

Metabolic responses to dietary protein/carbohydrate ratios in zebra sea bream (*Diplodus cervinus*, Lowe, 1838) juveniles

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Abstract This study aims to evaluate the effect of diets with different protein to carbohydrate ratios (P:C) on the omnivorous zebra sea bream (*Diplodus cervinus*) juveniles growth performance, feed efficiency, N excretion and metabolic response of intermediary metabolism enzymes. Four isoenergetic and isolipidic diets were formulated to contain increasing protein levels (25, 35, 45 and 55 %) at the expense of carbohydrates (43, 32, 21 and 9 %): diets P25C43, P35C32, P45C21 and P55C9. Growth performance, feed efficiency (FE), N intake [(g kg⁻¹ average body weight (ABW) day⁻¹)], N retention (g kg⁻¹ ABW day⁻¹) and energy retention

(kJ kg⁻¹ ABW day⁻¹) increased with the increase of P:C ratio. The best growth performance and FE were achieved with diet P45C21. Ammonia excretion (mg NH₄-N kg⁻¹ ABW day⁻¹) increased as dietary protein level increased. Alanine aminotransferase and glutamate dehydrogenase activities increased with the increase of dietary P:C ratio. The opposite was observed for malic enzyme activity. Aspartate aminotransferase, hexokinase, glucokinase, fructose-1, 6-bisphosphatase and fatty acid synthetase activities were unaffected by dietary treatments. Response of key amino acid catabolic enzymes and N excretion levels to dietary P:C ratio supports the metabolic adaptability of this species to dietary protein inclusion levels. Overall, zebra sea bream seems capable of better utilize dietary protein rather than dietary carbohydrates as energy source which may be an obstacle for using more economically diets and thus for reducing environmental N loads in semi-intensive aquaculture of this species.

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Introduction

In the Mediterranean region, risk of market saturation of gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) production encouraged the search of alternative species for aquaculture.

Among candidates, breams of *Diplodus* genus are considered as having great potential due to their high market price and demand, easy adaptability to captivity and farming characteristics similar to other sparides (Abellan and Basurco 1999). Regarding feeding habits, *Diplodus* sp. are omnivorous species (Sala and Ballesteros 1997) and therefore are expected to have lower dietary proteins requirements and use dietary carbohydrates as energy source more efficiently than carnivorous species (Stone 2003; Enes et al. 2009; Polakof et al. 2012). Such characteristics may be an added-value when considering dietary costs, one of the main costs in fish production. Also, diets with low protein levels will contribute to decrease N losses to the water with the consequent reduction of the environmental impact associated with feeding.

Sharpsnout sea bream (*D. puntazzo*) appears as one of the most promising bream species as it has growth rates similar to European sea bass (Divanach et al. 1993; Hidalgo and Alliot 1988; Coutinho et al. 2012). Despite its high dietary protein requirement for an omnivorous species (43 %), sharpsnout sea bream uses dietary lipids and carbohydrates efficiently as energy sources (Hernandez et al. 2001). White sea bream (*D. sargus*) juveniles are slower growers, but their lower protein requirement (27 %) and efficient use of dietary starch as energy source are positive aspects when considering its potential for semi-intensive aquaculture (Abellan and Garcia-Alcazar 1995; Sá et al. 2007, 2008a). Two-banded sea bream (*D. vulgaris*) juveniles are also considered slow growers and have higher dietary protein requirement (36 %) than white sea bream (Ozório et al. 2009). Zebra sea bream (*D. cervinus*) potential value for aquaculture is yet poorly studied. To the authors' knowledge, there is only one study conducted with juveniles of this species, which aimed to establish dietary protein requirement (Coutinho et al. 2014). Zebra sea bream dietary protein requirement (44 %) is similar to that estimated for sharpsnout sea bream, but in contrast it seems to be the slowest grower among the *Diplodus* species studied so far (Coutinho et al. 2014). This is somehow unexpected, as it is assumed that in fish, growth performance is positively related to dietary protein requirements (Tacon and Cowey 1985).

It is known that amino acid utilization is affected by many factors, including diet composition, and that metabolic utilization of absorbed glucose as well as

the amount of net energy that can be derived from digestible carbohydrate are limited and species dependent (Kaushik and Seiliez 2010). In this sense, alterations on fish intermediary metabolism induced by an increase in dietary protein at the expense of carbohydrate are expected and deserve to be investigated. However, in *Diplodus* sp., dietary nutrient effects on intermediary metabolism were only studied on white sea bream juveniles (Sá et al. 2006, 2007, 2008a, b; Enes et al. 2015). In agreement with their omnivorous habits, a higher glucose load increased liver glycolytic pathway while depressed gluconeogenic capacity (Enes et al. 2015). In contrast, lipogenesis stimulation by dietary carbohydrate levels was not observed, while the activity of amino acid catabolism-related enzymes was positively correlated with the increase in dietary protein level (Sá et al. 2007, 2008a, b). Daily ammonia excretion levels was also related to dietary protein level, while the absence of variation of urea excretion was assumed to be due to the lack of a functional urea cycle (Sá et al. 2008a).

To elucidate the utilization of dietary carbohydrates as energy source by zebra sea bream juveniles, the present trial was designed to evaluate the utilization of diets with different protein to carbohydrate ratios (P:C). With that purpose, the effect of dietary P:C ratio on N excretion and on the activity of key liver enzymes of glycolytic, gluconeogenic and lipogenic pathways was investigated.

Materials and methods

Diets composition

Four experimental diets were formulated to be isolipidic (18 % crude lipid) and to contain increasing protein levels: 25, 35, 45 and 55 %, at the expense of carbohydrate: 43, 32, 21 and 9 %; diets P25C43, P35C32, P45C21 and P55C9, respectively. Fish meal and cod liver oil were used as the main protein and lipid sources, respectively, and pregelatinized maize starch as carbohydrate source. Dibasic calcium phosphate was added to adjust dietary phosphorus level. All diet ingredients were thoroughly mixed and dry-pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA), through a 2.0-mm die. Pellets were dried in an oven at 50 °C for 24 h and then stored in airtight bags until use. Ingredients

and proximate composition of the experimental diets are presented in Table 1.

Animals and experimental conditions

The experiment was performed at the Marine Zoology Station, Porto University, Portugal, with zebra sea bream (*D. cervinus*) juveniles obtained from IPMA/CRIPSul, Olhão, Portugal. After transportation to the experimental facilities, fish were submitted to a quarantine period of 2 weeks and then acclimated for 1 month to the experimental system. During this time fish were fed a commercial diet (Sorgal S.A., Ovar, Portugal; 44 % crude protein). The trial was

performed in a semi-recirculating water system equipped with eight cylindrical fibreglass tanks of 100 l water capacity and thermo-regulated to 22.0 ± 1.0 °C. Tanks were supplied with continuous flow of filtered seawater ($2.5\text{--}3.5$ l min^{-1}) of 33.0 ± 2.0 g l^{-1} salinity and dissolved oxygen was kept near saturation (7 mg l^{-1}). Thereafter, 20 zebra sea bream with an initial mean body weight of 7.7 g were distributed to each tank and the experimental diets randomly assigned to duplicates groups. The trial lasted 14 weeks and fish were fed by hand, twice daily (9:00 am and 16:00 pm), 6 days a week, until apparent visual satiation. Utmost care was taken to avoid feed losses. The experiment was performed by accredited

Table 1 Ingredients composition and proximate analysis of experimental diets

Diets	P25C43	P35C32	P45C21	P55C9
Ingredients (% dry weight)				
Fish meal ^a	29.9	44.0	58.1	72.1
Soluble fish protein concentrate ^b	5.0	5.0	5.0	5.0
Cod liver oil	14.2	12.8	11.5	10.2
Pregelatinized maize starch ^c	43.0	32.0	21.0	9.2
Choline chloride (50 %)	0.5	0.5	0.5	0.5
Vitamin premix ^d	1.0	1.0	1.0	1.0
Mineral premix ^e	1.0	1.0	1.0	1.0
Binder ^f	1.0	1.0	1.0	1.0
Dibasic calcium phosphate	4.4	2.7	1.0	0.0
Proximate analysis (% dry weight)				
Dry matter	93.6	91.0	94.8	92.1
Crude protein	25.8	35.7	45.3	54.0
Crude lipids	18.3	17.6	17.3	17.3
Starch	43.7	32.8	19.1	8.6
Ash	12.1	13.1	14.1	16.2
Gross energy (kJ g^{-1})	20.2	20.3	20.4	21.1
P/E (g MJ^{-1})	12.8	17.6	22.2	25.7

DM dry matter, CP crude protein, CL crude lipid, P/E protein/energy

^a Pesquera Diamante, Steam Dried LT. Austral Group, S.A. Peru (CP: 71.1 % DM; CL: 9.5 % DM)

^b Sopropèche, France (CP: 74.1 % DM; CL: 20.1 % DM)

^c C-Gel Instant-12016, Cerestar, Mechelen, Belgium

^d Vitamins (mg kg^{-1} diet): retinol acetate, 18,000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamine-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400

^e Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.44 (g kg^{-1} diet)

^f Aquacube (guar gum, polymethyl carbamide, manioc starch blend, hydrate calcium sulphate) Agil, UK

scientists (following FELASA category C recommendations) and was conducted according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

Ammonia excretion

On week 13 of the growth trial, water samples were collected in the outlet of each tank at 0, 2, 4, 6, 8, 10 and 12 h after the morning meal during 2 consecutive days for total ammonia [(mg N-NH₄ kg⁻¹ average body weight (ABW) day (d)⁻¹)] measurements. For that purpose, water flow in the tanks was reduced to 0.2–0.5 l min⁻¹ the day before and during the sampling days. Water collection in the outlet of a tank without fish was used as a blank.

Sampling

Fish in each tank were bulk-weighed every 3 weeks, after 1 day of feed deprivation. For that purpose, fish were slightly anaesthetized with 0.3 ml l⁻¹ ethylene glycol monophenyl ether. After the final weighing, a random sample of five fish per tank were killed by a lethal dose of anaesthesia. Whole fish, viscera and liver weights were recorded for determination of hepatosomatic (HSI) and visceral indexes (VI). Liver samples were immediately frozen in liquid nitrogen and then stored at -80 °C until measurement of glycogen and lipid contents. In order to minimize manipulation stress, the remaining fish continued to be fed for 3 more days, after which nine fish from each tank were randomly sampled 3 h after the morning meal. Fish were killed with a sharp blow in the head and dissected on chilled trays. Three pools of three livers each were snap frozen in liquid nitrogen until measurement of key enzymes of intermediate metabolism.

Chemical analysis

Chemical analyses of the diets were performed following the Association of Official Analytical Chemists methods (AOAC 2000). Briefly: dry matter after drying in an oven at 105 °C until constant weight; ash by incineration in a muffle furnace at 450 °C for 16 h; protein content (N × 6.25) according to the Kjeldahl method, using Kjeltex digester and distillation units (Tecator Systems, Höganäs, Sweden; model

1015 and 1026, respectively); lipids by petroleum ether extraction in a Soxtec extraction system (Tecator Systems; extraction unit model 1043 and service unit model 1046) and gross energy by direct combustion in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261). Dietary starch was determined according to Beutler (1984). Hepatic glycogen and liver lipids were measured as described by Roehrig and Allred (1974) and Folch et al. (1957), respectively. Ammonia in water was measured by the indophenol method (Koroleff 1983).

Enzyme activity

Liver samples were homogenized in 9 volumes of ice-cold 100 mM Tris-HCl buffer containing 0.1 mM EDTA and 0.1 % (v/v) Triton X-100 (pH 7.8). All procedures were performed on ice. Homogenates were centrifuged at 30,000 g for 30 min at 4 °C, and the resultant supernatants were kept in aliquots and stored at -80 °C for further enzyme assays. All enzyme activities were measured at 340 nm in a microplate reader (model ELx808TM, Bio-Tek Instruments, USA), monitoring the changes in absorbance of substrate at 37 °C. The optimal substrate and protein concentrations for measurement of maximal activity for each enzyme were established by preliminary assays.

Alanine aminotransferase (ALAT, EC 2.6.1.2) and aspartate aminotransferase (ASAT, EC 2.6.1.1) activities were measured using commercial kits from Spinreact, Girona, Spain (ASAT/GOT, ref. 41273; ALAT/GPT, ref. 41283). Glutamate dehydrogenase (GDH, EC 1.4.1.2) activity was determined using a reaction mixture containing 50 mM imidazole-HCl buffer (pH 7.4), 0.2 mM NADH, 1 mM ADP, 100 mM ammonium acetate, 2 units ml⁻¹ LDH and 10 mM α-ketoglutarate (Morales et al. 1990).

Hexokinase (HK, EC 2.7.1.1) and glucokinase (HK-IV, EC 2.7.1.2) activities were determined as described by Vijayan et al. (1990). Reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.4), 2.5 mM ATP, 5 mM MgCl₂, 0.4 mM NADP, 2 units ml⁻¹ G6PDH and 1 mM (HK) or 100 mM (HK-IV) glucose.

Fructose 1,6-bisphosphatase (FBPase, EC 3.1.3.11) activity was determined using a reaction mixture consisting of 50 mM imidazole-HCl buffer (pH 7.4), 5 mM MgCl₂, 12 mM 2-mercaptoethanol, 0.5 mM

NADP, 2 units ml⁻¹ G6PDH, 2 units ml⁻¹ PGI and 0.5 mM fructose 1,6-bisphosphate (Morales et al. 1990).

Glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) activity was measured as described by Morales et al. (1990), using a reaction mixture containing 50 mM imidazole–HCl buffer (pH 7.4), 5 mM MgCl₂, 2 mM NADP and 1 mM glucose-6-phosphate. Malic enzyme (ME, EC 1.1.1.40) activity was measured using a reaction mixture containing 50 mM imidazole–HCl buffer (pH 7.4), 5 mM MgCl₂, 0.4 mM NADP and 2 mM l-malate (Singer et al. 1990). Fatty acid synthetase (FAS, EC 2.3.1.38) activity was quantified as described by Chang et al. (1967) and modified by Chakrabarty and Leveille (1969) using a reaction mixture containing 100 mM K₂PO₄H buffer (pH 6.5), 0.1 mM NADPH, 25 μM acetyl-CoA and a substrate solution with 100 mM KPO₄H₂ buffer (pH 6.5) and 0.6 mM malonyl-CoA.

All enzyme activities were expressed as milliunits per milligram of hepatic soluble protein (specific activity). One unit of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate per minute under the above assay conditions. Hepatic soluble protein concentration was determined according to Bradford (1976), using a commercial kit (Sigma-Aldrich protein Kit, ref. B6916) and bovine serum albumin as standard.

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA). Before analysis, data were tested for normality by the Shapiro–Wilk test and homogeneity of variances by the Levene’s test. When normal distribution of values was not verified, data were transformed prior to ANOVA. The probability level of 0.05 was used for rejection of the null hypothesis. Significant differences among groups were determined by the Tukey’s multiple range test. Statistical analysis was performed using an IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp, Armonk, NY, USA).

Results

Data on growth performance and feed utilization efficiency of zebra sea bream fed the experimental diets are presented in Table 2. Final body weight and daily growth index increased with the increase of P:C ratio ($R = 0.944$ and $P = 0.000$; $R = 0.943$ and $P = 0.000$, respectively). Significantly higher values for both parameters were recorded in fish fed diets P45C21 and P55C9 than in fish fed the P25C43 diet. No significant differences on growth performance were noticed in fish fed P35C32, P45C21 and P55C9

Table 2 Growth performance and feed utilization efficiency of zebra sea bream fed the experimental diets

Diets	P25C43	P35C32	P45C21	P55C9	<i>P</i> value	SEM
Final body weight (g)	13.5 ^a	16.5 ^{ab}	19.3 ^b	20.4 ^b	0.010	1.05
Daily growth index ¹	0.43 ^a	0.60 ^{ab}	0.75 ^b	0.80 ^b	0.007	0.06
Feed intake (g kg ⁻¹ ABW ² day ⁻¹)	15.7	15.2	14.7	16.1	0.545	0.33
Feed efficiency ³	0.36 ^a	0.50 ^b	0.62 ^c	0.59 ^{ab}	0.002	0.04
Mortality (%) ⁴	12.5	12.5	15.0	5.0	0.547	2.63
N Intake (g kg ⁻¹ ABW ² day ⁻¹)	0.65 ^a	0.87 ^{ab}	1.07 ^b	1.39 ^c	0.002	0.10
N Retention (g kg ⁻¹ ABW ² day ⁻¹)	0.14 ^a	0.20 ^b	0.25 ^c	0.26 ^c	0.002	0.02
E Intake (kJ kg ⁻¹ ABW ² day ⁻¹)	317.2	307.9	300.1	338.4	0.359	7.5
E Retention (kJ kg ⁻¹ ABW ² day ⁻¹)	55.0 ^a	69.4 ^{ab}	75.8 ^b	81.2 ^b	0.012	3.9

SEM pooled standard error of the mean

Means ($n = 2$) in the same row with different superscript letters are significantly different ($P < 0.05$)

¹ DGI: $([\text{final body weight}]^{1/3} - [\text{initial body weight}]^{1/3}) / (\text{time in days}) \times 100$

² ABW: average body weight (initial body weight + final body weight)/2

³ Feed efficiency (FE) = (wet weight gain/dry feed intake)

⁴ Mortality = (number of dead fish/number of initial fish)

diets. Feed intake, mortality and energy intake ($\text{kJ kg}^{-1} \text{ABW d}^{-1}$) were unaffected by diet composition. Feed efficiency was higher in fish fed P45C21 diet than the other diets. N intake ($\text{g kg}^{-1} \text{ABW d}^{-1}$), N retention ($\text{g kg}^{-1} \text{ABW d}^{-1}$) and energy retention ($\text{kJ kg}^{-1} \text{ABW d}^{-1}$) increased with the increase of P:C ratio ($R = 0.979$ and $P = 0.000$; $R = 0.944$ and $P = 0.000$; $R = 0.929$ and $P = 0.001$, respectively). N intake was higher in fed diet P55C9 than the other diets, while N retention was identical in fish fed diets P45C21 and P55C9. Energy retention was higher in fish fed diets P45C21 and P55C9 than in fish fed diet P25C43.

HSI and VI indexes and liver composition of zebra sea bream fed the experimental diets are presented in Table 3. HSI decreased with the decrease in dietary carbohydrate content and was lower in fish fed diet P55C9 than in fish fed diet P25C43. In contrast, VI was similar among groups. Liver glycogen levels were higher in fish fed diet P35C32 compared with fish fed diets P45C21 and P55C9. Liver lipid levels were higher in fish fed diets P25C43 and P45C21 than in fish fed diet P55C9.

A positive linear correlation ($R = 0.986$ and $P = 0.000$) was found between daily ammonia excretion ($\text{mg NH}_4\text{-N kg}^{-1} \text{ABW d}^{-1}$) and dietary protein incorporation level (Fig. 1).

Activities of key liver enzymes of intermediary metabolism in fish fed the experimental diets are presented in Table 4. ALAT and GDH activities increased with the increase of P:C ratio, while ASAT was not affected by diet composition. Higher ALAT activity was found in fish fed P55C9 diet than in other

groups. GDH activity was higher in fish fed diets P45C21 and P55C9 than in fish fed the lower protein diets. Glycolytic (HK, GK) and gluconeogenic (FBPase) enzymes activities were unaffected by dietary treatments. FAS activity was also unaffected by dietary treatments, but G6PD activity was higher in fed diet P45C21 than in fish fed diet P25C43. ME activity decreased with the increase of P:C ratio.

Discussion

Zebra sea bream growth performance was closely related to that observed in white sea bream and two-banded sea bream which are both considered slow-growers species (Sá et al. 2008a, b; Ozório et al. 2009). Final body weight and daily growth index pointed to higher growth performance of fish fed diets P45C21 and P55C9. Higher N and E retention was also recorded in fish fed these diets. This is in line with the dietary protein requirement of zebra sea bream for maximum weight which was estimated to be 43.8 % (Coutinho et al. 2014). As other animals, fish eat to cover their energy needs (Bureau et al. 2002), and results from this study as well as previous observations in white sea bream, two-banded sea bream and sharpsnout sea bream, back up this feed intake regulation to cover energy demands (Vivas et al. 2006; Sá et al. 2008a; Ozório et al. 2009; Coutinho et al. 2012). Results also suggest a more efficient use of protein for energy proposes than carbohydrates, which is supported by the improved FE with the increase of P:C ratio until reaching the optimum

Table 3 Hepatosomatic (HSI) and visceral (VI) indexes and liver composition ($\text{g } 100 \text{ g}^{-1}$ liver) of zebra sea bream fed the experimental diets

Diets	P25C43	P35C32	P45C21	P55C9	P value	SEM
Indexes						
HSI ¹	2.8 ^b	2.3 ^{ab}	2.2 ^{ab}	1.6 ^a	0.019	0.18
VI ²	8.7	8.2	7.0	7.2	0.094	0.30
Liver composition						
Glycogen	6.1 ^{ab}	7.0 ^b	4.9 ^a	5.0 ^a	0.007	3.43
Lipids	31.2 ^b	20.0 ^{ab}	29.1 ^b	14.8 ^a	0.023	26.80

SEM pooled standard error of the mean

Means ($n = 6$) in the same row with different superscript letters are significantly different ($P < 0.05$)

¹ HSI = (liver weight/body weight) \times 100

² VI = (viscera weight/body weight) \times 100

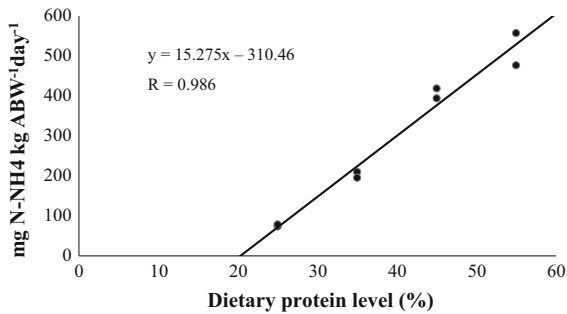


Fig. 1 Ammonia excretion of zebra sea bream fed the experimental diets

dietary protein level. Such result was somewhat unexpectedly for an omnivorous species (Hemre et al. 2002; Stone 2003; Polakof et al. 2012). It is, however, in agreement with the high dietary protein requirement of zebra sea bream, which is also unexpected for an omnivorous fish. In contrast, white sea bream, another omnivorous bream of the same genus efficiently uses dietary starch as energy source and, according to Sá et al. (2007), dietary P:C ratio can be reduced from 64:0 to 38:36 without impairing growth and feed utilization.

A more efficient use of protein than carbohydrates for energy purposes was also observed in sharpsnout sea bream (Coutinho et al. 2012) which also has a similar dietary protein requirement (42.9 %).

However, sharpsnout sea bream grows faster (thermal growth coefficient of 0.64 for sharpsnout sea bream vs. 0.35 for zebra sea bream) and it is known that faster growing fish have higher dietary protein requirements (Tacon and Cowey 1985). Thus, a lower dietary protein requirement of zebra sea bream was to be expected (Coutinho et al. 2014).

Mortality during this experiment ranged from 5 to 15 % and was not significantly different among groups. Considering that no clinical signs were noticed, it is plausible to infer that mortality was related with stress as this species is not yet domesticated. In fact, fish were bulk-weighted every 3 week during the trial, and mortality normally occurred after handling.

Transaminase and deaminase activities are considered valuable tools for assessing the response of fish to diet modifications, even for fish growing at reduced rate as is the case of zebra sea bream (Dean et al. 1986). In the current study, an increase in ALAT and GDH activities due to an increase of P:C ratio was noticed indicating that zebra sea bream was to some extent capable of adapting protein catabolism to protein intake, as previously observed in white sea bream (Sá et al. 2006, 2007, 2008a, b). The similar GDH activities observed in fish fed the lower protein diets (P25C43 and P35C32 diets) correlate with similar low N retention levels, confirming that fish

Table 4 Hepatic amino acid catabolic, glycolytic, gluconeogenic and lipogenic enzyme activities (mU mg protein⁻¹) in zebra sea bream fed the experimental diets

Diets	P25C43	P35C32	P45C21	P55C9	P value	SEM
Amino acid catabolism						
Alanine aminotransferase	298.7 ^a	300.1 ^a	337.1 ^a	479.1 ^b	0.000	18.4
Aspartate aminotransferase	943.2	925.9	930.1	1066.6	0.181	26.6
Glutamate dehydrogenase	62.8 ^a	58.9 ^a	141.4 ^b	162.5 ^b	0.000	10.9
Glycolysis						
Hexokinase	5.69	4.76	4.30	4.63	0.088	0.21
Glucokinase	1.61	0.94	1.24	1.02	0.103	0.11
Gluconeogenesis						
Fructose-1,6-bisphosphatase	37.8	45.7	43.6	38.2	0.107	1.42
Lipogenesis						
Glucose-6-phosphate dehydrogenase	91.0 ^a	102.9 ^{ab}	128.7 ^b	120.4 ^{ab}	0.023	5.00
Malic enzyme	88.3 ^c	82.2 ^{bc}	69.2 ^{ab}	57.5 ^a	0.001	3.31
Fatty acid synthetase	1.56	1.34	1.79	1.45	0.755	0.15

SEM pooled standard error of the mean

Means ($n = 6$) in the same row with different superscript letters are significantly different ($P < 0.05$)

were fed on dietary protein levels below requirements (Coutinho et al. 2014). ASAT activity was higher than ALAT activity in this species but, contrary to ALAT, it was not induced by dietary protein levels. In white sea bream, and although higher ASAT activity values were also observed compared with ALAT, contradictory results on the effects of dietary protein levels on ASAT and ALAT activities were reported (Sá et al. 2006, 2007, 2008a, b). Sá et al. (2007) observed a decrease in ASAT activity but not in ALAT with a decrease of P:C ratio. In a later study, both amino acid catabolism enzymes activities were positively correlated with the increase in dietary protein level (Sá et al. 2008a). However, an absence of ASAT and ALAT response in relation to dietary protein variation was reported in other studies (Sá et al. 2006, 2008b). Thus, in white sea bream it is difficult to conclude which amino acid catabolism enzyme, ASAT or ALAT, better response to dietary protein variations.

The increase in dietary protein levels and consequent increase in dietary protein to energy ratios, usually leads to higher ammonia excretion rates (Peres and Oliva-Teles 2001). Present data corroborate this, as a positive linear correlation between ammonia excretion and dietary protein levels was observed in zebra sea bream. GDH is considered the key enzyme responsible for amino acid deamination and ammonia production (Cowey and Walton 1989). The observed increase in GDH activity in fish fed diets with 45 and 55 % protein levels compared with fish fed the lower protein diets are in concordance with the higher N excretion recorded in these fish. Our results are also in line with those obtained by Sá et al. (2008a) with white sea bream fed diets containing increasing protein levels (from 6 to 49 %).

Excess glucose in high carbohydrate diets is directly used for energy purposes or for glycogen and lipid synthesis. As indicated above, zebra sea bream seems to use dietary protein more efficiently than carbohydrates for energy purposes. This is further corroborated by the excess of dietary glucose being directed towards glycogen and lipid deposition in the liver.

Curiously, in the present trial, independently of dietary carbohydrate level, no differences were observed in GK activity, a key enzyme of both glycogenesis and glycolytic pathways. Further, GK activity was lower than that of HK activity, which is a constitutive hexokinase that it is not under nutritional

regulation (Panserat et al. 2000; Enes et al. 2006, 2008; Moreira et al. 2008). The lack of GK response to dietary carbohydrate is contradictory to general observations in several fish species, including white sea bream, which belongs to the same genus as zebra sea bream (Panserat et al. 2000; Borrebaek and Christophersen 2001; Enes et al. 2006, 2008, 2015). This apparent lack of GK response to dietary carbohydrate level deserves to be further analysed in future experiments, but may at least in part explain the inefficient carbohydrate utilization for energy purposes and the high protein requirements of this species.

On the other hand, higher hepatic lipid content was noticed in fish fed diet P25C43 comparatively to fish fed diet P55C9, which is in line with the higher ME activity observed in these fish, and seems to reveal that excess dietary carbohydrates were mainly directed towards lipid synthesis. ME together with G6PD are the main enzymes responsible for the reductive power supply (NADPH) needed for lipogenesis in fish. The relative contribution of both enzymes to NADPH production is species-specific (Leaver et al. 2008) and in this species these enzymes seemed to contribute equally to NADPH production. In white sea bream, no differences in NADPH-generating enzymes in relation to dietary P:C ratio were observed (Sá et al. 2007, 2008a). Unexpectedly, and in contrast to the ME results, FAS activity was unaffected by dietary P:C ratio.

Higher dietary carbohydrate levels may lead to a reduction of gluconeogenesis pathway in some fish species (Shikata et al. 1994; Panserat et al. 2002; Enes et al. 2015), although an absence of regulation of both FBPase activity and/or gene expression has been reported for several carnivorous and omnivorous fish species (Tranulis et al. 1996; Borrebaek and Christophersen 2000; Panserat et al. 2002; Enes et al. 2006, 2008). Present data indicate no effect of dietary carbohydrates on FBPase activity suggesting that also in this species endogenous glucose synthesis was not depressed even at high dietary carbohydrate intake. In contrast, in white sea bream hepatic FBPase activity was depressed by an increase in dietary starch intake (Enes et al. 2015). Moreover, our data also showed no effect of dietary protein level on FBPase activity, in contrast to previous studies in other species that suggested that the major factor involved in gluconeogenic enzyme regulation is dietary protein levels

(Walton 1986; Kirchner et al. 2003, 2005; Enes et al. 2006). Overall, present results sustain the hypothesis of a lack of regulation of hepatic glucose utilization and production, which contributes to explain the low efficiency of dietary glucose utilization by zebra sea bream juveniles.

In conclusion, present results indicate that the best growth performance and feed utilization efficiency were achieved a diet with a P:C ratio of 45:21. The activity of key amino acid catabolic enzymes and N excretion levels in relation to dietary P:C ratio supports the metabolic adaptability of this species to dietary protein inclusion levels. Overall, zebra sea bream seems capable of better utilize protein rather than dietary carbohydrates as energy source which may be an obstacle for using more economically diets and thus for reducing environmental N loads in semi-intensive aquaculture of this species.

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