Goldspink G (1977) Energy metabolism of carp swimming muscles. J Comp Physiol 114:203–216

Johnston IA, Alderson D, Sandham C et al (2000) Muscle fiber density in relation to the colour and texture of smoked Atlantic salmon (*Salmo salar* L.). Aquaculture 189:335–349. doi:10.1016/S0044-8486(00)00373-2

Johnston IA, Manthri S, Alderson R et al (2002) Effects of dietary protein level on muscle cellularity and flesh quality in Atlantic salmon with particular reference to gaping. Aquaculture 210:259–283. doi:10.1016/S0044-8486(01)00862-6

Kiessling A, Storebakken T, Asgard T et al (1991) Changes in the structure and function of the epaxial muscle of the rainbow trout (*Oncorhynchus mykiss*) in relation to ration and age I-growth dynamics. Aquaculture 93:335–356. doi:10.1016/0044-8486(91)90225-V

Koumans JTM, Akster HA, Booms GHR et al (1993) Growth of carp (*Cyprinus carpio*) white axial muscle; hyperplasia and hypertrophy in relation to the myonucleus/sarcoplasm ratio and the occurrence of different subclasses of myogenic cells. J Fish Biol 43:69–80. doi:10.1111/j.1095-8649.1993.tb00411.x

Larsen JO, Gundersen HJG, Nielsen J (1998) Global spatial sampling with isotropic virtual planes: estimators of length density and total length in thick, arbitrarily orientated sections. J Microsc 191:238–248. doi:10.1046/j.1365-2818.1998.00365.x

López-Albors O, Ayala MD, Gil F et al (2003) Early temperature effects on muscle growth dynamics and histochemical profile of muscle fibers of sea bass *Dicentrachus labrax* L., during larval and juvenile stages. Aquaculture 220:385–406. doi:10.1016/S0044-8486(02)00532-X

Meyer-Rochow VB, Ingram JR (1993) Redwhite muscle distribution and fibre growth dynamics: a comparison between lacustrine and riverine populations of the Southern smelt *Retropinna retropinna* Richardson. P Roy Soc Lond B Bio 25:85–92

Rowlerson A, Scapolo PA, Mascarello F et al (1985) Comparative study of myosin present in lateral muscle of some fish: species variations in myosin isoforms and their distribution in red, pink and white muscle. J Muscle Res Cell Motil 6:601–640. doi:10.1007/BF00711917

Sá R, Pousão-Ferreira P, Oliva-Teles A (2006) Effect of dietary protein and lipid levels on growth and feed utilization of white sea bream (*Diplodus sargus*) juveniles. Aquacult Nutr 12(4):310–321. doi:10.1111/j.1365-2095.2006.00434.x

Santinha PJM, Gomes EFS, Coimbra JO (1996) Effects of protein level of the diet on digestibility and growth of gilthead seabream, *Sparus aurata* L. Aquacult Nutr 2:81–87. doi:10.1111/j.1365-2095.1996.tb00012.x

Savnik A, Bliddal H, Nyengaard JR et al (2002) MRI of the arthritic finger joints: synovial membrane volume determination, a manual vs. a stereologic method. Eur Radiol 12:94–98. doi:10.1007/s003300100986

Silva P, Andrade CAP, Timóteo VMFA et al (2006) Dietary protein, growth, nutrient utilization and body composition of juvenile blackspot seabream, *Pagellus bogaraveo* (Brunnich). Aquacult Res 37:1007–1014. doi:10.1111/j.1365-2109.2006.01520.x

Stickland NC (1981) Muscle development in the human fetus as exemplified by m sartorius: a quantitative study. J Anat 132: 557–579

Suresh AV, Sheehan RJ (1998) Muscle fiber growth dynamics in diploid and triploid rainbow trout. J Fish Biol 52:570–587. doi:10.1111/j.1095-8649.1998.tb02018.x

Tibaldi E, Beraldo P, Volpelli LA et al (1996) Growth response of juvenile dentex (*Dentex dentex* L.) to varying protein level and protein to lipid ratio in practical diets. Aquaculture 139:91–99. doi:10.1016/0044-8486(95)01150-1

Valente LMP, Rocha E, Gomes EFS et al (1999) Growth dynamics of white and red muscle fibers in fast and slow growing strains of rainbow trout. J Fish Biol 55:675–691. doi:10.1111/j.1095-8649.1999.tb00710.x

Vergara JM, Robainà L, Izquierdo M et al (1996) Protein sparing effect of lipids in diets for fingerlings of gilthead seabream. Fish Sci 62:624–628

Weatherley AH, Gill HS, Rogers SC (1979) Growth dynamics of muscle fibers, dry weight, and condition in relation to somatic growth rate in yearling rainbow trout (*Salmo gairdneri*). Can J Zool 57:2385–2392. doi:10.1139/z79-310

Weatherley AH, Gill HS, Rogers SC (1980) The relationship between mosaic muscle fibers and size in rainbow trout (*Salmo gairdneri*). J Fish Biol 17:603–610. doi:10.1111/j.1095-8649.1980.tb02792.x

Weatherley AH, Gill HS, Lobo AF (1988) Recruitment and maximal diameter of axial muscle fibers in teleosts and their relationship to somatic growth and ultimate size. J Fish Biol 33:851–859. doi:10.1111/j.1095-8649.1988.tb05532.x

Zar JH (1996) Biostatistical analysis. Prentice-Hall International, London

Zhang G, Swank DM, Rome LC (1996) Quantitative distribution of muscle fiber types in the scup *Stenotomus chrysops*. J Morphol 229:71–81. doi:10.1002/(SICI)1097-4687(199607)229:1<71::AID-JMOR4>3.0.CO;2-S