Effect of diet composition and ration size on key enzyme activities of glycolysis–gluconeogenesis, the pentose phosphate pathway and amino acid metabolism in liver of gilthead sea bream (*Sparus aurata)*

I. Metón[1], D. Mediavilla[1], A. Caseras[1], E. Cantó[1]*, F. Fernández[2] and I. V. Baanante[1]†

[1]*Departament de Bioquímica i Biologia Molecular, Facultat de Farmàcia, Universitat de Barcelona, Barcelona, Spain*
[2]*Departament d'Ecologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain*

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The effects of diet composition and ration size on the activities of key enzymes involved in intermediary metabolism were studied in the liver of gilthead sea bream (*Sparus aurata*). High-carbohydrate, low-protein diets stimulated 6-phosphofructo 1-kinase (*EC* 2.7.1.11), pyruvate kinase (*EC* 2.7.1.40), glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49) and 6-phosphogluconate dehydrogenase (*EC* 1.1.1.44) enzyme activities, while they decreased alanine aminotransferase (*EC* 2.6.1.2) activity. A high degree of correlation was found between food ration size and the activity of the enzymes 6-phosphofructo 1-kinase, pyruvate kinase, glucose-6-phosphate dehydrogenase (positive correlations) and fructose-1,6-bisphosphatase (*EC* 3.1.3.11) (negative correlation). These correlations matched well with the high correlation also found between ration size and growth rate in starved fish refed for 22 d. Limited feeding (5 g/kg body weight) for 22 d decreased the activities of the key enzymes for glycolysis and lipogenesis, and alanine aminotransferase activity. The findings presented here indicate a high level of metabolic adaptation to both diet type and ration size. In particular, adaptation of enzyme activities to the consumption of a diet with a high carbohydrate level suggests that a carnivorous fish like *Sparus aurata* can tolerate partial replacement of protein by carbohydrate in the commercial diets supplied in culture. The relationship between enzyme activities, ration size and fish growth indicates that the enzymes quickly respond to dietary manipulations of cultured fish.

Sparus aurata: **Glycolysis**: **Gluconeogenesis**: **Amino acids**

The use of nutrients by carnivorous fish differs from that of the rat and other carnivorous mammals. Glycaemia is less well regulated than in mammals, and the capacity to metabolize glucose is diminished (Cowey & Walton, 1989; Steffens, 1989). Most key enzymes for carbohydrate metabolic pathways are apparently found in fish, and the basis for the low ability of carnivorous fish to utilize carbohydrates remains unclear (Wilson, 1994). Omnivorous fish such as carp (*Cyprinus carpio*) (Furuichi & Yone, 1981) have a higher capacity to metabolize glucose than carnivorous fish, although this is lower than in mammals.

We have previously shown that starvation–refeeding and differences in diet composition promote changes in the levels of enzyme activities involved in intermediary metabolism in the liver of gilthead sea bream (*Sparus aurata*) (Baanante *et al*. 1991). Nowadays *Sparus aurata* is one of the most extensively cultured marine fish in the Mediterranean countries. The cost of the diets supplied to gilthead sea bream in culture is high because of the high protein content required, usually provided by fish meal.

Somatic factors, such as body and liver weight, and metabolic factors, such as metabolite and enzyme activity determinations, are currently used to determine the capacity for metabolic adaptation to dietary supply in fish. In this regard, the liver–somatic index (LSI), hepatic glycogen content and key liver enzyme activities of intermediary metabolism have been shown to match well with the nutritional status and the growth rate in fish (Bonamusa *et al*. 1989, 1992; Bastrop *et al*. 1992; Brauge *et al*. 1994; Pelletier *et al*. 1994). Important in this respect are 6-phosphofructo 1-kinase (PFK-1; *EC* 2.7.1.11) and pyruvate kinase (PK; *EC* 2.7.1.40), key enzymes for glycolysis; fructose-1,6-bisphosphatase (FBPase-1; *EC* 3.1.3.11), in relation to gluconeogenesis; glucose-6-phosphate dehydrogenase (G6P-DH;

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BW, body weight; FBPase-1, fructose-1,6-bisphosphatase; G6P-DH, glucose-6-phosphate dehydrogenase; LSI, liver–somatic index; PFK-1, 6-phosphofructo 1-kinase; 6PG-DH, 6-phosphogluconate dehydrogenase; PK, pyruvate kinase.

* **Present address:** Departament d'Immunologia, Hospital de la Santa Creu i Sant Pau, 08025 Barcelona, Spain.
† **Corresponding author**: Dr Isabel V. Baanante, fax +34 3 402 1896, email baanante@farmacia.far.ub.es

EC 1.1.1.49) and 6-phosphogluconate dehydrogenase activity (6PG-DH; *EC* 1.1.1.43), involved in the pentose phosphate pathway; alanine aminotransferase (ALT; *EC* 2.6.1.2) and aspartate aminotransferase (AST; *EC* 2.6.1.1), related to amino acid metabolism.

The aim of the present study was to evaluate in detail the changes promoted in intermediary metabolism in the liver of *Sparus aurata* following feeding or refeeding after starvation with diets containing different levels of protein, carbohydrate and lipids, or with different amounts of a given diet. In addition, as growth was determined at different daily ration sizes, the present work may also contribute to establishing a relationship between biochemical variables and growth rates.

Materials and methods

Gilthead sea bream fish were obtained from Aquadelt S.A. (Deltebre, Tarragona, Spain), and were maintained in 250-litre aquaria supplied with running sea-water at 20° in a closed system with active pump filter and u.v. lamps. Photoperiod was regulated as a dark–light cycle of 12 h.

The effect of diet composition supplied to *Sparus aurata* was studied using the diets shown in Table 1. To study the effects of diet composition on key enzymes involved in glycolysis–gluconeogenesis, the pentose phosphate pathway and amino acid metabolism in the liver, five different diets were supplied to the fish. Proportions of ingredients in the diets were selected to cover a wide range above and below the levels in diets available commercially. Analyses of the diets were performed following standard procedures (Windham, 1997). Three levels of protein were selected (380, 480 and 580 g/kg) and the proportions of gelatinized starch and fish-liver oil were adjusted to achieve two energy levels (about 19 and 20 kJ/g) for each level of protein, except for the 580 g protein/kg level, for which only one level of energy (19 kJ/g) was designed. The diets are ordered by carbohydrate content in all tables. The fish were fed on the different diets at 20 g/kg body weight (BW) for 18 d (fed fish), then starved for 19 d and finally refed for 8 d with the corresponding diet at 20 g/kg BW. Two aquaria were used for each diet treatment, each stocked with twenty-five fish with an average weight of 25 (SD 3·6) g.

In order to study the nutritional regulation due to the quantity of diet supplied to animals, fish were starved for 8 d and then refed for 22 d. Refed fish were divided into four groups, receiving diet 3 (Table 1) once daily at 5, 10, 20 or 35 g/kg BW respectively. Two aquaria were used for each treatment, with thirty-six to thirty-eight fish per aquarium with an average weight of 9·4 (SD 1·6) g.

All fish in each aquarium were weighed at the start and end of a given feeding regimen. They were also weighed at intervals of 7–8 d, to readjust ration size, when the total feeding period was longer than 8 d.

To obtain tissue samples, fish were anaesthetized with 3-aminobenzoic acid ethyl ester (1 : 12 500) and were then killed by cervical section. The liver was dissected out, immediately frozen in liquid N_2 and kept at −80° until use. Six to twelve fish were killed per treatment and sampling period.

The LSI was calculated as liver weight/BW × 100, where BW represents fresh BW of fish.

Liver glycogen was determined spectrophotometrically at 620 nm using the anthrone reaction method according to García de Frutos *et al.* (1990). The glycogen content is expressed in mg glucose equivalent per 100 mg fresh liver tissue.

Crude extracts for assaying enzyme activities were

Table 1. Composition (g/kg diet) of the different types of diet provided to *Sparus aurata**

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Formulation					
Brown fish meal†	91·40	81·74	81·74	64·35	64·35
Fish oil‡	2·50	6·58	2·33	9·06	4·82
Starch§	3·60	9·18	13·43	24·09	28·33
Vitamin mixture‖	0·47	0·47	0·47	0·47	0·47
Carrageenan	2·00	2·00	2·00	2·00	2·00
BHT	0·03	0·03	0·03	0·03	0·03
Chemical analysis¶					
Moisture	2·6	3·0	2·8	3·1	3·4
Protein	58·5	47·7	47·6	37·9	37·4
Fat	8·7	16·2	12·5	16·9	11·6
Carbohydrate	9·9	13·1	17·1	26·0	31·6
Ash	20·3	20·0	20·0	16·1	16·0
Gross energy (kJ/g)**	19·1	20·0	19·2	20·1	18·9

BHT, butylated hydroxytoluene.

* Diets used in this study were designed by us and manufactured by Dalgety España S.A. (Castellbisbal, Barcelona, Spain).

† Whole fish and trash fish meal from Induxtra (Banyoles, Girona, Spain).

‡ Fish oil with added all-*trans* retinol (300 μg/g) and cholecalciferol (2·5 μg/g) from Bonafont Química (Barcelona, Spain).

§ Gelatinized maize starch from Campo Ebro (Zaragoza, Spain).

‖ Vitamin mixture provided (/kg diet): all-*trans* retinol 11·28 mg, cholecalciferol 117·5 μg, choline chloride 1·88 g, ascorbic acid 1·18 g, nicotinic acid 160 mg, all-*rac*-tocopherol acetate 118 mg, thiamin-HCl 118 mg, calcium pantothenate 94 mg, riboflavin 71 mg, pyridoxine-HCl 24 mg, menadione-$HNaSO_3$ 19 mg, pteroylmonoglutamic acid 14 mg, cyanocobalamin 0·37 mg.

¶ Analysis of the diets followed standard procedures (Windham, 1997); carbohydrate was calculated by difference.

** Calculated from gross composition (protein 24 kJ/g, lipid 39 kJ/g, carbohydrate 17 kJ/g).

obtained by homogenization of the powdered frozen liver (1 : 5, w/v) in 50 mM-Tris-HCl pH 7·5, 4 mM-EDTA, 50 mM-NaF, 0·5 mM-phenylmethylsulfonyl fluoride, 1 mM-1,4-dithiothreitol and 250 mM-sucrose using a PTA-7 Polytron (Kinematica GmbH, Littau-Luzern, Switzerland) (position 3, 30 s), and centrifugation at 20 000 ***g*** for 30 min at 4°.

Assays for PFK-1, PK, FBPase-1, G6P-DH and 6PG-DH activities and total protein were adapted for automated measurement using a Cobas Mira S spectrophotometric analyser (Hoffman-La Roche, Basel, Switzerland), based on previously described methods (Bonamusa *et al*. 1992). ALT and AST were assayed with kits from Roche for routine determinations in Cobas Mira S. All enzyme assays were carried out at 30° and followed at 340 nm.

PFK-1 activity was assayed in a final volume of 200 μl containing: 100 mM-Tris-HCl pH 8·25, 5 mM-$MgCl_2$, 50 mM-KCl, 0·15 mM-NADH, 4 mM-ammonium sulfate, 12 mM-2-mercaptoethanol, 10 mM-fructose 6-phosphate, 30 mM-glucose 6-phosphate, 0·675 U/ml fructose bisphosphate aldolase (*EC* 4.1.2.13), 5 U/ml triose-phosphate isomerase (*EC* 5.3.1.1), 2 U/ml glycerol-3-phosphate dehydrogenase (*EC* 1.1.99.5) and 4 μl crude extract. The PFK-1 reaction was measured after addition of 1 mM-ATP.

The specific assay conditions for PK activity were: 250 μl of final volume containing 70 mM-glycylglycine pH 7·4, 10 mM-$MgCl_2$, 100 mM-KCl, 0·15 mM-NADH, 2·8 mM-phosphoenolpyruvate, 21 U/ml lactate dehydrogenase (*EC* 1.1.1.27) and 2·5 μl sample. ADP (2·5 mmol/l) was added to monitor the PK reaction.

FBPase-1 activity was assessed in a final volume of 200 μl with 85 mM-imidazole-HCl pH 7·7, 5 mM-$MgCl_2$, 0·5 mM-NADP, 12 mM-2-mercaptoethanol, 0·05 mM-fructose-1,6-bisphosphate, 2·5 U/ml phosphate-glucose isomerase (*EC* 5.3.1.9), 0·48 U/ml G6P-DH and 4 μl crude extract.

G6P-DH activity was measured using 4 μl sample in a final volume of 200 μl containing 78 mM-imidazole-HCl pH 7·7, 5 mM-$MgCl_2$, 1 mM-NADP and 1 mM-glucose 6-phosphate.

The assay conditions for 6PG-DH activity were: 83 mM-imidazole-HCl pH 7·7, 3 mM-$MgCl_2$, 0·5 mM-NADP, 2 mM-6-phosphogluconate and 4 μl crude extract in a final volume of 200 μl.

The total protein content was determined in Cobas Mira S at 600 nm by the Bradford (1976) method at 30° in liver crude extracts using bovine serum albumin as a standard.

Specific growth rates (SGR) were calculated for each aquarium (fish were not marked individually, to avoid possible stress and/or infections), according to the expression: SGR (%/d) = (lnBWf − lnBWi) × 100/number of days, where BWi and BWf were the mean initial and final fresh weight of fish.

Data were analysed by one-factor ANOVA using a computer program (SuperANOVA, Abacus Concepts, Inc., Berkeley, CA, USA). Differences were determined according to Duncan's multiple range test, with significance levels at $P < 0{\cdot}05$ and $P < 0{\cdot}01$. Figures and correlations were constructed with the help of the KaleidaGraph program (KaleidaGraph, Synergy Software, PCS Inc., Reading, PA, USA).

Results

Effect of diet composition

The LSI was dependent on the type of diet supplied (Fig. 1(a)). After the initial 18 d feeding period, fish fed on diet 1, which had the highest protein content and the lowest carbohydrate level, showed the lowest LSI values, whereas fish fed on diets 3 and 5, with the same energy content as diet 1 but with a higher carbohydrate : protein ratio showed the highest LSI values. Diets 2 and 4 with higher energy (and lipid) contents than the other diets, presented intermediate values, even though diet 4 contained more carbohydrate than diet 3. Comparison of the groups fed on diets with similar protein levels but different energy contents indicated that higher LSI values were observed in fish fed on the lower energy diet (with high carbohydrate : lipid ratio). On the other hand, a 19 d period of starvation resulted in a decrease in the LSI in all groups of fish studied. In starved fish, the higher decrease appeared in fish previously fed on diets containing the highest carbohydrate : lipid ratio at a similar protein level (diets 3 and 5 compared with diets 2 and 4 respectively). After 8 d refeeding, higher LSI values were found again in the groups of fish refed on diets 3 and 5, although complete recovery or even higher values than those observed in fed fish were achieved only by the fish fed on diets 1 and 4 (Fig. 1(a)).

Fed fish showed a dependence of the hepatic glycogen levels on the type of diet supplied (Table 2). Carbohydrate content of the diets seemed to be the main conditioning factor. Those animals fed on high-carbohydrate, low-protein diets (diets 4 and 5) showed the highest levels of glycogen, whereas diets 1 and 2, with the lowest carbohydrate : protein ratios, led to the lowest glycogen levels. Moreover, fish fed on diet 2 (130 g carbohydrates/kg), showed a lower level of hepatic glycogen than diet 3 even though energy (or lipid) content of diet 2 was higher than diet 3 and both contained the same amount of protein. In all groups of fish studied, starvation for 19 d was also accompanied by a highly significant decrease in liver glycogen. Refeeding restored the values to those observed in fed fish (control), however the time-course of recovery depended on the composition of the diet. One day of refeeding was sufficient for fish to recover the levels of glycogen when refed on low-carbohydrate diets (diets 1 and 2). Those refed on high-carbohydrate, low-protein diets (diets 4 and 5) did not reach control values until day 3 (Table 2).

The liver PFK-1, PK, G6P-DH and 6PG-DH activities showed a dependence on the levels of carbohydrate and protein in the diets. In the liver of fish fed on diet 5 (316 g carbohydrate and 374 g protein/kg) all values were between 1·6- and 2-fold higher than those observed in fish fed on diet 1 (115 g carbohydrate and 583 g protein/kg) (Table 3).

After 19 d starvation, glycolytic (PFK-1, PK) and pentose phosphate pathway (G6P-DH, 6PG-DH) enzyme activities had decreased significantly. PFK-1 enzyme activity showed an almost full recovery after 1 d of refeeding. However, only a slight recovery was observed in PK activity values.

G6P-DH and 6PG-DH activities decreased in fish starved for 19 d and, after 8 d of refeeding, higher activities were found in fish fed on high-carbohydrate, low-lipid diets (diets 3 and 5) than in those fed on diets of high lipid content (diets 2 and 4).

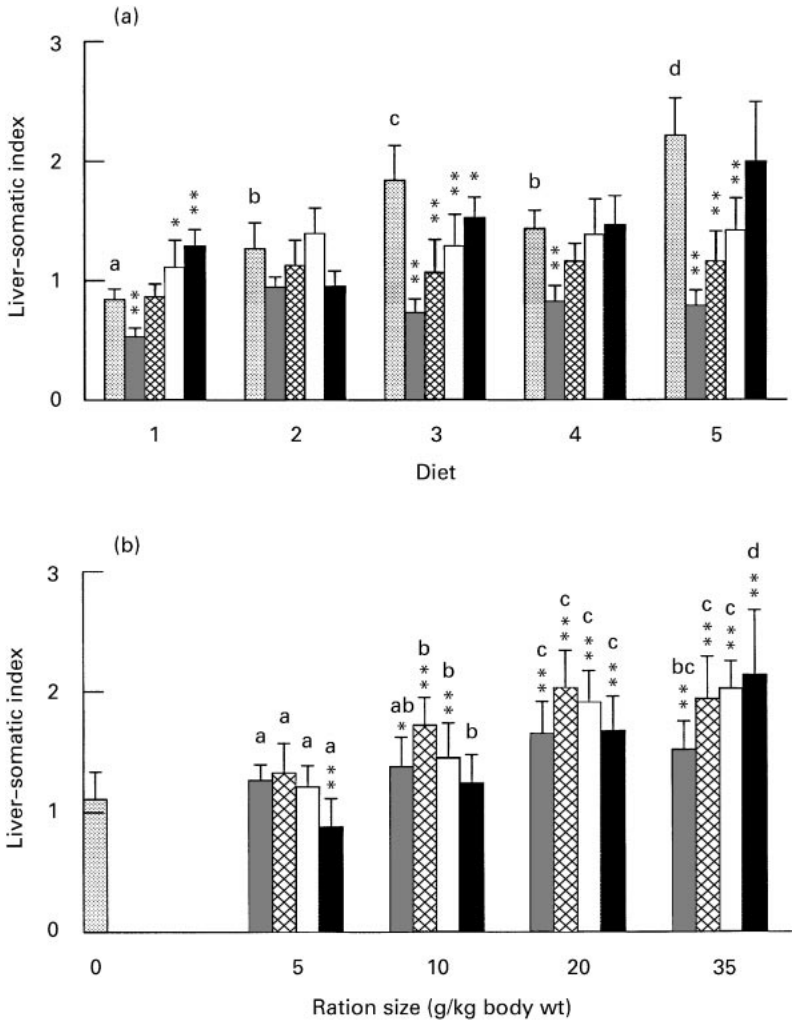

Fig. 1. (a) Liver–somatic index values (liver weight/body weight×100) in gilthead sea bream fed, starved and subsequently refed on diets of different composition (diets 1–5, Table 1). (▨), Control; (■), starved 19 d; (▩), refed 1 d; (□), refed 3 d; (■), refed 8 d. Values are means for at least four fish, with their standard errors indicated by vertical bars. Statistical significance was determined by Duncan's multiple range test. [a,b,c,d] Mean values for control groups with unlike letters were significantly different, $P<0{\cdot}05$. Mean values were significantly different from those for the corresponding control: *$P<0{\cdot}05$, **$P<0{\cdot}01$. (b) Liver–somatic index values in gilthead sea bream refed on diet 3 (Table 1) at 5, 10, 20 or 35 g/kg body weight after an 8 d period of starvation. (▨), Starved 8 d; (■), refed 1 d; (▩), refed 3 d; (□), refed 8 d; (■), refed 22 d. Values are means for at least four fish, with their standard errors indicated by vertical bars. [a,b,c,d] Mean values not sharing a common letter were significantly different, $P<0{\cdot}05$ (Duncan's multiple range test). Mean values were signifcantly different from those for starved fish: *$P<0{\cdot}05$, **$P<0{\cdot}01$.

Table 2. Liver glycogen levels (mg/g fresh liver) in gilthead sea bream fed, starved and subsequently refed on diets of different composition†
(Mean values and standard deviations for three fish)

	Fed		Starved 19 d		Refed 1 d		Refed 3 d		Refed 8 d	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Diet 1	62·6[ab]	13	2·7[b]**	1·0	74·4[b]	28 (*n* 5)	103·2[b]*	20 (*n* 4)	102·1[b]	3
Diet 2	37·6[a]	9	0·7[a]**	0·2	28·6[a]	15 (*n* 4)	56·8[a]*	4	45·1[a]	7
Diet 3	84·3[bc]	7	0·7[a]**	0·2	38·3[a]**	2	96·9[b]	14	138·2[c]**	4
Diet 4	140·2[d]	36	1·1[a]**	0·2	20·9[a]**	6	126·0[b]	19	180·3[d]*	9
Diet 5	117·2[cd]	21	1·0[a]**	0·1	35·7[a]**	23	109·0[b]	24	146·6[c]	11

[a,b,c,d] Mean values within a column not sharing a common superscript letter were significantly different, $P<0{\cdot}05$.
Mean values were significantly different from those for the corresponding fed fish: *$P<0{\cdot}05$, **$P<0{\cdot}01$.
† For details of diets and procedures, see Table 1 and pp. 224–225.

Table 3. Activities (mU/mg protein) of 6-phosphofructo 1-kinase (PFK-1), pyruvate kinase (PK), fructose-1,6-bisphosphatase (FBPase-1), glucose-6-phosphate dehydrogenase (G6P-DH), 6-phosphogluconate dehydrogenase (6PG-DH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the liver of gilthead sea bream fed, starved and subsequently refed on diets of different composition†

(Mean values and standard deviations for three fish)

		Condition									
		Fed		Starved 19 d		Refed 1 d		Refed 3 d		Refed 8 d	
Enzyme	Diet	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PFK-1	1	30·7^{a}	2·4	8·8**	3·8	23·0^{a}*	1·3	34·3^{ab}	4·5	30·7^{a}	2·2
	2	36·0^{ab}	4·4	8·3**	6·0	32·3^{ab}	2·7	32·6^{ab}	4·6	33·3^{a}	10
	3	33·0^{a}	3·6	8·7**	2·3	30·6^{ab}	4·0	31·4^{a}	2·7	41·5^{ab}*	5·6
	4	42·5^{ab}	3·4	9·3**	0·7	30·8^{ab}	8·6	38·8^{b}	2·6	53·6^{bc}	9·9
	5	49·2^{b}	15·2	6·1**	1·0	37·0^{b}	6·3	39·1^{b}	2·9	65·5^{c}*	17
PK	1	300^{a}	33	197^{ab}**	6	205^{a}**	38	268^{a}	36	273^{a}	34
	2	382^{a}	51	146^{a}**	21	297^{b}*	29	283^{a}*	25	338^{ab}	45
	3	513^{b}	91	231^{ab}**	101	327^{b}*	66	329^{ab}*	33	392^{bc}	31
	4	784^{c}	40	305^{b}**	43	426^{c}**	34	427^{c}**	33	486^{cd}**	36
	5	567^{b}	85	242^{ab}**	68	360^{bc}**	37	384^{bc}*	31	524^{d}	84
FBPase-1	1	123	10	137^{a}	34 (*n* 5)	156^{a}	2·3	194^{ab}**	35	178*	8·9
	2	119	13	219^{c}**	12	151^{a}	16	157^{ab}*	26	180**	16
	3	144	11	193^{bc}*	6	211^{b}*	42	145^{a}	21	194	41 (*n* 4)
	4	140	29	171^{ab}	34 (*n* 5)	168^{a}	21	207^{b}*	36	207*	9·1
	5	142	29	185^{bc}	23	164^{a}	13	156^{ab}	6·1	190	83
G6P-DH	1	138^{a}	50	84*	10	86^{a}*	13	145	32	187^{b}*	24
	2	132^{a}	34	113	17	117^{ab}	9·9	134	6·1	124^{a}	41
	3	201^{bc}	8·0	114**	31	118^{ab}**	6·9	120**	12	209^{bc}	33
	4	163^{ab}	3·0	116**	13	117^{ab}*	9·9	118*	21	153^{ab}	25
	5	246^{c}	17	105**	8	126^{b}**	19	122**	6·3	222^{c}	64
6PG-DH	1	24·4^{a}	4·0	13·8^{a}	2·3	19·3^{a}	3·2	28·6	10	29·9^{a}	0·6
	2	34·8^{ab}	12	28·2^{b}	1·2	23·3^{ab}	2·5	26·9	0·8	27·6^{a}	8·0
	3	44·5^{b}	11	25·1^{b}**	4·0	22·5^{ab}**	2·3	24·0**	4·4	37·6^{a}	2·8
	4	40·3^{ab}	6·5	29·6^{b}*	3·3	24·6^{ab}**	2·3	30·3*	3·6	34·4^{a}	5·1
	5	44·6^{b}	1·4	28·5^{b}**	4·9	26·1^{b}**	4·9	28·1**	1·7	56·3^{b}	2·3
ALT	1	1085^{b}	179	575^{ab}**	19	847^{b}	49	1058^{c}	132	1408^{c}*	186
	2	1040^{b}	109	567^{ab}**	8	748^{b}**	73	795^{b}*	112	799^{a}*	74
	3	1154^{b}	81	858^{b}*	274	867^{b}	184	796^{b}	35	1109^{b}	204
	4	671^{a}	77	467^{a}**	29	521^{a}*	79	471^{a}**	78	526^{a}*	36
	5	695^{c}	198	526^{ab}	16	490^{a}	92	490^{a}	63	538^{a}	50
AST	1	1745^{b}	59	1838	29	1457	142	1967^{c}	138	1786^{b}	279
	2	1489^{a}	19	1804**	140	1608	68	1498^{b}	58	1655^{ab}*	26
	3	1693^{b}	139	1972	405	1631	116	1597^{b}	130	1780^{b}	204
	4	1387^{a}	156	2014**	338	1512	106	1120^{a}	21	1312^{a}	30
	5	1567^{ab}	20	1833	245	1341	284	1076^{a}**	20	1544^{ab}	96

^{a,b,c} Mean values within a column not sharing a common superscript letter were significantly different, $P < 0{\cdot}05$.
Mean values were significantly different from those for the corresponding fed fish: *$P < 0{\cdot}05$, **$P < 0{\cdot}01$.
† For details of diets and procedures, see Table 1 and pp. 224–225.

Starvation resulted in a significant increase in FBPase-1 activity in the fish previously fed on diets 2 and 3, and a tendency to increase was observed in the other groups studied. After 8 d of refeeding the values remained elevated, regardless of the diet.

Moreover, as the carbohydrate content of the diets increased, higher PFK-1 : FBPase-1 and PK : FBPase-1 ratios were observed. For example, at the end of the refeeding period PFK-1 : FBPase-1 values rose from 0·17 for diet 1 to 0·26 and 0·34 for diets 4 and 5 respectively. After 8 d of refeeding the PK : FBPase-1 ratio rose from 1·53 for diet 1 to 2·35 and 2·76 for diets 4 and 5 respectively (Table 3).

In fish fed on high-protein diets (diets 1–3) for 18 d, ALT values were significantly higher than in those fish fed on low-protein diets (4 and 5) (Table 3). Starvation for 19 d caused a decrease in the enzyme activity in all groups of fish studied, particularly in those previously fed on high-protein, low-carbohydrate diets. Refeeding fish for 8 d on high-protein, low-carbohydrate diets promoted high ALT levels except for diet 2. However, refeeding on low-protein, high-carbohydrate diets (diets 4 and 5) produced levels of enzyme activity slightly below those previously found in the liver of fed fish.

In contrast, a slight increase in AST enzyme activity was observed in all groups of starved fish, with differences being significant for diets 2 and 4 (Table 3). The highest AST enzyme activity was found in fish fed on the highest protein, low-carbohydrate diet (diet 1), as for ALT. In addition, the highest AST values were observed when fish were fed on the diets with the lower energy content (1, 3 and 5).

Effect of ration size

Four groups of fish were refed on different quantities of diet after a starvation period of 8 d. Diet 3, with a composition close to that of a standard commercial diet, was provided at

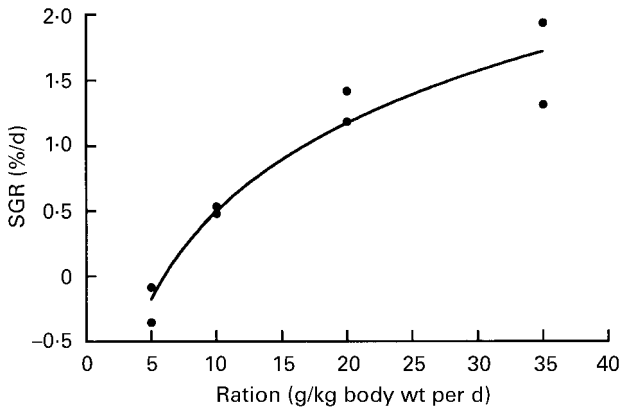

Fig. 2. Specific growth rate (SGR) of gilthead sea bream as a function of ration size. Each point represents the mean growth rate (as % SGR per d) of the fish per aquarium. Two aquaria were used for each treatment (thirty-six to thirty-eight fish per aquarium). For procedures to calculate SGR, see p. 225. The solid line indicates the fitted logarithmic relationship, constructed by regression analysis ($y = 0{\cdot}50 + 2{\cdot}25 \log x$; r 0·96).

5, 10, 20 and 35 g/kg BW per d, respectively. After 22 d of refeeding the BW and the LSI correlated well with the quantity of diet provided. Moreover, fish refed on the 5 g/kg BW ration size showed negative growth and their LSI values were significantly lower than in any other group (Fig. 1(b)). The specific growth rate of the fish was also calculated, and Fig. 2 shows that a good correlation between specific growth rate and ration size could be established. Meanwhile at 20 and 35 g/kg BW significant increases in BW were found; fish fed at 5 g/kg BW lost weight compared with starved animals.

After 1 d of refeeding, liver glycogen was significantly higher in fish fed at 20 and 35 g/kg BW per d than in fish starved or fed on 5 and 10 g/kg BW rations. As might be expected, after 22 d of refeeding, the lowest values were found in fish refed at 5 g/kg BW, and those refed at 20 g/kg BW showed the highest values, although these were not significantly different from those obtained with 10 and 35 g/kg (Table 4).

PFK-1, PK, G6P-DH, 6PG-DH and ALT enzyme activities were all positively correlated with ration size after 22 d of refeeding whereas a negative correlation was found for FBPase-1. Fig. 3 shows regression analysis corresponding to PFK-1, PK, FBPase-1 and G6P-DH activities. PFK-1 and PK activity values showed a certain tendency to plateau at a ration size of 20 g/kg BW, as for 6PG-DH and ALT activities (results not shown). PFK-1 and ALT activities, in fish refed for 22 d at 5 g/kg BW, were not significantly higher than those observed in the starved fish, while the PK, G6P-DH, 6PG-DH and AST values were even lower than those in the starved fish. FBPase-1 activity was similar to that seen in starved fish (Table 5).

At 8 and 22 d of refeeding, a clear relationship was found between PFK-1 : FBPase-1 and PK : FBPase-1 ratios and the quantity of diet supplied. Thus, at 22 d PFK-1 : FBPase-1 was 0·12 for a ration size of 5 g/kg BW and 0·34 for 35 g/kg BW, and similarly the PK : FBPase-1 value changed from 0·98 to 3·3 (Table 5). In addition, in fish fed at 5 g/kg BW both values were similar to those of starved fish.

AST activity was very variable and it was difficult to compare between starved and refed fish or between fish fed at different ration sizes. However, it is clear that there was a strong decrease in enzyme activity after only 1 d of refeeding (significant in the case of 5 and 35 g/kg BW ration sizes), with lower values being maintained afterwards with minor oscillations. Even though AST was lower in fish refed for 22 d compared with starved fish, no significant differences were found in relation to the ration size (Table 5).

Discussion

Nutritional regulation of key enzyme activities of glycolysis–gluconeogenesis, the pentose phosphate pathway and amino acid metabolism by diet composition

In the present study the activities of PFK-1 and PK, both key enzymes in the regulation of the glycolysis, decreased after prolonged starvation, while FBPase-1 activity seemed to increase. We have previously observed that starvation promotes a decrease in the hepatic content of fructose 2,6-bisphosphate in *S. aurata* (García de Frutos *et al*. 1990, 1991; Metón *et al*. 1995, 1999; Metón, 1996). The fall in the frutose 2,6-bisphosphate levels halts stimulation of PFK-1 and allows the increase in FBPase-1 (Bonamusa *et al*. 1992). This process shows analogies with that found in mammals (Hue & Rider, 1987; Baanante *et al*. 1991; Pilkis & Claus, 1991).

The present results indicate that after refeeding the starved fish with different types of diet, levels of glycogen in the liver recovered in a gradual manner within 3–8 d, with the highest values found in fish fed on high-carbohydrate, low-protein diets. On the basis of enzyme activity measurements, gluconeogenesis seems to predominate over glycolysis in fish refed for 1–3 d (Table 3), suggesting that the initial recovery of glycogen in the liver of refed *S. aurata* may also derive from three-C compounds (Baanante *et al*. 1991). In favour of this possibility, French *et al*. (1981) and Cowey & Walton (1989), indicated that the low carbohydrate and high protein content of the natural fish diet makes the synthesis of glucose and glycogen from non-carbohydrate compounds, such as lactate, amino acids and glycerol, more feasible.

In the present study, the highest hepatic PFK-1 and PK activities were found in *S. aurata* fed with high-carbohydrate, low-protein diets (diets 4 and 5). Moreover, after 8 d of refeeding the PFK-1 : FBPase-1 ratio showed differences between diets, with values ranging from 0·17 for fish fed on diet 1 to 0·34 for those fed on diet 5. The PK : FBPase-1 values also ranged from 1·53 for diet 1 to 2·75 for diet 5 (Table 3). The increased ratio values of glycolytic enzymes (PFK-1 and PK) over FBPase-1 gluconeogenic enzyme activity would suggest an increase in glycolysis and thus metabolic adaptation to high-carbohydrate, low-protein diets. In other fish, such as the European eel (*Anguilla anguilla*), the administration of high-carbohydrate diets also increases the liver PK activity (Suárez *et al*. 1995). This process is related to the increase in the production of pyruvate, which together with NADP, is a precursor for biosynthesis of fatty acids. The same is found in the rat, in which, in addition to increased PFK-1 and PK liver activities, the respective mRNA increase following feeding on high-carbohydrate diets (Noguchi *et al*. 1985; Gehnrich *et al*. 1988). In contrast, no nutritional regulation of PK

Table 4. Liver glycogen levels (mg/g fresh liver) in gilthead sea bream refed on diet 3 (see Table 1) at 5, 10, 20 and 35 g/kg body weight for 1, 3, 8 or 22 d after an 8 d period of starvation†

(Mean values and standard deviations for four fish)

Starved 8 d		Ration size	Refed 1 d		Refed 3 d		Ref 8 d		Refed 22 d	
Mean	SD	(g/kg body wt)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
		5	11[a]	9	49[a]**	18	30[a]*	10	21[a]	18
2	1	10	12[a]	13	53[a]**	22	68[b]**	12	92[b]**	13
		20	55[b]**	15	75[a]**	19	109[c]**	13	128[b]**	28 (*n* 5)
		35	71[b]**	19	69[a]**	27 (*n* 6)	76[b]**	29 (*n* 5)	102[b]**	38 (*n* 6)

[a,b,c] Mean values within a column not sharing a common superscript letter were significantly different, $P < 0{\cdot}05$.
Mean values were significantly different from that for starved fish: *$P < 0{\cdot}05$, **$P < 0{\cdot}01$.
† For details of procedures, see pp. 224–225.

activity has been observed in rainbow trout (*Salmo gairdneri*) (Guderley & Cardenas, 1980; Hilton & Atkinson, 1982) nor in the American eel (*Anguilla rostrata*) (Roberts & Anderson, 1985). High-protein diets promoted a decrease in liver PFK-1 in rainbow trout (Fideu *et al*. 1983), as we observed in *S. aurata*. Besides, Cowey *et al.* (1977*a*,*b*) reported a stimulation of gluconeogenesis through an increase in FBPase-1 enzyme activity in rainbow trout fed on high-protein diets. In the European eel, high-carbohydrate diets promoted a decrease in the rate of gluconeogenesis (Suárez *et al.* 1995).

In fish, knowledge of the regulation of the pentose phosphate pathway is limited. Nevertheless it is clear that this pathway plays a key role in glucose utilization (Christiansen & Klungsøyr, 1987; Cowey & Walton, 1989). Lipids appear to be the main energy source in teleost fish (Cornell *et al*. 1986). The activities of enzymes that control the pentose phosphate pathway, G6P-DH and 6PG-DH, decreased in starved *S. aurata* (Table 3), as observed in *Salvelinus fontinalus* (Yamauchi *et al.* 1975) and rainbow trout (Barroso *et al*. 1993). Moreover, our results indicate that refeeding promoted a gradual recovery of both G6P-DH and 6PG-DH activities, depending on the carbohydrate content of the diet. This indicates that glucose may be

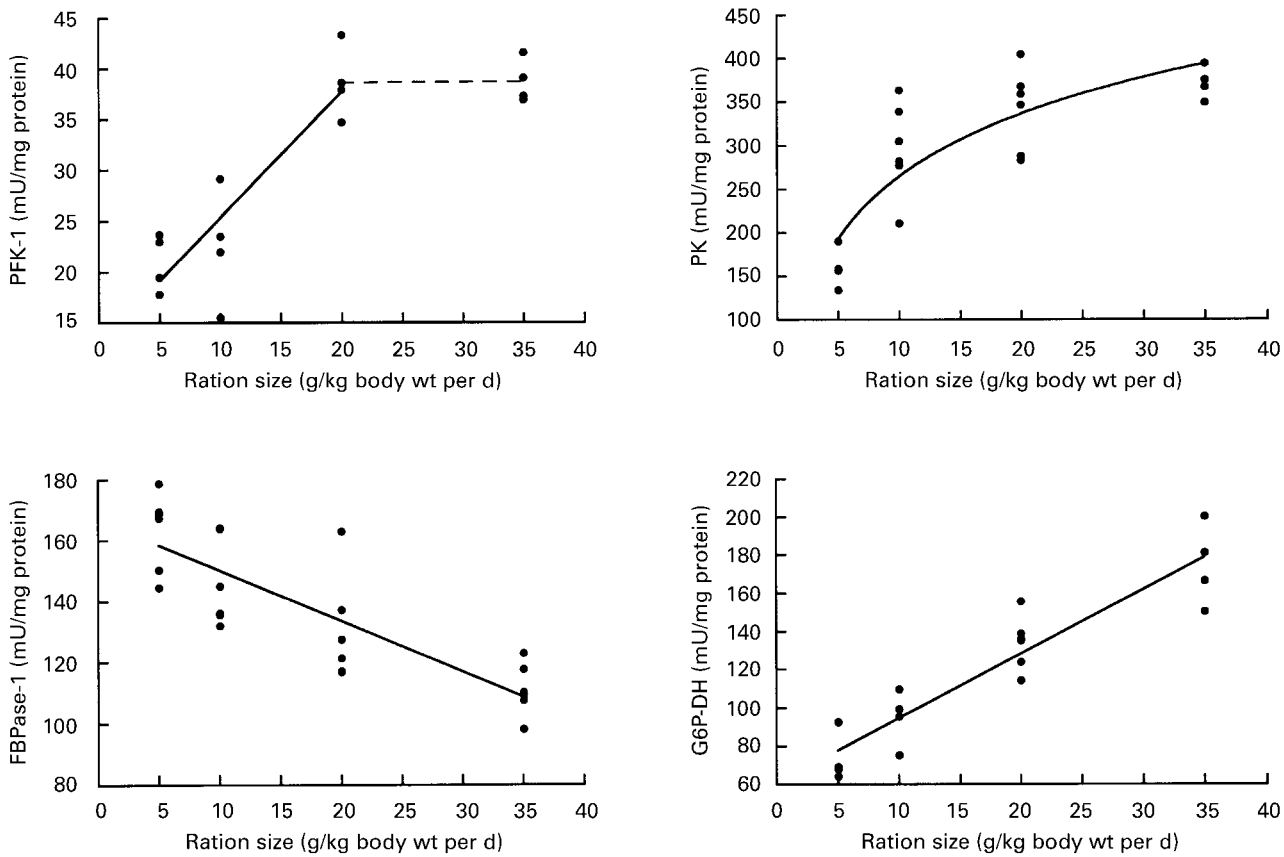

Fig. 3. Relationship between hepatic enzyme activity and ration size in gilthead sea bream. Each point represents the liver enzyme activity of an individual fish after refeeding for 22 d at 5, 10, 20 or 35 g/kg body weight per d with diet 3. PFK-1, 6-phosphofructo 1-kinase; PK, pyruvate kinase; FBPase-1, fructose-1,6-bisphophatase; G6P-DH, glucose-6-phosphate dehydrogenase. For details of procedures, see pp. 224–225. Linear and logarithmic relationships were tested using regression analysis. PFK-1, (—) $y = 12{\cdot}963 + 12{\cdot}368x$, R 0·88; (---) $y = 38{\cdot}492 + 0{\cdot}066x$, R 0·02; PK (—) $y = 264{\cdot}78 + 237{\cdot}4 \log(x)$, R 0·838; FBPase-1, (—) $y = 166{\cdot}58 + -16{\cdot}502x$, R 0·821; G6P-DH, (—) $y = 61{\cdot}195 + 33{\cdot}609x$, R 0·928.

used to provide NADPH for lipogenesis in *S. aurata*, as reported for other fish species (Likimani & Wilson, 1982; Shimeno *et al*. 1993; Suárez *et al*. 1995). Furthermore, at equivalent protein levels supplied, partial replacement of lipid by carbohydrate in the diet (diet 2 compared with diet 3, and diet 4 compared with diet 5) resulted in increased G6P-DH and 6PG-DH activity values. Likewise, activity of the rat hepatic G6P-DH, mRNA levels and gene transcription are promoted by high-protein, low-lipid diets and also by high-carbohydrate diets. In addition, both G6P-DH enzyme activity and mRNA levels decrease following starvation, and recover their normal levels after refeeding (Iritani, 1992; Towle *et al*. 1997; Stabile *et al*. 1998). As in *S. aurata*, the channel catfish (*Ictalurus punctatus*) fed on high-carbohydrate diets, showed increased liver G6P-DH and 6PG-DH enzyme activities (Likimani & Wilson, 1982). In contrast both activities were found to be decreased in coho salmon (*Oncorhynchus kisutch*) (Lin *et al*. 1977).

It appears that carnivorous fish make more efficient use of protein than carbohydrates. However, the fact that *S. aurata* fed on high-carbohydrate, low-protein diets showed stimulation of key liver enzymes for glycolysis and the pentose phosphate pathway suggests its ability to utilize high-carbohydrate diets and thus spare protein.

ALT and AST enzyme activities are quantitatively the most important aminotransferases in the teleostean fish liver (Cowey & Walton, 1989). We found that ALT activity decreased following starvation, whereas AST activity showed a slight tendency to increase. The effect of starvation on ALT and AST activities in other fish species is variable, as either increases or no changes after starvation have been reported (Moon & Johnston, 1981; Cowey & Walton, 1989; Kim *et al*. 1992; Fynn-Aikins *et al.* 1995). The increase in AST after starvation appears to indicate its more prominent role in protein mobilization compared with ALT in *S. aurata*. The increase observed in liver ALT when the fish were fed on high-protein diets may denote an efficient use of the dietary amino acids either for growth or as a substrate for gluconeogenesis. As for ALT, the highest AST enzyme activity was found in fish fed on the highest protein, low-carbohydrate diet (diet 1) or in fish fed on diets of the lowest energy content (1, 3 and 5). In Atlantic salmon (*Salmo salar*), Fynn-Aikins *et al.* (1995) found that AST was not dependent on the type of energy source supplied to fish, while high-protein diets

Table 5. Activities (mU/mg protein) of 6-phosphofructo 1-kinase (PFK-1), pyruvate kinase (PK), fructose-1,6-bisphosphatase (FBPase-1), glucose-6-phosphate dehydrogenase (G6P-DH), 6-phosphogluconate dehydrogenase (6PG-DH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the liver of gilthead sea bream refed on diet 3 (see Table 1) at 5, 10, 20 and 35 g/kg body weight for 1, 3, 8 or 22 d after an 8 d period of starvation†

(Mean values and standard deviations for three fish)

	Starved 8 d		Ration size (g/kg body wt)	Refed 1 d		Refed 3 d		Refed 8 d		Refed 22 d	
Enzyme	Mean	SD		Mean	SD	Mean	SD	Mean	SD	Mean	SD
PFK-1	15·0	5·5	5	16·4^{a}	5·6 (*n* 6)	20·7	7·5 (*n* 5)	13·0^{a}	4·4	21·0^{a}	2·8
			10	16·3^{a}	7·4 (*n* 6)	28·4*	0·3	25·6^{b}*	9·3 (*n* 5)	22·5^{a}*	5·6
			20	32·4^{b}**	1·4	28·7*	2·1	40·7^{c}**	9·5 (*n* 6)	38·6^{b}**	3·5
			35	25·5^{b}**	4·6	30·2**	8·7	32·3^{bc}**	3·3	38·7^{b}**	2·2
PK	231	29	5	216	25	176*	30	181^{a}*	15	160^{a}*	23
			10	191	16	221	32	226^{a}	33	297^{b}**	53
			20	195	38	194	21	291^{b}*	61 (*n* 6)	341^{bc}**	36
			35	227	9	228	36	292^{b}*	39 (*n* 6)	371^{c}**	19
FBPase-1	162	19	5	153^{ab}	12	100^{a}**	14	130^{b}*	6	162^{a}	13 (*n* 6)
			10	156^{ab}	17	106^{a}**	14 (*n* 6)	95^{a}**	10	146^{ab}	15 (*n* 6)
			20	160^{b}	17	156^{b}	19	97^{a}*	25 (*n* 7)	131^{b}**	18 (*n* 6)
			35	130^{a}*	18	149^{b}	11	121^{ab}**	24 (*n* 6)	111^{c}**	9 (*n* 6)
G6P-DH	133	34	5	120	15	105	22	78^{a}*	13	73^{a}**	13
			10	111	14	107	15	105^{ab}	33	95^{a}*	14
			20	134	13	107	14	126^{b}	30 (*n* 5)	134^{b}	55 (*n* 6)
			35	115	15	99	26	175^{c}*	20	175^{c}*	21
6PG-DH	35	2	5	25^{a}**	0·2	29	6	29	5	24^{a}*	5
			10	26^{a}**	4	29	5	28	6	28^{ab}	4
			20	34^{b}	2	29	6	41	13	38^{b}	10 (*n* 7)
			35	32^{b}	3	46	3 (*n* 6)	36	8	38^{b}	9 (*n* 6)
ALT	342	106	5	570^{a}**	115	472	55	427^{a}	65	233^{a}	43
			10	570^{b}**	92	526*	78	580^{bc}**	97	629^{b}*	81
			20	427^{a}	52	511*	87	525^{ab}*	14	1071^{c}**	236
			35	436^{ab}	28	452	133	671^{c}**	43	831^{bc}**	132
AST	2218	344	5	1515^{ab}*	459	2074^{b}	98	1539^{a}**	59	1438**	304
			10	2071^{b}	505	1752^{ab}*	206	2049^{b}	238	1438*	102
			20	1663^{ab}	223	1615^{a}**	48	1690^{a}**	216	1891	272
			35	1433^{a}*	170	1846^{ab}	358	1862^{ab}	169	1502**	195

^{a,b,c} Mean values within a column not sharing a common superscript letter were significantly different, $P < 0{\cdot}05$.
Mean values were significantly different from those for starved fish: *$P < 0{\cdot}05$, **$P < 0{\cdot}01$.
† For details of procedures, see pp. 224–225.

promoted stimulation of the liver ALT. Lupiáñez *et al.* (1989) also observed an increase in ALT activity in rainbow trout fed on high-protein diets. Both aminotransferases increase with the increase in the protein content of the diet in *Mugil capito* (Alexis & Papaparaskeva-Papoutsoglou, 1986). However, no variation in aminotransferase activity resulting from high-protein diets has been reported in other fish (Nagai & Ikeda, 1973*a*,*b*; Cowey & Walton, 1989).

Effect of energy restriction on key enzyme activities of glycolysis–gluconeogenesis, the pentose phosphate pathway and amino acid metabolism

Restricted feeding resulted in a decreased LSI. *S. aurata* refed with a ration of 5 g/kg BW showed similar values to those of starved fish, reflecting the low availability of nutrients for growth (Fig. 1).

The hepatic levels of glycogen also depended on ration size. After 22 d of refeeding, the glycogen content in the group fed on the 5 g/kg BW ration was extremely low, although in all cases values were above those found in starved fish. On the other hand, the glycogen values observed in the group fed on the 20 g/kg BW ration were not significantly different from those of the group fed at 35 g/kg BW. This suggests saturation of the stimulating effect over the synthesis of glycogen in the group of fish fed at 20 g/kg BW (Table 4).

We have previously observed that feeding *S. aurata* at 5 g/kg BW promotes low levels of fructose 2,6-bisphosphate in the liver (Metón, 1996). This finding could at least partially explain the decrease in PFK-1 enzyme activity found in this group of fish, which together with the decrease in PK activity and the increase in the FBPase-1 activity, could lead to a rate of glycolysis–gluconeogenesis similar to that for starved fish.

In addition, the activity of the enzymes involved in the control of the pentose phosphate pathway, G6P-DH and 6PG-DH, decreased as a consequence of the energy restriction. After 22 d of refeeding at 5 and 10 g/kg BW, G6P-DH activity was even lower than that in starved *S. aurata*. This may be related to the low availability of glucose from the diet to be derived as a substrate for the pentose phosphate pathway. Consequently, the biosynthetic capacity of fatty acids would also decrease. These findings are consistent with those found in rainbow trout (Bastrop *et al*. 1992), striped bass (*Morone saxatilis*) (Hung *et al*. 1993) and carp (Shimeno *et al*. 1997). Moreover, as food supplied to *S. aurata* increased to 20 or 35 g/kg BW, the activity of these enzymes increased, which is consistent with long-term regulation by either dietary manipulation of food quantity or diet composition. In fact, the best correlation between ration size and enzyme activity was found for G6P-DH (Fig. 3).

After 22 d of refeeding the liver AST activity decreased, regardless of the ration size. In contrast, liver ALT enzyme activity showed a positive correlation with ration size. Refeeding at 5 g/kg BW led to values similar to those obtained in starved *S. aurata*. Other ration sizes, mainly 20 and 35 g/kg BW, led to increases in this enzyme activity. Thus, hepatic ALT appears to be more sensitive than AST to changes in ration size in *S. aurata*. Similarly, glutamate dehydrogenase (*EC* 1.4.1.2), another enzyme involved in amino acid catabolism, decreases under restricted protein and energy diets in the European eel (Suárez *et al*. 1995).

All data presented here indicate a high level of metabolic adaptation to both diet type and ration size. In particular, adaptation of enzyme activities to the consumption of a diet with high carbohydrate levels indicates that a carnivorous fish like *S. aurata* can tolerate partial substitution of protein by carbohydrate in the commercial diets supplied in culture.

The relationship between enzyme activities, ration size and growth suggests that the enzymes studied respond quickly to dietary manipulation and could be used as indicators of nutritional conditions and growth performance of cultured fish.

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