Zn, Cu, Pyruvate Kinase and Myosin in White Muscle of Gilthead Seabream (*Sparus aurata*) Fed A Zn Enriched Diet

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Abstract

Two groups of young gilthead sea bream were fed two different diets: a control diet containing Zn at 120 mg/kg feed and a supplemented diet containing Zn at 240 mg/kg feed. White portions of lateral muscle were analyzed for Zn and Cu concentrations, myofibrillar composition and pyruvate kinase (PK) activity in order to determine the influence of Zn on these biochemical parameters during the growing period and a subsequent starvation phase. Levels of Zn in liver and muscle were ten times higher than those of Cu. No significant variations in metal concentrations were observed between the two diets. Myofibrillar proteins extracted from the white portion of lateral muscle were mostly constituted by myosin heavy (HC) and light (LC) chains that, as for other fish, had the following order of electrophoretic mobility: LC1F<LC3F<LC2F. Changes in LC2F and LC3F amounts were found during the experimental period. PK activity showed a significant increase at the end of the experiment, when maximal growth rate occurred. The starvation period resulted in a decrease of metal concentration and PK activity. In conclusion, Zn supplementation did not influence significantly metal concentration, protein composition and PK activity of white lateral muscle. It remains therefore a crucial target to determine the amount of Zn to be added to the diet of gilthead sea bream, to obtain both a rapid growth and a good quality of flesh.

Key words: Zn, muscle, myosin, pyruvate kinase, gilthead sea-bream.

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Trace elements, like Zn, Cu and Fe, are essential for the physiological metabolism of fish and metal deficiency or excess can cause biochemical, physiological and pathological changes, which are dependent on the metal and fish species [6]. Although a large number of studies have been made on nutritional requirements of trace elements in fish [11, 29], the research carried out on gilthead sea bream is still fragmentary [4, 25]. Metal requirements in fish are difficult to determine because these animals can utilize trace elements from dietary sources as well as from ambient water. However, many essential metals, such as Zn and Cu, are not taken up from water in sufficient amounts to satisfy physiological requirements and must therefore be supplied by the diet to prevent deficiency. In particular, Zn is a cofactor of over 300 enzymes and is involved in several metabolic pathways [28]; it has also a structural role in maintaining protein structure and it plays an important function during the development. Fish may present a Zn requirement higher than other animals due to the continuous growth of lateral muscle. Previous studies [4,25] demonstrated that gilthead sea bream fed a diet supplemented with 900 mg Zn/Kg feed had a significantly lower weight than fish fed with a control diet (60.9 mg Zn/Kg); however, no evident sign of toxicity was present, indicating that possible molecular damages could be present at a sub lethal level.

Therefore, the present study was undertaken to determine if the content of Zn in the diets may influence different parameters of muscle physiology and to obtain new information regards to the interaction between myofibrillar proteins, metabolic pathways, Zn and Cu tissues concentrations and the functioning of muscle machinery. For this purpose, we investigated the myofibrillar composition and pyruvate kinase activity in the white portion of lateral muscle of gilthead sea bream fed two commercial diets supplemented in different

concentration of Zn (120 mg Zn/Kg feed and 240 mg Zn/Kg feed) during juvenile growth; Zn and Cu concentration in white muscle were also measured.

Material and methods

Fish

Specimens of 0+ age gilthead sea bream (*Sparus aurata* L.), initial weight 0.9 ± 1 g, supplied by Cà Zuliani Porto Tolle (Rovigo), were randomly distributed in six polyethylene tanks containing 500 litres of natural sea water continuously recycled through separate biological filters. Fish, at a density of 50 specimens in each tank, were reared maintaining the natural photoperiod at a temperature of 19 ± 2°C and at a salinity of 29 ‰. Fish were fed a commercial diet containing 120 ± 5 mg Zn/kg and 4 ± 0.5 mg Cu/kg feed from 25th of February to 18th of March 1999; then they were divided in two groups: control fish were fed this commercial diet, whereas the remaining were fed a diet containing 240 mg Zn /kg feed. Gilthead sea bream were fed daily with 2% feed respect to the total fish weight of each tank. The feeding experiment started the 18th of March and ended the 21th of July, while the remaining fish were subjected to starvation until the end of August. Three specimens were monthly sampled from each tank. Fish were killed by a blow on the head, measured and weighed. The liver and portions of white muscle were sampled and stored until analysis.

Metal analysis

To avoid contamination, all reagents were handled carefully, polyethylene disposables were thoroughly washed with HCl 1 N under a fume and disposable gloves were worn during the procedure. All reagents were from Merck, Darmstadt (Germany); acids were of Suprapur grade. Samples from reserved tissues (100-200 mg of fresh tissue for muscle and 50-200 mg of liver) were placed in individual acid-washed Teflon jars and were digested in 2 ml 65% HNO_3 and 0.5 ml 30% H_2O_2 in a microwave oven, Milestone, model 1200, for 5 min at 250W, 5 min at 400W, 5 min at 500W and 1 min at 600W. Cooled samples were transferred into 5-10 ml polyethylene volumetric flasks and directly aspirated into the flame of an atomic absorption spectrophotometer, Instrumentation Laboratory, Model IL11, equipped with a deuterium lamp background correction. The instrumental wavelengths were: 213.9 for Zn and 324.7 for Cu. Metal concentrations were reported in µg/g wet weight.

Two blanks were digested simultaneously during each run. Blank values (n=10) were: 0.010±0.002 µg/ml for Zn and 0.006±0.003 µg/ml for Cu. Trace metal standards were run every 50 samples.

The accuracy of the method was evaluated by calibration with an international standard (CRM 278: lyophilised mussels). The analyses gave the subsequent results: 79 ± 3 for Zn and 9.7 ± 0.3 for Cu; the certified values were respectively 76 ± 2 and 9.6 ± 0.16. The concentrations (>80%) found with the method used in this study fell within the confidence interval given by the Community Bureau of Reference - BCR (Brussels).

Northern blotting

Total RNA was isolated from approximately 75 mg of frozen tissue by TriReagent isolation kit, following the Chomczynski and Sacchi procedure [5]; the extracted RNA was applied on 1% formaldehyde-agarose gel, transblotted onto HyBond_XL membrane (Amersham Pharmacia Biotech) and subjected to Northern hybridisation with ^{32}P-randomly labelled cDNAs of metallothionein (MT) and β-actin synthesised form *S. aurata* as described in [14].

Myofibrillar preparations

Minced muscle samples of epaxial lateral muscle (0.2-0.5 g) were washed 3 times with 40 mM NaCl; then the tissue, diluted in 10 volumes of the saline medium, was homogenised with a teflon pestle. The homogenate was centrifuged at 1800 g at 4°C, to give a pellet that was then resuspended, washed and centrifuged again 3 times. The final pellet was dissolved in 1 ml of 62.5 mM Tris-HCl buffer, pH 6.8, containing 2.3% SDS, 10 % glycerol and 5% 2-mercaptoethanol. The myofibrillar solution was heated for 3 min at 100 °C, centrifuged at 1,800 g at 4°C for 5' and stored at -20 °C.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Analytical SDS-PAGE separations were performed on gels containing 12.5% acrylamide according to [17]. Running buffer was made of Tris 50 mM, SDS 0.1% w/v, glycine 200 mM, pH 8.6 and separation was achieved using 150V and 20 mA in 2h. About 30 µg of myofibrillar proteins were loaded per lane. Gels were stained with Coomassie Brilliant Blue R-250. Purified rabbit myosin was used as a standard.

Western blotting

The electrophoretic transfer of myofibrillar proteins fron polyacrylamide gels to nitrocellulose sheets was performed under the general conditions of Towbin [27]. Blotted proteins were tested with specific polyclonal antibodies raised against gilthead sea bream myosin.

Determination of pyruvate kinase activity

Sample extracts were prepared by homogenising muscles (0.3-0.5 g) in 20 volumes of 50 mM imidazole-HCl buffer, pH 7.0, containing 5 mM EDTA, 5 mM EGTA, 100 mM NaF, 0.1 mM phenyl-methil sulphonide fluoride (PMSF), 1 mM DTT using an Ultra-Turrax homogeniser. The homogenate was centrifuged at 30,000 g for 30 min in a Kontron-Centrikon T-1160 refrigerated centrifuge at 4°C; the resulting supernatants were filtered and passed through a column of Sephadex G-25, equilibrated with 100 mM imidazole-HCl buffer, pH 7.0, containing 1.5 mM EDTA, 1 mM EGTA, 30 mM NaF, 0.1 mM PMSF, 0.5

mM DTT. Supernatants were immediately used for the enzyme assays. Pyruvate kinase activity was measured at 25°C using an UV/Vis Beckman mod. DU530 Spectrophotometer. Preliminary experiments established the optimal substrate and cofactor concentrations and pH value. Standard assay conditions utilised 100 mM imidazole-HCl buffer, pH 7.5, 67 mM KCl, 8.3 mM $MgSO_4$, 0.2 mM NADH, 3 mM ADP, 5 IU LDH, and 2 mM phosphoenolpyruvate (PEP). Enzyme activity is expressed as U/g wet weight of muscle.

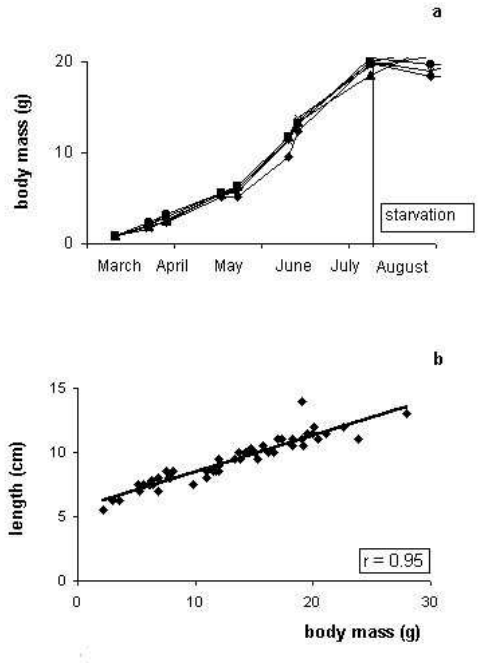

Figure 1 Increase in body mass of gilthead sea bream during the experimental trial (n=6) (a); correlation between length and fish weight (n=50) (b).

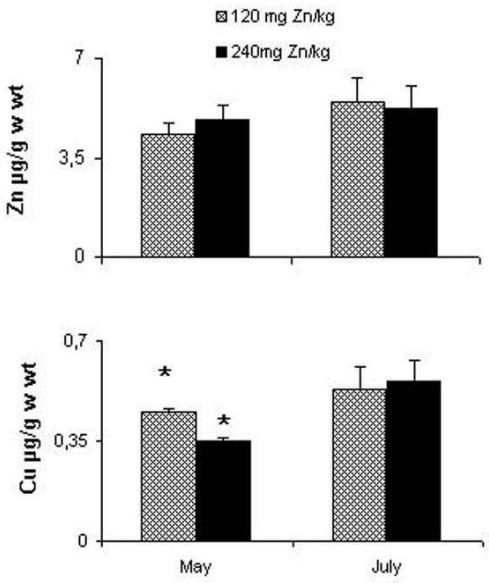

Figure 2 Zinc and copper concentrations in the white muscle of Sparus aurata *fed two experimental diets containing respectively 120 and 240 mg Zn/kg and sampled in May and July. Data are reported as mean (n=3± standard deviation).* Indicates significant differences (t test,* p<*0.05.)*

Statistical analysis

Values are reported as mean ± standard deviation. Significant differences between data of May and July were tested by Student t test ($p<0.05$, or $p>0.01$).

Results

Increase in body mass

Gilthead sea bream with an initial weight 0.9 ± 0.1g in February presented a continuous increase in body weight, that in July reached 20.05 ± 4 g for fish fed the control diet and 19.4±5 for fish fed the Zn enriched diet (Fig. 1a). No significant differences were found between the two experimental groups. As expected, a positive correlation between weight and length was found (Fig. 1b). During the starvation period no significant variation in body weight was observed.

Zn and Cu concentrations

Zn and Cu concentrations were determined in the white muscle (Fig. 2) and in the liver (Fig. 3); Zn concentrations were about ten times higher than Cu in both tissues. The presence of MTmRNA was shown only in the liver (Fig.4). In the white muscle, a slight increase in Zn and Cu was present at the end of the feeding trial, whereas in the liver a significant decrease in Zn was found from February to June, followed by an increase in July; the starvation period resulted in a decrease of the metal concentration. No significant variations in Cu were measured. We did not observe

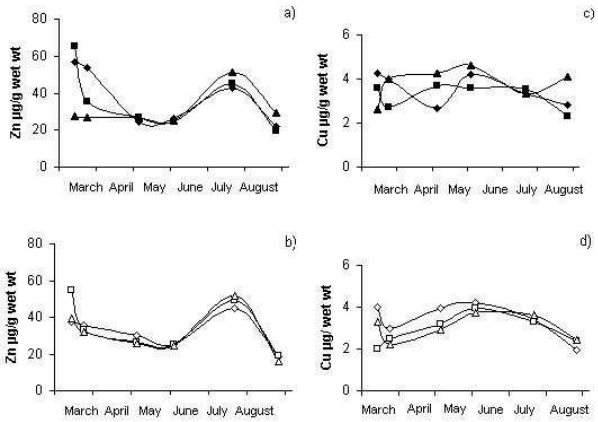

Figure 3 Zinc and copper concentrations in liver of Sparus aurata*, fed a 120 mg Zn/kg diet (a, c) and 240 mg Zn/kg diet (b, d). Each value is reported as a mean of three different analyses.*

significant differences in hepatic Zn and Cu concentrations between fish fed the two diets.

Myofibrillar proteins

Myofibrillar proteins extracted from the white muscle of gilthead sea bream were mostly constituted by myosin heavy (HC) and light (LC) chains; in particular three light chains were identified, namely LC1F, LC2F and LC3F (Fig. 5). The identity of LCs was confirmed by Western blot with a specific anti-myosin antibody (Fig. 6). Myofibrillar extracts also contained tropomyosin, actin, minor bands that could be related to troponins, and other unidentified proteins. No contamination by myosin light chains of red muscle was found. The electrophoretic migration of LCs was LC1F<LC3F<LC2F. An increase of LC3F and LC2F amount was found between March and July. Zn supplementation and starvation did not affect significantly the electrophoretic myofibrillar pattern of white muscle.

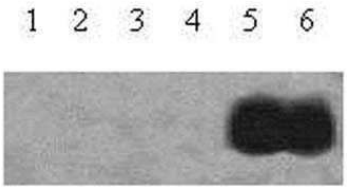

Figure 4 Northern hybridization of MT cDNA probe to RNA extracts from muscle (lanes 1-4) and liver (lanes 5,6) of Sparus aurata *fed the 120 mg Zn/kg diet.*

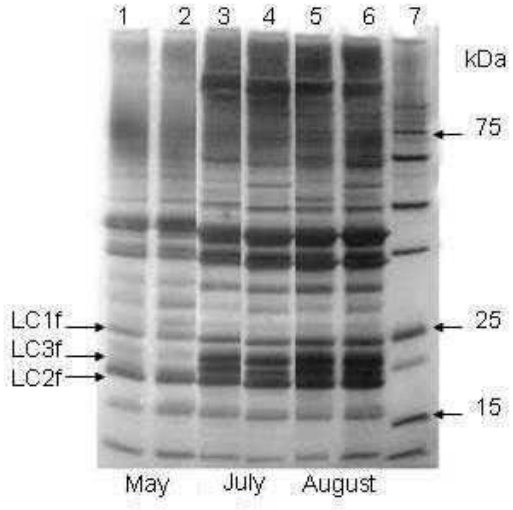

Figure 5. SDS-PAGE of myofibrillar proteins from lateral white muscle of Sparus aurata *fed the 120 mg Zn/kg diet (lanes 2,4,6) and the 240 mg Zn/kg diet (lanes 1,3,5) Markers proteins were loaded on lane 7. LC1f = myosin light chain 1 fast; LC2f = myosin light chain 2 fast; LC3f = myosin light chain 3 fast.*

Pyruvate kinase (PK) activity

In the white muscle of gilthead sea bream PK was characterized by high activity, low Km and hyperbolic saturation kinetics. Maximal activities ranged between 187 and 401 U/g wet wt (Fig. 7), and K_m between 0.05 and 0.16 mM. At the end of feeding trials an increase in PK activity was measured: in fish fed the control diet significant for two replicates ($p<0.05$), while in fish fed the Zn supplemented diet significant for one replicate ($p<0.05$). After the starvation a significant decrease in PK activity ($p<0.05$) was evident in both experimental groups.

Correlation between PK activity and weight

In Fig. 8a,b,c the relationship between muscle PK activity and body weight is reported. No significant correlation was evident. A significant inverse correlation was evident ($p<0.05$) in August, after the starvation phase (Fig. 8d).

Discussion

The present work aims to obtain more knowledge about the effects of Zn dietary supplementation on growth rate and selected biochemical parameters of young gilthead sea bream white muscle during post-larval phase.

The same increase in body weight of the two groups of young gilthead sea bream fed diets containing respectively 120 and 240 mg Zn/kg feed demonstrates

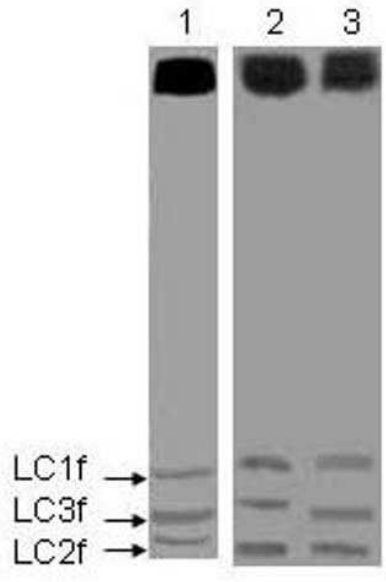

Figure 6 Western blotting of myofibrillar proteins from lateral white muscle of Sparus aurata *after electrophoresis on 12% SDS-PAGE. Muscle were sampled in May, lane 2 and July, lane 3. 40 μg of purified myosin from lateral white muscle of* Sparus aurata *were loaded as standard, lane 1.*

that the two diets should equally satisfy the metal requirement during the post-larval phase without signs of deficiency or toxicity; this conclusion is reinforced by the lack of significant differences in hepatic and muscular Zn concentrations between the two groups of fish. Accordingly, in a previous work on adult gilthead sea bream it was demonstrated that dietary Zn concentrations in the range of 60.9-387 mg/kg feed produced similar growth, whereas 983 mg/kg feed had a negative effect [25].

The lack of a dietary and seasonal influence on muscle Zn concentrations could be related to a strict homeostatic control, which can regulate the tissue metal level through the activity of specific influx/efflux systems, as it was demonstrated for bivalent metal cations in yeast [12]. The failure of metal accumulation in the white muscle is favored by the absence of MT biosynthesis as demonstrated by the complete lack of MTmRNA by Northern blot. However, previously we have observed a significantly higher Zn concentration in white muscle of wild gilthead sea bream respect to farmed fish, probably due to a different diet and a higher locomotory activity [3]. Hepatic metal concentrations, that are one order of magnitude higher than in the muscle should be explained on the basis of liver function, related to the presence of specific storage proteins, e.g. ferritin for Fe and MT for Zn and Cu. The isolation of MTmRNA in the liver of young gilthead sea bream is confirmatory of a previous research on adult sea bream [14]. Differently from muscle, evident seasonal variations in metal concentrations were observed in the liver. The initial decline in metal concentrations may be attributed to dilution by growth, as reported for bluefish and morids [8] and gilthead sea bream [4]. On the other hand, the increase of Zn in July, when fish present the major gain of body weight, could be related either to a low hepatic fat content or to enhanced needs of the Zn-enzyme systems involved in nucleic acid and protein biosynthesis. Moreover, also seasonal fluctuation of MT induction cannot be excluded.

Myofibrillar protein pattern is the second biochemical parameter investigated in the present work. In several fish species there is a continuous increase in body mass, due to hyperplastic [2] and hypertrophic processes of skeletal lateral muscle [23]; these processes could be regulated by several factors, including myostatin [22]. Tissue development is associated with sequential synthesis of a range of myofibrillar protein isoforms [10-26]. In this respect myosin is one of the most represented and studied. As concerns the order of myosin LCf migration, the present results obtained in young gilthead sea bream are confirmatory of previous data on adult [3] and young specimens [13], with LC3F displaying a lower electrophoretic mobility than LC2F. In fish LC3F has a molecular weight higher than that reported for mammals and is encoded by a separate gene [9]. In the present research the most relevant result was the low expression of LC3F during the month of May, as shown by SDS PAGE, even if the correspondent immunoblots were less indicative. Our data point out for gene regulation acting at different time on LC1F, LC2F and LC3F expression [24]. In *S. aurata*, LCs exhibited a different age-related pattern of

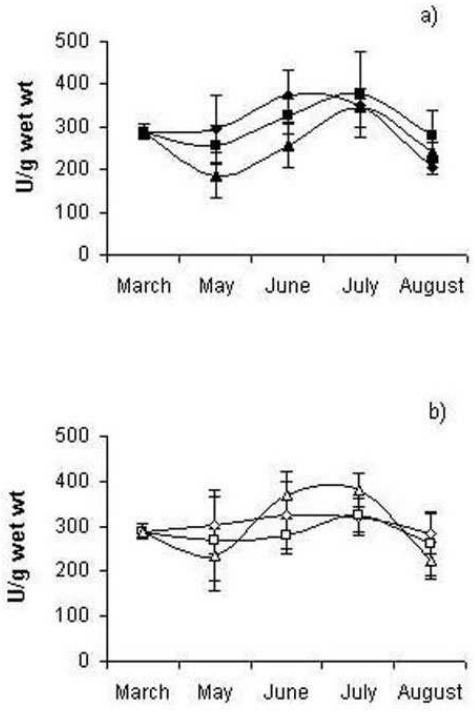

Figure 7. Pyruvate kinase activity from white muscle extracts of fish fed respectively 120 mg Zn/kg diet (a) and 240 mg Zn/kg diet (b). Data are reported as mean (n = 3 ± standard deviation).

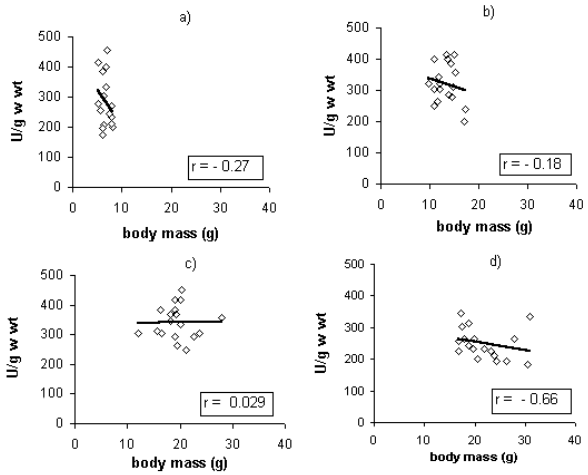

Figure 8. Correlation between body mass and pyruvate kinase activity in the white muscle of fish samled in May (a), June (b), July (c) and August (d). Data are obtained from pooled fish.

response to varied thyroidal states, suggesting that different regulatory mechanisms exist for different LCs [19].

The third biochemical parameter investigated in the present research is related to the energy production partially used for protein synthesis and swimming. The high energy mechanical output of skeletal muscle originates from ATP hydrolyzed by myosin ATP-ase. In the white muscle the ATP is mainly produced by the glycolytic pathway, which is controlled by PFK and PK. The significant increase in PK activity, measured especially in fish fed the 120 mg Zn/kg feed, is probably related to the increased energy demand of growing muscle. Indeed, it has been reported in *Gadus morhua* that PFK, PK and LDH activities are positively correlated to growth rate [7, 20, 21].

Finally, we have studied the effect of starvation on the above-mentioned parameters. Many species of wild fish undergo natural periods of starvation due to temperature and food supply fluctuations or spawning. Several physiological and metabolic responses to starvation have been reported, including a general down regulation of metabolic and locomotory activity [18]. This work showed that in *S. aurata* starvation is accompanied by a lack of growing, a decrease in muscular PK activity and hepatic Cu and Zn concentrations, while myofibrillar proteins of white muscle did not show any significant variations. The decline in glycolytic enzymes in fish white muscle is a general response to starvation [16] and is related to a decrease of locomotory activity. In *Gadus morhua* no quantitative changes in the electrophoretic pattern of the myofibrillar fraction was observed, while a differential decrease of solubile proteins was present [1].

In conclusions, the present data suggest that a diet containing 120 mg Zn/kg feed should satisfy the metal requirement in young gilthead sea bream, maintaining an optimal muscular Zn level, which together with muscle fiber density [15] will contribute to the nutritional characteristics of the flesh for human nutrition.

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