www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 13: 305-313 (2013) DOI: 10.4194/1303-2712-v13_2_12

Sea Cucumber Meal as Alternative Protein Source to Fishmeal in Gilthead Sea Bream (*Sparus aurata*) Nutrition: Effects on Growth and Welfare

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Received 19 November 2012
Accepted 15 April 2013

Abstract

The aim of the present study was to evaluate the effect of sea cucumber meal on gilthead sea bream growth. Two diets were used: a fishmeal based diet (control) and a diet containing 18% of sea cucumber meal inclusion (HM). A 100-days growth trial was carried out (120 fish, initial mean body weight of 35.28±9.31 g). The experimental plan used was monofactorial, balanced with three replicates for two experimental treatments (fish diet). The diets were isolipidic (CL 15.80 ±0.5%), isonitrogenous (CP 49.70±1.0%) and isoenergetic (GE 20.00±1.0 MJ kg^{-1}) and feeding rate was 1.5% of biomass. At the end of the trial, growth performances parameters, digestive enzymes (liver and intestinal), haematological parameters, Na^+/K^+ ATPase activity were measured. Oressigenic and anoressigenic brain neuropeptides were also analyzed in order to investigate other effects of food intake in fish. Productive parameters, blood plasma parameters, enzyme activities and neuropeptides expression were not affected by the introduction of sea cucumber meal in fish feed thus showing that HM can be used as a partial substitute of fish meal in sea bream nutrition

Keywords: Fish nutrition; fishmeal; protein sources; fish feeds.

Introduction

Fish meal replacement in fish feeds is a argument of primary interest for the future of aquaculture (Gatlin *et al.*, 2007) and new feedstuffs either vegetal or animal are continuously proposed as potential alternative to fish meal. Several animal protein sources have been proposed for fish meal replacement in fish feeds, mainly coming from by product for their high protein content, enhancing diet palatability and low price (Allan *et al.*, 2000; Millamena, 2002; Oliva-Teles *et al.*, 1999; Toppe *et al.*, 2006, Rathbone *et al.*, 2001, Shapawi *et al.*, 2007), in this context, sea cucumber meal could be interesting as a potential ingredient for fish meal substitution. Sea cucumbers are echinoderms harvested for human consumption, primarily in China, but they are present in several areas of the world, including Mediterranean Sea (Çhakly *et al.*, 2004; Lovatelli *et al.*, 2004; Toral-Granda *et al.*, 2008; Purcell, 2010; Sicuro *et al.*, 2012). Several studies have been conducted on nutritional value of different species of sea cucumber: *Holothuria forskali* (Rodrìguez *et al.*, 2000), *Holothuria tubulosa* (Chackly *et al.*, 2004), *Cucumaria frondosa* (Zhong *et al.*, 2007) and *Stichopus chloronotus* (Fredalina *et al.*, 1999). These researches showed low fat and high protein content in these species, moreover *H. tubulosa* and *H. polii*, which are typical species from Mediterranean sea, showed an interesting amino acid composition (Sicuro *et al.*, 2012). Sea cucumbers are normally present in gilthead bream natural diet and their utilisation in fish feeds is particularly promising for other bentophagous fish potentially interesting for aquaculture, as common pandora (*Pagellus erythrinus*) and shi drum (*Umbrina cirrosa*). In the present study the effect of the partial substitution of fishmeal with sea cucumber meal has been investigated on juvenile gilthead sea breams, by evaluating growth performances, feed utilization, digestive enzyme activities and haematological parameters. In addition, since it has been demonstrated that in fish nutrients can affect food intake by acting on the expression of oressigenic and anoressigenic brain neuropeptides (Narnaware and Peter, 2002; Volkoff *et al.*, 2005; Volkoff, 2006; Kulczykowska and Sánchez Vázquez, 2010), the expression of NPY (neuropeptide Y) and cocaine CART (cocaine and amphetamine regulated transcript) RNA messengers were also measured.

Materials and Methods

Sea Cucumber Meal and Diets

Sea cucumbers were collected along the coast of Adriatic Sea, near Lecce (Apulia, South East Italy). Sea cucumbers were washed with distilled water, gutted, dried in oven (at 60°C until constant weight) and transformed in a sea cucumber meal (HM) (Table 1). The experimental diet containing 18% of HM was tested against a control diet fishmeal based. Diets and sea cucumber meal were analyzed for proximate composition, according to standard methods (AOAC, 1995). A nitrogen analyser (Rapid N III, Elementar A-nalysnsysteme GmbH, Hanau, Germany) was used to determine the total nitrogen content. The crude protein was calculated as the total N x 6.25. The gross energy content was determined using an adiabatic calorimetric bomb (IKA C7000, Staufne, Germany).

Growth Trial

The growth trial was carried out at the Research Centre for Fisheries and Aquaculture of the University of Salento (Frigole, Lecce, Italy). At the beginning of the trial 120 juvenile gilthead sea breams (*Sparus aurata*) with an initial mean body weight of 35.3 □ 9.3 g, were randomly stocked (20 fish for tanks) in six fibreglass tanks (80 L) supplied by an open water circuit: three for control diet (CTRL) and three for sea cucumber meal (HM). The experimentation lasted 100 days, started on March 2009 and finished on June 2009. All fish were singly weighted, every 15 days, in order to check the fish biomass gain and regulate the feeding rate. The initial feeding rate was 1.5% of initial biomass and was successively modified according the water parameters. The fish were fed by hand twice a day, 6 days per week. Water flow was 1,2-1,5 L/min, temperature and dissolved oxygen concentration were daily measured.

Fish Sampling

At the beginning of the trial five fish were used for initial sampling. In order to control biomass gain, all fish were weighted every two weeks; six fish for experimental treatment were sampled at days 7, 21, 36 and 100 for gut and brain sampling. Liver, kidney, gill and blood sample were taken at beginning and at the end of the experimentation.

Table 1. Proximate analysis of sea cucumber meal (mean ± S.D., n = 3)

Proximate analyses (% dry weight)	Sea cucumber meal
Dry matter	19.11 ± 3.42
Crude protein	40.79 ± 1.19
Crude lipid	0.63 ± 0.17
Ash	47.22 ± 1.20
Gross Energy (MJ kg^{-1})	8.90 ± 0.29

Productive Indexes

At the end of experimentation, hepato-somatic index (HSI), biomass growth (BG), specific growth rate (SGR), feed conversion rate (FCR) and protein efficiency ratio (PER) were measured, with the following formulas (Palmegiano *et al.*, 2006):

Survival rate (%) = (number of fish at the end / number of fish at the beginning) x 100;

Hepato Somatic Index (%) = (liver/body weight) x 100;

Biomass gain (g) = final individual weight – initial individual weight;

Specific Growth Rate (%) = (ln final weight – ln initial weight) ·100/feeding days;

Feed Conversion Rate (FCR) = total feed supplied /BG;

Protein Efficiency Ratio (%) = ((BG)/ (total utilized food x food protein)) x100.

Haematology

Blood was collected from the caudal vein with heparinised syringes, centrifuged (20 min at 2500 g; 4°C) and the plasma samples stored at -20°C until use. Blood protein concentration was determined by the Lowry protein assay, using bovine serum albumin as standard. Cholesterol and cortisol plasma levels were measured by colorimetric enzymatic method CHOD-PAP (SGMitalia) and cortisol EIA, Enzyme Immunoassay (Cayman Chemical Company), respectively, while plasma osmolarity was determined by osmometer (Vapro 5520 Wescor).

Enzymatic Activities

Intestine, liver, kidney and gills were sampled in order to determine the enzymatic activities. The organs were rinsed with cold distilled water, homogenized in solution containing 1.1% NaCl and PMSF 0.5 mM, and filtered with a mesh of 100 µm. A glass homogenizer was used for intestines, livers and kidneys, while a polytron PT 10/35 for gills. All procedures were performed at 0-4 °C. The enzymatic activities were measured following the methods of Burlina (1970) for amylase, Bessey *et al*. (1946) for alkaline phosphatase, Storelli et al. (1986) for Na^+/K^+-ATPase (NKA), Nagel *et al*. (1964) for leucine amino peptidase (LAP) and Albro *et al*. (1985), for lipase. Enzymatic activities were investigated at the days 7, 21, 36 and 100.

NPY and CART mRNA Expression

Brains were excised and homogenised for extraction of total RNA using Trizol RNA isolation reagent (Life Technologies, Gaithersburg, MD) based on the acid guanidium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987). Total RNA from tissues was fractionated by electrophoresis in a denaturing agarose gel (1.5%) with formaldehyde. Total RNA (2 μg) isolated from brain was reverse-transcribed to first-strand cDNA. Partial sequences of NPY and CART were amplified by PCR using primers designed from regions with high degree of identity between several fish CART (Blomqvist *et al.*, 1992; Cerda-Reverter *et al.*, 2000; Leonard *et al.*, 2001; Volkoff and Peter, 2001; GenBank accession no. BQ480503):

NPY-F	GGATACCCGGTGAAACCGGAGA
NPY-R	GTGTCCAGAATCTCAGGACTGGA
CART-F	GTCGTCCATGGAGCTGATCT
CART-R	GAACCACGTTCCCATTTCAC

The PCR parameters were 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, with an additional initial 15-min denaturation at 95 °C and a 10-min final extension at 72 °C. The PCR products were separated on a 2% agarose gel and stained with ethidium bromide. The gels were then photographed and analyzed by densitometric scanning (AlphalmagerTM, Alpha innotech, USA). The relative NPY and CART mRNA abundance among different sample was determined by semi-quantitative RT-PCR within the exponential phase, using beta-actin as an external control, designed from a partial sequence of sea bream β-actin (GenBank accession no X89920). Negative controls were performed for each primer sets where cDNA was omitted from the PCR reactions. The PCR products, obtained for each primer pair, were sequenced to confirm the target gene was being amplified. The relative tissue NPY and CART cDNA were expressed as the ratio NPY or CART/beta-actin cDNA. Gene expression was investigated at the beginning of the experiment and at the days 7, 21 and 36.

Experimental Plan and Statistical Analysis

The experimental design was monofactorial, balanced with randomized blocks, 3 replicates per treatment (3x2) and the experimental factor was the diet with 2 levels of treatment (HM and CTRL). The data were analyzed by one-way analysis of variance (ANOVA) and Kruskall Wallis test (non parametric ANOVA), using SYSTAT and R statistical packages. After ANOVA, the differences among means were determined by the Tukey multiple comparisons of means test, using the significance level of $P<0.05$. Results are presented as means ± S.D.

Results

During the experimentation water temperature ranged between 17 and 26 °C and the salinity between 16 and 35 ppt. Dissolved oxygen ranged between 4.5 and 8 mg/L. Water parameters fluctuations were related with natural characteristic of supplied water. All the measured water parameters were resulted in the physiological range for this fish species. Diets were isoenergetic, isoproteic and isolipidic (Table 2).

Productive Indexes

There were not statistically significant differences on productive indexes between the experimental treatments, even if a better utilisation of sea cucumber proteins can be noted (Table 3). A noticeable fish mortality was registered at the end of experimentation that was comparable in the experimental groups (73.33±6.7% CTRL group; 62.22±10.2% HM group) and it was related to the equipments and fish facilities used for the first time for this experimentation.

Enzyme Activities

Analysis of the digestive enzymes revealed (Figure 1) that HM diet did not affect the intestinal α-amilase (1a) and lipase (1c) activities while had a significant ($P<0.05$) stimulatory effect on both leucine amino peptidase and alkaline phosphatase activities measured at 100 days. Interestingly, HM diet did not stimulate the activity of the liver alkaline phosphatase. Moreover, our results demonstrated that fish fed HM diet showed no significant different values of Na^+/K^+ ATPase activity in kidneys, liver and gills (Table 4).

Blood Plasma Parameters

The measured values of osmolarity, cholesterol and protein plasmatic concentrations of fish did not show differences at the end of the experimentation (Table 5). Cortisol concentration was measured during the experimentation resulted statistically different at the end.

NPY and CART Expression

RT-PCR of mRNA was used to determine the change in NPY and CART mRNA expression between control and HM group. A 332 bp region of NPY and 319 bp region of CART were amplified using primer sets NPY-F/NPY-R or CART-F/CART-R, respectively. For all tissues examined, PCR using β-actin gene specific primers was performed as an internal control, and a 230 bp fragment of β-actin was detected in all tissues. As a negative control, a reaction with no template was performed using each

Table 2. Ingredients and proximate composition (mean ± S.D.; n = 3) of the experimental diets

	Control Diet (CTRL)	Experimental Diet (HM)
Ingredients (% dry weight)		
Fish meal	65.1	45
Fish oil	9.5	11.5
Holoturian meal	0	18
Maize starch	21.4	15.5
Blood meal	0	6
Celite	0.5	0.5
Lignum sulphite	1	1
Vitamin mixture[a]	1	1
Mineral mixture[b]	0.5	0.5
Liver-protector integrator[c]	1	1
Proximate analyses (% dry weight)		
Dry matter	95.59 ± 0.20	94.99 ± 0.05
Crude protein	50.4 ± 0.03	48.96 ± 0.05
Crude lipid	15.41 ± 0.26	16.12 ± 0.10
Ash	10.87 ± 0.02	16.39 ± 0.19
Gross Energy (MJ kg^{-1})	20.70 ± 0.33	19.30 ± 0.33

[a] Mineral mixture (g or mg/kg diet): bicalcium phosphate, 500 g; calcium carbonate, 215 g; sodium salt, 40 g; potassium chloride, 90 g; magnesium chloride, 124 g; magnesium carbonate, 124 g; iron sulphate, 20 g; zinc sulphate, 4 g; copper sulphate, 3 g; potassium iodide, 4 mg; cobalt sulphate, 20 mg; manganese sulphate, 3 g; sodium fluoride, 1 g (Granda Zootecnica, Cuneo, Italy).
[b] Vitamin mixture (IU or mg/kg diet): dl-a-tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15 000 IU; dlcholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium pantothenate, 50 mg; choline chloride, 2000 mg (Granda Zootecnica, Cuneo, Italy).
[c] Liver-protector integrator: INVE TECHNOLOGIES, DENDERMONDE, BELGIUM

Table 3. Productive (n=3) and hepato-somatic (n=9) indexes (mean ± S.D.)

	CTRL	HM
Biomass Gain (g)	29.40 ± 1.50	29.00 ± 6.60
Specific Growth Rate (%)	1.12 ± 0.04	1.11 ± 0.19
Feed Conversion Rate	1.31 ± 0.11	1.09 ± 0.10
Protein Efficiency Ratio (%)	1.59 ± 0.13	1.98 ± 0.17
Hepato-Somatic Index (%)	0.97 ± 0.25	0.86 ± 0.11

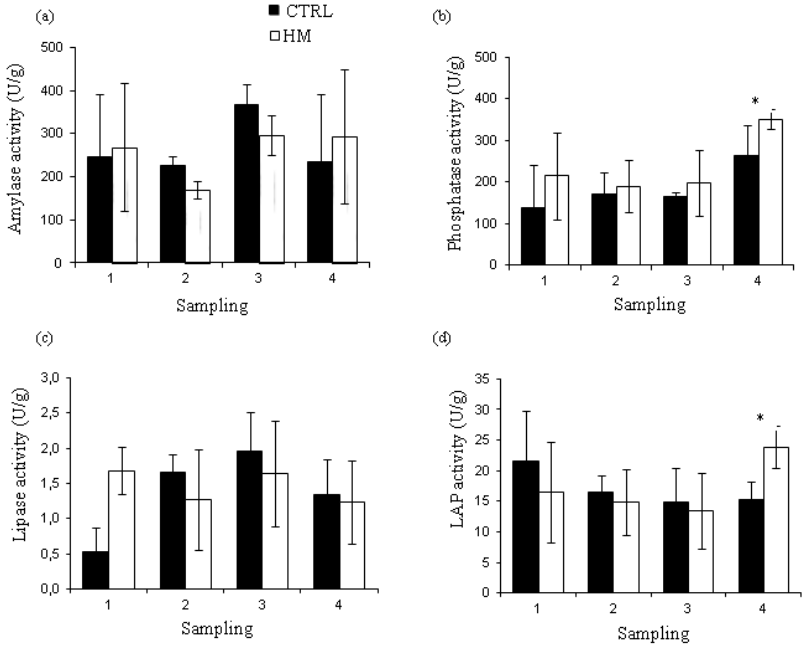

Figure 1. Values of intestinal enzymatic activity (a) amylase, (b) alkaline phosphatase, (c) lipase, (d) leucine amino peptidase activities. (* = P < 0.05) (1= 7th day; 2 = 21th day, 3= 36th day; 4 = 100th day)

primer pair. There were no amplification products in these reactions, verifying the absence of contamination. Fish from HM and control group showed, at brain level, similar amount of mRNA of both neuropeptides (Figure 2).

Discussion

The results of the present study demonstrated that fish meal can be replaced with a 18% of HM meal inclusion in gilthead sea bream diet. Sea cucumber is rich in protein, but it is also rich in ashes and contains a repulsive compound, called holoturin, that "*in vivo*" is a natural defence and could exert an antinutritional or distasteful effects in fish feeds. For these reasons, before the preparation of experimental fish feeds, a mechanical separation of ossicles of calcareous endoskeleton was attempted, in order to decrease ash content in sea cucumber meal, but it was unsuccessful as the ash content of sea cucumber meal did not decrease after sieving. Consequently, during the fish feed formulation and preparation, the inclusion of sea cucumber meal was limited mainly by its high ash content. High protein and low fat diets promote good growth rates and feed utilisation in marine fish (Toppe *et al.*, 2006) and in particular in

Table 4. Na^+/K^+ ATPase activity in gills, liver and kidney, detected at the beginning and end (n=6) of experiment (mean ± S.D.)

	Initial	CTRL	HM
Kidney (U/g)	$9.12 \pm 3.66_a$	$11.68 \pm 2.92_a$	$8.19 \pm 2.74_b$
Liver (U/g)	$26.34 \pm 14.34_a$	$10.59 \pm 3.76_b$	$11.66 \pm 5.39_b$
Gills (U/g)	$19.64 \pm 7.70_a$	$37.10 \pm 12.67_b$	$27.04 \pm 7.10_b$

Notes: In the rows, different letters mean statistical difference at P< 0.05.

Table 5. Plasma parameters measured before and at the end (n=12 for cortisol, otherwise n=3) of trial (mean ± S.D.). Significant differences (P<0.05)

	Initial	CTRL	HM
Cortisol (pg/ml)	1015.00 ± 130.24	$804.6 \pm 248.96_a$	$935.0 \pm 149.68_b$
Osmolarity (mmol/kg)	341.67 ± 44.23	$368.67 \pm 32.01_{n.s.}$	$363.00 \pm 4.36_{n.s.}$
Cholesterol (mg/dl)	140.58 ± 29.31	$125.53 \pm 1.92_{n.s.}$	$134.04 \pm 14.10_{n.s.}$
Prot. concentration (mg/ml)	26.18 ± 11.70	$32.41 \pm 15.25_{n.s.}$	$17.18 \pm 7.92_{n.s.}$

Notes: In the rows, different letters mean statistical difference at P< 0.05; n.s.= not significant

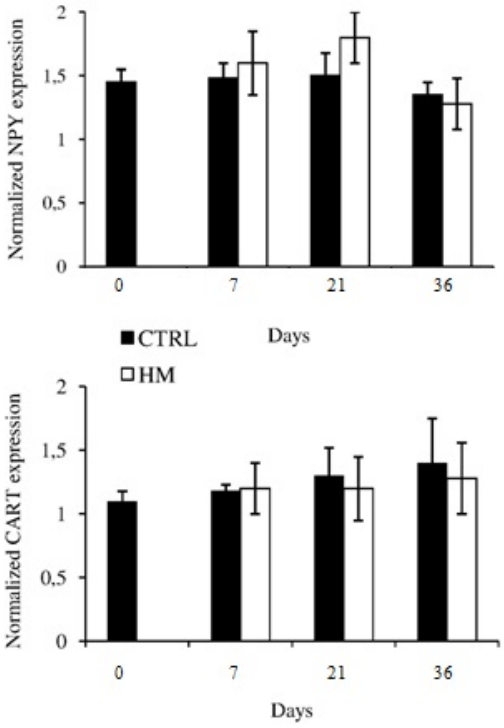

Figure 2. The influences of sea cucumber meal on NPY and CART gene expression. (mean ± S.D; n=6).

gilthead seabream (Atienza *et al*., 2004) and high ash content increases clearly dietary Ca in fish. Between farmed fish in the Mediterranean area, gilthead sea bream is certainly the most suitable species to high dietary Ca, considering that molluscs are primary food source in the wild, but the bioavailability of dietary minerals depends on its solubility. Marine ingredients for fish feeds that contain high ash content will evidently provide high Ca levels that sometimes have been found to inhibit absorption of other essential minerals like phosphorus, magnesium and zinc (Toppe *et al.*, 2006). Similarly to other marine invertebrates already used in fish feeds, basically crustaceans (Suontama *et al.*, 2007; Toppe *et al.*, 2006), HM meal is not variable in composition because it is obtained from entire body animal, not from by product. In this experimentation fish growth was less than expected, considering that fish only doubled initial body weight at the end of experimentation. We consider that this was caused by experimental tanks that was used for the first time in this experimentation. Even considering low growth, it was similar between fish meal and HM based diet, PER was similar in the experimental groups thus suggesting that the protein quality of the two diets was comparable. In gilthead sea bream lower values of PER were found when fishmeal was replaced by soybean, lupin seed meals (Robaina *et al.*, 1995) or corn gluten, meat and bone meals (Robaina *et al.*, 1997). In other researches on fishmeal substitution similar PER values were obtained with the introduction of essential amino acids to the feed (Gomez-Requeni *et al.*, 2004; Sitja-Bobadilla *et al.*, 2005). A preliminary trial on fish feed digestibility (unpublished data) suggested similar feed digestibility for the considered groups, confirming that the antinutritional substances were inactivated during the processing in sea cucumber meal. It is well documented that the activity of proteases, alkaline phosphatase, amylase (Saito and Suda, 1975; McCarthy *et al.*, 1980; Naz and Turkmen, 2009) and those of enzymes not "directly" involved in the digestive processes, such as Na^+/K^+ ATPase, can be modulated by the diet composition (Mecocci *et al.*, 1997). The data reported here demonstrated that only the activity of two digestive enzymes (alkaline phosphatase and leucine amino peptidase) were affected by different diets at the end of the experimentation, while amylase and lipase were not affected. At the end of the trial, intestinal phosphatase and leucine amino peptidase activities resulted significant higher in fish fed HM than in group fed fishmeal. To date, no information exists concerning the use of *Holothuria* meal for the preparation of fish food while Taboada *et al*. (2003) demonstrated that rats fed with *Holothuria forskali* meal, showed both, intestinal alkaline phosphatase and leucine amino peptidase activities significantly higher than that measured in casein fed rats. Alkaline phosphatase is found primarily in cell membranes where active transports take place and it is considered as a general marker of nutrient absorption. Furthermore alkaline phosphatase is involved in the intestinal absorption of calcium (Brun *et al.*, 2008). The higher activity of the alkaline phosphatase measured in HM fed fish could be explained by the higher calcium contents of HM meal. Since the two diets were isoproteic, the observed higher intestinal leucine amino peptidase activity in HM fed fish could be explained by the different amino acid composition of the two diets. From the physiological point of view, haematic cortisol is commonly used to evaluate fish welfare (Campbell, 2004; Sadler *et al.*, 2000; Wagner and Congleton, 2004; Haukenes *et al.*, 2008; Sicuro *et al.*, 2009) and provides a good indication of the severity and duration of the eventual stress response in gilthead sea bream (Jentoft *et al.*, 2005; Syriou *et al.*, 2011). Interestingly, the final values of haematic cholesterol and cortisol were less than initial ones and this was one of more clear evidences suggesting the absence of negative effect of experimental conditions. Cortisol at the end of the experimentation resulted higher in the HM meal group. Osmolarity and cholesterol blood levels were similar in the experimental treatments so confirming the absence of diet-dependent stress. Furthermore, cortisol and osmolarity concentration was comparable to those measured in a previous research in sea bream (Sicuro *et al.*, 2010) where similar values were considered indicators of good conditions of farmed fish. Blood cholesterol and cortisol values measured in this experimentation also showed that the registered final fish mortality was not caused by experimentation. Plasma protein concentration was the only haematological parameter found under the physiological range of sea bream (Pavlidis *et al.*, 1997). Changes in the concentration of serum protein, albumin and globulin have been used as indicators of stress response in fish (Sala-Rabanal *et al.*, 2003) and considering that the greater part of plasma proteins (albumin, fibrinogen and globulins) are synthesized in the liver (Sala-Rabanal *et al.*, 2003), the decrease of total protein in HM fed fish may be explained as to a deficient hepatic operation. This fact is also supported by the decrease of liver size in the HM fed fish. No other negative consequences of TP descent were found in HM fed fish and it is presumable that different plasma protein fractions should be investigated in order to clarify this low TP level in the HM fish.

Brain neuropeptide studies represent an unusual aspect for fish nutrition and in this research have been introduced as a supplementary aspect of traditional studies on fish nutrition. Neuropeptide Y (NPY) regulates food intake in fish and it is the most potent orexigenic factor in fish whereas cocaine- and amphetamine-regulated transcript is a potent anorexigenic agent (Volkoff, 2006). It has been shown that diet composition influenced the brain expression of NPY both in mammals (Beck *et al.*,

1990; Wilding *et al.*, 1992; Giraudo *et al.*, 1994; Widdowson *et al.*, 1999) and goldfish (Bernier *et al.*, 2004; Narnaware and Peter 2002). The observation that brain NPY and CART mRNA expression in these experimental conditions were similar in control and HM fed fish further confirmed productive and physiological results. In conclusion, this study considered several nutritional and physiological aspects that can be influenced by HM meal introduction in sea bream feeds and none of them resulted affected by the presence of HM meal in fish feed. Actually we found a noticeable final mortality and fish growth inferior to expected, but physiological parameters measured on fish make us confident on obtained results. We did not see a clear antinutritional effect of HM meal at the end of experimentation on gilthead sea bream and this indicated its potential inclusion in future fish feeds. However the ash separation and potential contamination with heavy metals should be solved in HM meal in order to make it an effective fish meal substitute. Even with the compositional limitations depicted in this research, HM meal has an evident advantage: the great availability of sea cucumbers along Mediterranean coasts and their great potentiality for integrated farming.

Acknowledgments

The authors thank Dr. Elena Sparasci and Dr. Roberta Comi for their technical help. They wish to express their gratitude to Dr. Francesco Gai (National Council of Researches, Torino, Italy) for his scientific support.

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