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Effect of Sodium Alginate Dietary in Body Parameters and Muscle Growth of Gilthead Sea Bream, *Sparus aurata* L.

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Abstract

Specimens of gilthead sea bream, *Sparus aurata* L., of ≈ 98 g (sampling I) were fed with three types of dietary regime: diet I or standard diet, diet II and diet III, these two latter being supplemented with sodium alginate at 2 and 5%, respectively. Both body and muscle growth were analysed after 2 and 4 months from the beginning of the experiment (samplings II and III, respectively).

Body growth significantly increased throughout the experiment, thus reaching ≈ 200 and ≈ 300 g after 2 and 4 months, respectively. Similarly, muscle parameters (size and number of white fibres) increased throughout the experiment. The supplementation of sodium alginate in the diet showed no significant effects on body growth, such that length and weight were similar among different feeding groups for each sampling points. Muscle parameters were also similar among the experimental groups within each stage, with no significant effects of sodium alginate in dietary regime. Survival was higher in supplemented groups, although it was not significant.

Keywords: Sodium alginate, muscle parameters, body growth.

Introduction

Sparus aurata is a marine teleost intensively farmed and of high commercial value in Mediterranean countries. In most teleost species, the axial musculature is formed by a series of myotomes each one including a superficial layer of red muscle, an intermediate layer of pink fibres and a deep stratum of white muscle. The white muscle comprises up to 80 % of the trunk musculature thickness. White muscle is constituted by fibres with a great variability of sizes as a consequence of a double mechanism of muscle growth: hypertrophy and hyperplasia of white muscle fibres. Hypertrophy is an increase in the size of the muscle fibres, whereas hyperplasia depends on the recruitment of new muscle fibres. Both parameters show considerable plasticity with respect to feeding regime, diet composition, environmental and genetic factors (Johnston and Mclay, 1997; Johnston, 1999). Also, the timing of the histochemical maturity of the myotome can be influenced by these factors (Nathanailides *et al*., 1995; López-Albors *et al*., 2003).

Some studies have shown that the growth of fish is improved by effect of sodium alginate dietary (Nakagawa *et al*., 1997). Similarly, sub-adult specimens of hybrid striped bass, *Morone chrysops x Morone saxatilis* improved the growth when adding hydrolized levadures to the dietary regime (Peng and Gatlin, 2005). However, there are not any studies about the effect of prebiotics on the axial musculature of fish. According to these precedents the present work includes the study of the myotome (muscle parameters and histochemical maturity) by effect of feeding regime when supplementing with sodium alginate. Also, body parameters (weight and length), food conversion rate, standard growth rate and survival have been quantified in this study.

Materials and Methods

Experimental Diets

The present experiment was carried out at the Instituto Español de Oceanografía (Centro Oceanográfico de Murcia, Mazarrón). 413 specimens of gilthead sea bream, *Sparus aurata* L., were maintained under similar environmental conditions until that they reached ≈ 98 g. At this stage (sampling I) the specimens were classified into 3 experimental groups: One control group (C) was fed with standard diet (48% protein, 21% fat, 9.1% ashes and 1.4% cellulose); one second group (2A) was supplemented with sodium alginate at 2% (2 g. of additive for 100

kg of fish) and one third group was supplemented with sodium alginate at 5% (5A)). 2 experimental tanks (1300 litres/tank) were studied per group in each sampling point. Hence, 6 tanks (≈ 69 fish/tank) were used throughout the experiment. The effect of the feeding regime was studied after 2 and 4 months from the beginning of the experiment (samplings II and III, respectively).

The initial temperature was 24.5ºC; the photoperiod was natural and pH was 8.18. The water renewal rate was of 100% of tank volume/hour. Oxygen levels were always close to the saturation level.

Muscle Growth Parameters and Survival

From the sampling I until the sampling II, the feeding regime was carried out according to the recommendations of the manufacturer of the food. Fish were fed 3 times per day. The amount of food was related to temperature of the water and weight of the fish. From the sampling II until the sampling III, the feeding regime was "*ad libitum*", 3 times per day in all the tanks, since an increasing of appetite was observed in the supplemented fish.

In each sampling point, all fish in each tank were anaesthetized with clave oil (Guinama^{R}) (40 ppm). Subsequently they were measured (fork length) and weighed (Table 1) in all the stages (sampling I-III). The specific growth rate (SGR) {(ln final weight - ln initial weight)/time (days)} x 100 as well as the feed conversion rate per cage (FCR) {amount of ingested feed/(final biomass-initial biomass+dead fish biomass)} were calculated in both sampling II and III. Survival rate was obtained at the end of the experiment.

Sampling Processing For Muscle Parameters

At the beginning of the experiment (sampling I), the muscle parameters were measured in 8 specimens per each feeding group (4 fish/tank). In the following samplings points (II and III), 12-14 specimens per group (6-7 fish/tank) were randomly chosen in each stage in order to study the effect of the feeding regime.

In each specimen, a steak of 0.5-0.7 cm thickness was obtained at the level of anal opening. Then, the cross-section of the white muscle was traced onto acetate sheets using a fine permanent pen. The white muscle from the left side of the section was trimmed in 3-4 blocks of ≈ 0.5 cm^2. Muscle blocks were frozen in 2-methyl butane cooled to near its freezing point (-159ºC) in liquid nitrogen. Frozen blocks were stored at -65ºC until sectioning. Subsequently, after temperature equilibration to -20ºC within the cryostat chamber (Leica CM 1850), 8 µm thick sections were obtained from each block and then stained with both Haematoxilin/eoxin and mATPase techniques. The myosin ATPase (mATPase) technique was performed according to Mascarello *et al*. (1986), which is very useful to highlight the histochemical mosaic of the white muscle after acid preincubations (Ramírez-Zarzosa *et al*., 1995, 1998; López-Albors *et al*., 1998). Randomly selected fields of white muscle fibres from all the muscle blocks were measured using an image analysis system (SigmaScan Pro 5.0, SPSS Inc). The area and diameter of the muscle fibres were recorded. The total number of measured fibres per fish was 500. Muscle fibres cross-sectional area values were used to calculate the muscle fibre density (muscle fibre number/mm^2) for each fish. The cross-sectional area of the white muscle (mm^2) was quantified from the acetates by image analysis, and the number of white muscle fibres was estimated from values of muscle fibre density and the cross-section area of the white muscle, according to methodology described by Johnston *et al*. (2000) and López-Albors *et al*. (2008).

Statistical Analysis

Muscle parameters as well as body length and body weight were statistically analysed for each sampling point by Analysis of Variance (Anova, $P<0.05$) and post hoc Tukey test with Statistical Package SPSS 15.0. The mean and standard error from each group of data were calculated. Specific

Table 1. Body length and body weight mean values throughout the experiment.

Sampling point	Tank	Body weight (g)	Body length (cm)
Sampling I	C	99.26±1.62	18.5±01
	2A	95.4±1.5	18±0.1
	5A	98.1±1.5	18.1±0.1
Sampling II	C	186.13±4.5	22.58±0.17
	2A	194.5±4.23	20.3±0.4
	5A	191.8±4.2	22.7±0.2
Sampling III	C	281.35±5.09	25.3±0.16
	2A	280.3±5.3	25.3±0.15
	5A	286.3±6	25.4±0.16

Sampling I: start of the experiment. Sampling II and III: 2 and 3 months after the beginning of the experiment, respectively. C: control group. 2A and 5A: supplemented groups with sodium alginate at 2% and 5% levels, respectively. Mean values±SEM were obtained from all the specimens proceeding of each feeding regime. In each sampling point the body parameters values were similar in all the feeding regimes ($P>0.05$).

Growth Rate (SGR) and Feed Conversion Rate (FCR) were statistically analysed in sampling II and III. Survival was analysed at the end of the experiment.

Non-parametric statistical techniques were used to fit smoothed probability density functions (pdfs) to the measured diameters of white muscle fibres using a kernel approach (Johnston *et al*., 1999). Authors obtained the particular software for this study from I.A. Johnston after request. The programs are written in the PC language R, which is a dialect of Splus. Values for the smoothing parameter *h* were in the range 0.105 and 0.167 with no systematic differences among populations. Bootstrap techniques were used to distinguish underlying structure in the distributions from random variation. The kolmogorov-Smirnov two sample statistical tests were used to test the null hypothesis that the probability density functions of each group were equal over all diameters ($P_{k-s} \geq 0.05$).

Results

Body Growth Parameters and Survival

Table 1 shows the body growth of the specimens throughout the experiment. After 2 months from the beginning of the experiment (sampling 2), both body weight and body length showed a significant increasing ($P<0.001$) in all the tanks. Similarly, after 4 months (sampling 3), the growth increased in all the tanks ($P<0.05$). However, as shown in Table 2, standard growth rate (SGR) was higher at sampling II than at sampling III in all the tanks ($P<0.05$). Similarly, feed conversion rate (FCR) was better at sampling II than at sampling III ($P>0.05$). From sampling I until the sampling II, feeding regime was applied according to the recommendations of the manufacturer of the food (to see material and methods). Later on (between the second and the third sampling points), feeding regime was "*ad libitum*", since the supplemented fish showed a higher appetite. However, as described above, growth rate decreased at the end of the experiment.

When comparing among the different feeding groups within each sampling point, length and weight values were similar among the experimental groups ($P>0.05$) (Table 1), that evidences no effects of alginate dietary on the growth of gilthead sea bream during this experiment. Similarly, SGR and FCR parameters showed no significant differences when comparing the feeding groups in each stage.

Survival was 91.7, 95.8 and 100 % in C, 2A and 5A groups, respectively, thus showing a higher survival in supplemented groups than in control groups, although it was not significant.

Muscle Parameters

As shown in Table 3 it can be observed an increasing of the muscle growth throughout the experiment. Thus, muscle fibres diameter increased in all the groups after 2 and 4 months from the beginning of the experiment (samplings II and III, respectively). The increasing of the number of white fibres was only significant at the end of the experiment ($P<0.05$) (sampling III), parallel to an increasing of the white muscle total area ($P<0.05$) (Table 3).

On the other hand, when comparing the effect of feeding regime among groups in each sampling point, all the muscle parameters (white muscle total area, number and size of the fibres as well as the muscle fibres density) were similar among the experimental groups ($P>0.05$).

Probability density function (Figures 1 and 2) shows the distribution of the size of white muscle fibres throughout the cross-section of the myotome at the end of the experiment. The size of the muscle fibres shows a normal distribution, with a low percentage of both small (<25μm) and big fibres (>125μm). The higher percentage of fibres corresponds with fibres of 25-125 μm. The results showed no significant differences among groups in each sampling point. This result was expected since the muscle parameters were similar among feeding groups, as described above (Table 3).

Histochemical Profile of the White Muscle Fibres

The results of the present study show that the myotomal maturity was gradually reached by all the specimens throughout the experiment, with no differences among groups by effect of the feeding regime.

In the mature histochemical mosaic, the small

Table 2. SGR and FCR mean values in sampling II and III

Sampling point	Tank	SGR	FCR
Sampling II	C	0.98	2.2
	2A	1.11	1.85
	5A	1.17	1.73
Sampling III	C	0.6	2.39
	2A	0.6	2.62
	5A	0.67	2.42

Sampling II and III: 2 and 3 months after the beginning of the experiment, respectively. C: control group. 2A and 5A: supplemented tanks with sodium alginate at 2% and 5% levels, respectively. In each sampling point the SGR and FCR values did not show significant differences among the feeding regimes ($P>0.05$).

Table 3. Muscle parameters mean values (± SEM) throughout the experiment

Sampling point	Tank	White muscle total area (mm^2)	White muscle fibres diameter (µm)	Number of white muscle fibres	White muscle fibre density (number/mm^2)
Sampling I	C	596±18.43	54.83±0.48	207981.5±14083.74	350.25±24.09
	2A	625.5±22.27	64.22±0.54	172370.8±17601.91	273.25±21.56
	5A	566.25±19.65	54.73±0.47	201670.3±17276.38	354.5±25.45
Sampling II	C	761.66±37.66	69.99±0.44	165748.8±11940.18	217±9.85
	2A	771.53±15.05	66.96±0.41	180404.7±4897.14	234.38±6.49
	5A	694.66±29.08	68.14±0.62	156927.2±9012.69	226±9.45
Sampling III	C	1376.2± 38.92	75.61± 0.45	271422.9± 11399.82	196.95± 5.87
	2A	1379.8±53.54	74.65±0.45	264208.7±9512.49	192.35±3.56
	5A	1193.8±63.17	77.28±0.65	219472.3±12224.21	184.5±5.97

Sampling I: start of the experiment. Sampling II and III: 2 and 3 months after the beginning of the experiment, respectively. C: control tanks. 2A and 5A: supplemented tanks with sodium alginate at levels of 2% and 5%, respectively. Mean values±SEM from 12-14 specimens/feeding regime. The muscle parameters values were similar among the different feeding groups within each sampling point (P>0.05).

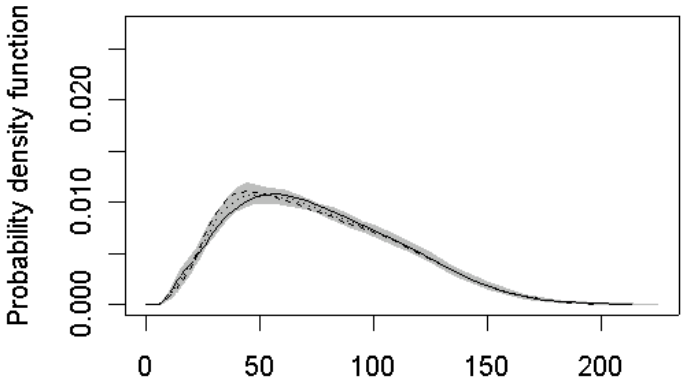

Figure 1. Mean probability density functions (pdf) of white muscle fibre diameter (µm) for experimental groups at sampling III. Solid and discontinuous lines correspond with control and 2A groups, respectively. The shaded polygon represents 100 bootstrap estimates of the combination of pooled groups, and the dotted line its mean probability density function. As observed in the figure, pdfs of both control and 2A groups fall inside shaded polygon, thus evidencing no significant differences between both groups. The kolmogorov-Smirnov statistical test evidences these results, such that P=0.6

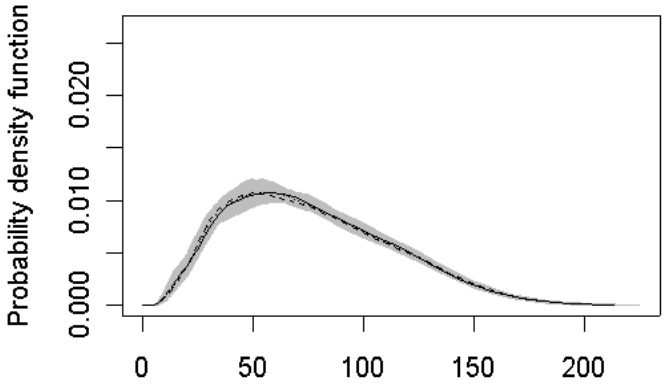

Figure 2. Mean probability density functions (pdf) of white muscle fibre diameter (µm) for experimental groups at sampling III. Solid and discontinuous lines correspond with control and 5A groups, respectively. The shaded polygon represents 100 bootstrap estimates of the combination of pooled groups, and the dotted line its mean probability density function. As observed in the figure, pdfs of both control and 5A groups fall inside shaded polygon, thus evidencing no significant differences between both groups. The kolmogorov-Smirnov statistical test evidences these results, such that P=0.94

white muscle fibres had very high or high mATPase activity after acid preincubation, whereas larger fibres had moderate or low mATPase activity (Fig 3a, 3c, 3e). However, all the specimens of the present study showed some zones of the myotome with no mATPase activity (Figure 3b, 3d, 3f), thus reflecting a gradual transition of the histochemical activity throughout of the myotome.

Discussion

Body Growth Parameters and Survival

Both body weight and body length showed a significant increasing throughout the experiment in all the tanks, as expected in the fattening phase (Nathanailides *et al*., 1996; López-Albors *et al*., 2008). Both parameters were similar among feeding groups in each sampling point, that evidences no effects of alginate dietary on the growth of gilthead sea bream during this experiment. Similarly, Skjermo *et al*. (2006) supplemented the diet of larvae of Atlantic cod, *Gadus morhua*, with alginate and high content of mannuronic acid (High-Malginate) and did not find significant effect on body weight when comparing with control group. Also, Cerezuela *et al*., (2008) studied effects of inulina (prebiotic) in the gilthead seabream (*Sparus aurata* L.) innate immune and did no find immunostimulant effects in this species. In contrast, red sea bream (*Pagrus major*) increased its body weight with supplemented diet with *Ascophyllum nodosum* at 5% level (Nakagawa *et al*., 1997). However, these authors did no find significant differences in muscle ratio by comparison with other no supplemented groups. Other studies in sub-adult hybrid striped bass (*Morone chrysops x M. saxatilis*) have found that dietary supplementation with brewers yeast and Grobiotic-A (a mixture of partially autolyzed brewers yeast, dairy ingredient components

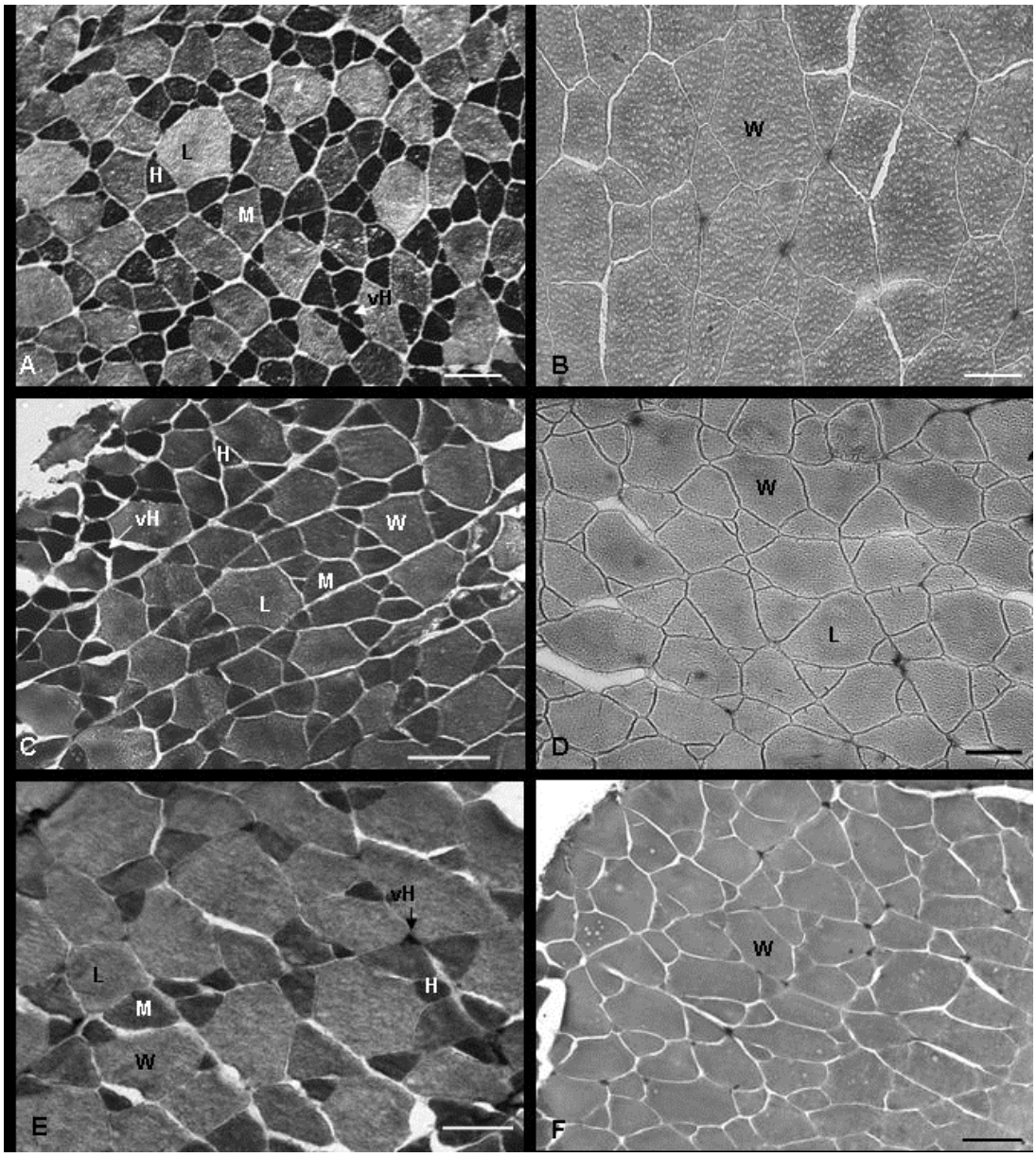

Figure 3. Cross-section of white muscle of sea bream (sampling III). mATPase reaction after acid preincubation pH 4.6 0.05M, 30 s. A,B: control tanks; C,D: supplemented groups with sodium alginate at 2% level; E,F: supplemented groups with sodium alginate at 5% level. Bars: A: 142 µm, B: 71.43 µm; C,D,F: 125 µm; E: 94.6 µm. W: white muscle fibres; vH, H, M, L: very high, high, moderate and low mATPase activity white muscle fibres, respectively.

and dried fermentation products) increased the weight gain as well as the survival against mycobacterial infection (Peng and Gatlin, 2005).

In relation with SGR and FCR parameters of the present study, there were not statistical differences among the experimental groups in each stage. In contrast, other experiments showed an increasing of these parameters in supplemented fish by comparison with no supplemented fish. Thus, the feed efficiency was higher in hybrid striped bass with supplemented diets (Peng and Gatlin, 2005).

These results show that the effect of the supplementation of prebiotics varies among species. This fact can be due to inter-specific variations in the required levels of the prebiotic and/or to different required period of supplementation in the different species. Hence, it would be necessary to wide the present experiment with different levels of sodium alginate and/or with the application of larger period of time.

Survival was higher in supplemented groups than in control tanks, although the differences were not significant. Similarly, in *Gadus morhua* larvae (Skjermo *et al*., 2006) and Atlantic halibut, *Hippoglossus hippoglossus* (Skjermo and Bergh, 2004) the survival increased when adding prebiotics in the diet. Also, Peng and Gatlin (2005) concluded that dietary supplementation with 2% GroBiotic-A enhanced survival in hybrid striped bass when exposing to mycobacterial infection. Peso *et al*. (unpublished observations) carried out a microbiological study in all the fish of the present work and found that supplemented groups presented the following lactic acid bacteriae: *Lactobacillus delbruekii* and *Lactobacillus acidophilus,* whereas both species were not found in the non-supplemented groups. Other works have shown that both species improved the immunostimulatory effects in the gilthead sea bream (Salinas *et al*., 2008) and had positive effects on growth of European sea bass (*Dicentrarchus labrax,* L.) (Carnevali *et al*., 2006).

Muscle Parameters

The white muscle growth was only significant at the end of the experiment, parallel to an increasing in the number of fibers. This fact can be due to that feeding regime was initially limited, whereas after sampling II, feeding regime was "*ad libitum*". Hence, a rapid growth was observed in the sampling III. Similarly, other authors have observed high rates of white muscle fibre hyperplasia and hypertrophy when favouring the maximum food availability (Nathanailides *et al*., 1996; López-Albors *et al*., 2003; Alami-Durante *et al*., 2007), that corresponds to a very active phase of mitotic activity and protein accretion.

On the other hand, data show that muscle tissue was not influenced by supplemented diets with sodium alginate, which coincides with results found with body parameters. Muscle cellularity (size distribution and number of fibres) varies according to genetic and environmental factors (nutrition, temperature, photoperiod, etc) (Nathanailides *et al*., 1996; Johnston *et al*., 1998; Johnston, 1999; Ayala *et al*., 2003; López-Albors *et al*., 2003, 2008). Hence, different muscle values among groups were expected by effect of different dietary regime. So far, muscle cellularity has not been studied in supplementary diets of fish by other authors and hence, it can no be compared with other studies. The results of the present work could be due to insufficient level of alginate sodium and/or to an insufficient period of time of the experiment, as suggested previously. Hence, it could be necessary other studies in this species.

Histochemical Profile of the White Muscle Fibres

The timing of the histochemical mATPase mosaic is an indicator of myotomal maturity as a consequence of postlarval muscle hyperplasia (Scapolo *et al*., 1988; Mascarello *et al*., 1995; Ramírez-Zarzosa *et al*., 1995). The results of the present study show that the myotomal maturity was gradually reached in all the specimens throughout the experiment, with no significant differences among groups by effect of the feeding regime. Gradual myotomal maturity has also been described in this species and in sea bass by other authors (Ramírez-Zarzosa *et al*., 1998; López-Albors *et al*., 1998, 2003).

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