

Biochemical Changes in Gilthead Sea-Bream White Muscle During Post-Larval Growth

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Abstract

In the present paper we present results of an investigation of the myofibrillar composition and pyruvate kinase (PK) activity in the white portion of lateral muscle of gilthead sea bream fed a commercial diet for five months during juvenile growth. The zinc and copper content of muscle and liver were also measured. Myofibrillar proteins extracted from the white muscle were mostly constituted by myosin as well as tropomyosin and actin, minor bands could be related to troponins. As reported for other fish, an apparent molecular weight of myosin LC3f higher than LC2f was found. Only minor changes in LC3f amounts were observed during the experimental period. A great variability in PK maximal activity was found, with values ranging from 175 to 453 U/g wet weight. The enzyme showed always hyperbolic saturation curves with the substrate phosphoenolpyruvate and was characterised by low K_m . At the end of the experiment, when body mass of fish increased approximately three times, a significant increase of PK activity was measured. Zn concentrations were about ten times higher than Cu in both liver and muscle; on the other hand, the metal concentrations in the liver were about ten times higher than that of the muscle. In the latter no significant variations in metal concentrations were found.

Key words: gilthead sea-bream, metals, muscle, myofibrillar proteins, pyruvate kinase.

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Fish growth, when considered as body weight increase, is commonly used to predict growth curves in fish management. However, this parameter might be not correlated with the metabolic changes which occur in different organs during fish ageing. Hyperplastic [3] and hypertrophic [28-31] processes of skeletal lateral muscle are a major contributor to fish weight increase. Fish lateral muscle presents a myomeric arrangement where red, pink and white fibres are distributed in separate layers with the white part constituting the main bulk. Fish undergo dramatic changes in the fibre type composition (myosin expression and organisation of fibre type) and in the isoforms of myofibrillar molecules during post-hatching growth [12-16]. Moreover biochemical parameters of lateral muscle could be influenced by several endogenous (e.g. growth rate and developmental stage, [12-22]) and exogenous factors (e.g. diet and temperature, [4-17]).

White muscle is recruited when a relatively high swim speed is necessary, e.g. escaping from predators, capturing a prey and migrating against water current [19]. This muscle layer is composed of fast anaerobic

fibres, which are responsible for bursts of rapid and vigorous activity [12].

In the white muscle, the ATP consumed in contraction is mainly produced by glycolysis. One of the key regulatory enzymes of this pathway is pyruvate kinase, which has been extensively studied in mammals. Less information is available about the structure and kinetic properties of PK in fish. PK isolated and purified from trout muscle showed the same subunit molecular weight as the cat M isoenzyme and had very similar aminoacid composition [8]. To our knowledge no other work has been reported on PK structure and sequence from fish muscle. Altogether there are 4 PK isoenzymes in fish, the predominant isoform of skeletal muscle is the K isoform, even though few species present in addition also the E isoform [25].

Several systems involved in the ATP production of muscle contraction are based on metalloproteins, e.g. Ca and parvalbumin, Cu and cytochrome c-oxidase. Moreover, gene expression is modulated by zinc-finger proteins, whose activity is based on Zn bioavailability. Therefore,

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the interactions between myofibrillar proteins, metabolic pathways (e.g. glycolysis), Zn and Cu status will determine the functioning of muscle machinery.

Gilthead sea bream is commonly farmed in the Mediterranean, is easily reared in experimental aquariums and its biology is well studied in some respect. However, the relationship between rearing conditions and many biochemical parameters of its muscle has been little studied. In the present paper we present results of an investigation of the myofibrillar composition and pyruvate kinase activity in the white portion of lateral muscle of gilthead sea bream fed a commercial diet during juvenile growth. The zinc and copper content of muscle and liver were also measured.

Material and Methods

Fish

Specimens of 0+ age gilthead sea bream (*Sparus aurata* L., Sparidae, Teleosts), supplied by Cà Zuliani Porto Tolle (Rovigo), were randomly distributed in three polyethylene tanks containing 500 litres natural sea water that was continuously recycled through separate biological filters. Fish, at a density of 50 specimens in each tank and of an initial weight of 0.9 ± 0.1 g, were reared maintaining the natural photoperiod at a temperature of $18 \pm 1^\circ\text{C}$ and at a salinity of 28 parts per thousands. Fish were fed a commercial diet containing 120 ± 5 mg Zn/kg and 4 ± 0.5 mg Cu/kg feed (Table 1). Gilthead sea bream were fed daily with 2% feed respect to the total fish weight of each tank. The experiment started the 25th of February and ended the 21th of July. Three specimens were monthly sampled from each tank. Fish were killed by a blow on the head, measured, weighed and different tissues were sampled and stored depending on the kind of analysis.

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 which was then resuspended, washed and centrifuged again 3 times. The final pellet was dissolved in 1 ml of a buffer solution of the following composition: 62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 10 % glycerol, 5% 2-mercaptoethanol. Thereafter the myofibrillar solution was heated for 3 min at 100°C and stored frozen at -20°C until used.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Analytical SDS-PAGE separations were performed on gels containing 12.5% acrylamide according to [14]. Gels were stained with Coomassie Brilliant Blue R-250.

Determination of pyruvate kinase activity

Samples of white muscle (0.3-0.5 g) were rapidly dissected epaxially the lateral line and immediately frozen and stored in liquid nitrogen until the analysis. Sample

Table 1 Composition^a of the experimental diet.

Component ^b	%	Minerals	mg/kg feed
Protein	55	Zinc	120
Fat	18	Copper	4
Carbohydrate	1		
Ash	10		

^a Vitamine mixture contained (as International Units or mg/kg feed): Vitamin A 18.000 I.U.; Vitamin D3 1.800 I.U.; Vitamin E 250 mg; Vitamin C 500 mg.

^b Data obtained from Hendrix S.p.A. (Mozzecane, Verona)

extracts were prepared by homogenising muscles 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-methyl sulphonide fluoride (PMSF), 1 mM DTT using an Ultra-Turrax for four 15 s periods. 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 column buffer, 100 mM imidazole-HCl, 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 a UV/Vis Beckman mod. DU 530 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 phosphoenolpyruvate (PEP) at varying concentration (0.05- 2 mM). The reaction was started by adding PEP solution. The effect of modulators on PK activity was tested at 0.05 and 2 mM PEP using 0.1 mM fructose-1,6-bisphosphate (FDP), 3.2 mM ATP and 4 mM alanine. Enzyme activity was expressed as $\mu\text{moles PEP converted/min/g wet weight of muscle}$ or as $\text{U/g wet weight of muscle}$.

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 of liver (100-200 mg of lyophilised tissues) and muscle (100-150 mg of fresh tissue) were placed in individual acid-washed Teflon jars and were digested by microwave oven (Milestone, Italy) for 5 min at 250 W, 5 min at 400 W, 5 min at 500 W and 1 min at 600 W in 2 ml 65 % HNO_3 and 0.5 ml 30% ml H_2O_2 . Cooled samples were transferred into 10 ml polyethylene volumetric flasks and directly aspirated into the flame of an atomic

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absorption spectrophotometer (Instrumentation Laboratory, Model IL 11, equipped with a deuterium lamp background correction).

Two blanks were digested simultaneously during each run. Blank values ($n = 10$) were respectively: $0.006 \pm 0.003 \mu\text{g/ml}$ for Cu; $0.020 \pm 0.002 \mu\text{g/ml}$ for Zn.

The accuracy of the method was evaluated by calibration with an international standard (CRM 278: lyophilised mussels). The concentrations ($> 80\%$) found with the method used in this study fell in the confidence interval given by Community Bureau of Reference - BCR (Brussels). Trace element concentrations measured in reference material were: $9.3 \pm 0.2 \mu\text{g/g}$ ($9.60 \pm 0.16 \mu\text{g/g}$) for Cu; $78.9 \pm 2.4 \mu\text{g/g}$ ($76 \pm 2.4 \mu\text{g/g}$) for Zn; certified values of reference material are reported in brackets. Detection limits found with this method for the analysis of tissues were: $0.04 \mu\text{g/g}$ for Zn; $0.05 \mu\text{g/g}$ for Cu.

Statistical analysis

Values are reported as mean \pm 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

During the experimental period gilthead sea bream (initial weight of $0.9 \pm 0.1 \text{ g}$ in February) presented a continuous increase in body weight, that in July reached a weight of $20.05 \pm 4 \text{ g}$ (Fig. 1).

Myofibrillar proteins

Myofibrillar proteins extracted from the white muscle of gilthead seabream in July, were mostly constituted by myosin as well tropomyosin and actin, minor bands could be related to troponins (Fig. 2); purified rabbit myosin was used as a control. Minor changes in LC3f amounts were found between May and July (data not shown).

Pyruvate kinase activity

Pyruvate kinase activity was measured in the white muscle. A great variability in PK maximal activity was found, with values ranging from 175 U/g wet weight to 453 U/g wet weight. Figure 3 shows an example of triplicate PEP saturation curves of PK extracted from white muscle of specimens sampled from the same aquarium: the enzyme showed hyperbolic saturation curves with the substrate PEP and was characterised by low K_m . When comparing PK activities of individuals from triplicate aquariums no significant differences were found. In Figure 4 a comparison between maximal enzyme activities in May and July is reported; the activity of PK in May was significantly ($p < 0.05$) lower ($247 \pm 70 \text{ U/g}$ w wt) than in July ($340 \pm 79 \text{ U/g}$ w wt).

In juvenile gilthead seabream no significant correlation between PK activity and body mass was found.

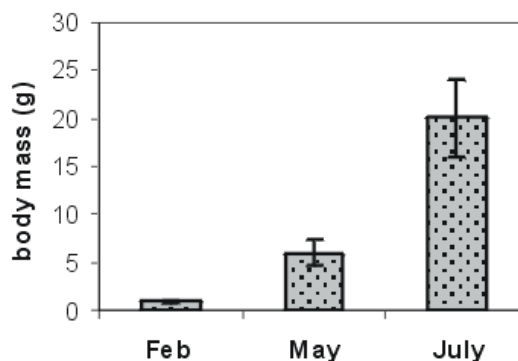


Figure 1. Body mass increase in specimens of *Sparus aurata*. Data are reported as mean ($n = 5$) \pm standard deviation.

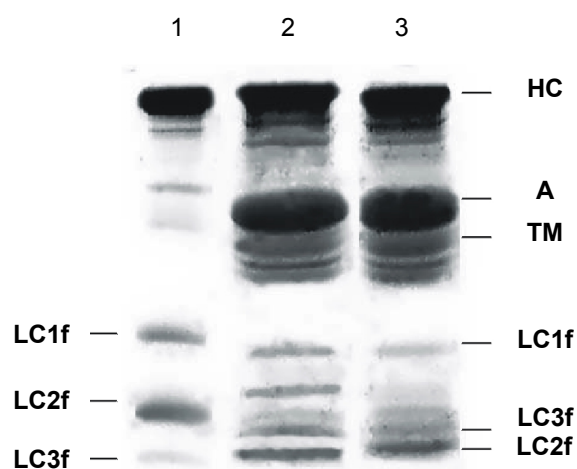


Figure 2. SDS-PAGE of purified myosin from fast rabbit muscle (*psaos*) (lane 1) and myofibrillar proteins from lateral white muscle of two specimens of gilthead seabream sampled during the month of July (lanes 2 and 3). HC: myosin heavy chain; A: actin; TM: tropomyosin; LC1f: myosin light chain 1 fast; LC2f: myosin light chain 2 fast; LC3f: myosin light chain 3 fast.

Zinc and copper concentrations

Zinc and copper concentrations were measured in the muscle and liver (Fig. 5a and 5b). Zn concentrations were about ten times higher than Cu in both tissues; on the other hand, the metal concentrations in the liver were about ten times higher than that of the muscle. In the muscle no significant variations in metal concentrations were found between May and July (Fig. 5a); differently, in the liver, Zn increased from 26 to $43 \mu\text{g/g}$ w wt ($p < 0.01$) and Cu decreased from 3.52 to $1.56 \mu\text{g/g}$ w wt ($p < 0.01$).

Discussion

Myosin is a hexameric protein composed of two heavy chains (HC) of approximately 200 kDa and four light chains (LC) of approximately $15\text{--}30 \text{ kDa}$ [15]. Myosin HCs and LCs are encoded by multigene families and the combination of these subunits give rise to

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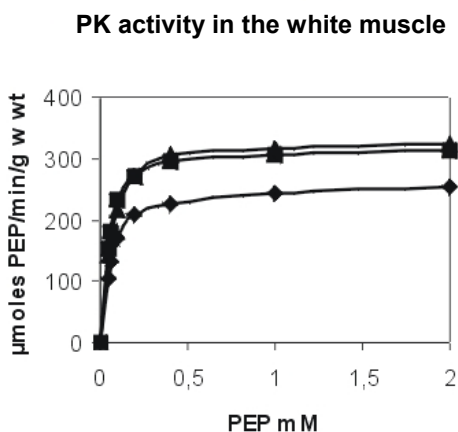


Figure 3 Pyruvate kinase activity at varying PEP concentrations. Saturation curves were obtained using preparations from white muscle of three different specimens of *Sparus aurata*.

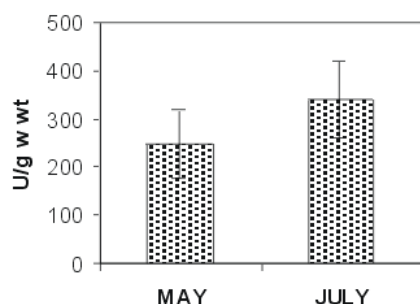


Figure 4. Comparison between pyruvate kinase activity in the white muscle of specimens sampled in May and July; data are reported as mean ($n = 9$) \pm standard deviation. Values resulted significantly different (t test, $p < 0.05$).

a large number of myosin isoforms [29]. In mammals fast skeletal muscle fibres are characterised by two isoforms of essential (or alkali) LCs, namely LC1f and LC3f, which derive from a single gene through alternative splicing and have an identical C-terminal sequence of 141 aminoacids [5-29]. The characterising N-terminal sequence derives from exons 1 and 4 for LC1f and from exons 2 and 3 for LC3f [24].

In fish LC3f is characterised in SDS gel by a low electrophoretic mobility, indicating a molecular weight higher than that reported for mammals [21]. Another characteristic makes fish LC3f different from that of mammals: in white lateral muscle this essential light chain is coded by a separate gene, as it has been demonstrated firstly by determining aminoacid sequence in *Mugil capito* [6] and subsequently by cDNA cloning in *Cyprinus carpio* [9]. Probably, due to the fact that LC3f is encoded by a specific gene, a marked variability of this light chain in fish has been previously pointed out by one dimensional [34] and by two dimensional gel-electrophoresis [21]. Interspecific variations in apparent

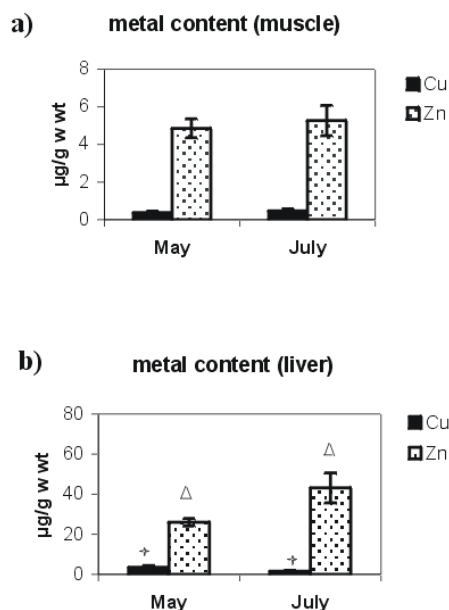


Figure 5. Zinc and copper concentrations in the white muscle (a) and liver (b) of specimens of *Sparus aurata* sampled in May and July. Data are reported as mean ($n = 9$ for the liver, $n = 6$ for the muscle) \pm standard deviation. Bars sharing the same symbol are significantly different (t test, $p < 0.01$).

molecular weights and order of migration of LCs has been reported in fish. Molecular weights of LC3f determined by SDS-PAGE range from 14 kDa to 19 kDa, depending on the species [21]. This marked variability in weight could be linked to the presence of specific aminoacids affecting surface charge, as it has been shown in mammals for LC1f; in this case several alanine-proline residues at the N-terminal caused an anomalous low electrophoretic mobility on SDS-PAGE [29]. In guppy, eel, short-horned sculpin and seabass the order of electrophoretic migration in SDS-PAGE is LC1f>LC2f>LC3f [1-2-11-27], whereas in goldfish (*Carassius auratus*) LC3f>LC2f [27]. In juvenile gilthead seabream the higher apparent molecular weight of LC3f respect to LC2f (Fig 2) confirms our previous findings [4]. In carp it has been demonstrated that the anomalous behaviour of LC3f is a function of the pH of the buffer used for the electrophoresis [10].

Even if fish adapt to different physical and biological conditions by expressing a different set of genes [1-32], in our conditions we observed only minor changes in LC3f amounts during the experimental period (data not shown).

As regards to the PK determination, in white muscle the activity is high relative to other glycolytic enzymes. The PK values found in this study fall in the range reported by authors for other species [13-18-33].

In fish, muscular PK shows a wide range of kinetic and regulatory characteristics, e.g. in *Mugil lisa* and

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Caetoditerus faber, PK is similar to the L-type form present in mammalian liver [20], whereas in other fish PK shows Michaelis-Menten kinetics and poor sensitivity towards modulators and is thus comparable to M1-PK expressed in skeletal muscle, heart and brain of mammals. In eel [26] and sea bass [7] muscle the enzyme has kinetic properties similar to the mammalian M2 type (FDP activation and inhibition by alanine and phenylalanine). By contrast, in farmed sea bass, white muscle PK showed hyperbolic saturation curves [13], indicating a possible influence of rearing conditions and experimental feeding on the enzyme activity; in the present study PK also failed to show cooperative kinetics (Fig. 3).

Recently, it has been reported that also enzyme activities are correlated to growth rate. In the white muscle of Atlantic cod, *Gadus morhua*, a strong positive correlation between glycolytic enzyme activities (PFK, PK, LDH) and growth rate was found [22-23]. Even if in our experiment we did not calculate the growth rate of each specimen, we have found a significant increase in PK activity in July (Fig. 4), when body mass of fish was approximately three times higher respect to May.

Zinc and copper concentrations in tissues of juvenile gilthead seabream confirm our previous data obtained in farmed and wild specimens of *Sparus aurata* [4-30]. It has been shown that Zn supplementation has little effects on metal accumulation in muscle and liver of gilthead seabream indicating the presence of an efficient homeostatic control [30]. The possible increase in protein synthesis occurring during the fish growth is not related to a relevant increase in tissue Zn concentration (Fig. 5a). On the other hand the hepatic increase found in July could be related to the induction of zinc-metallothionein.

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