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The effect of dietary methionine and white tea on oxidative status of gilthead sea bream (*Sparus aurata*)

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Abstract

Free radicals are continuously generated during an organism's lifetime. In order to understand the involvement in the oxidative status of fish, methionine and white tea were assayed as antioxidant supplements in diets for gilthead sea bream (*Sparus aurata*). For the purpose of this study, four isonitrogenous and isolipidic diets were formulated to contain 45 % of protein and 18 % lipid and 0·3 % methionine (Met diet), 2·9 % white tea dry leaves (Tea diet) and 2·9 % of white tea dry leaves + 0·3 % methionine (Tea + Met diet). An unsupplemented diet was used as the control. Key enzymatic antioxidant defences, superoxide dismutase (SOD) isoenzyme profile, total, reduced and oxidised glutathione and oxidative damage markers were determined. The results showed that dietary methionine supplementation increased liver SOD activity, while white tea induced higher hepatic catalase activity. Dietary white tea induced a notable increase in Mn-SOD isoenzyme. This is the first study to provide evidence that dietary tea inclusion in fish feeding could be an important source of Mn with metabolic repercussions on antioxidant mechanisms.

Key words: Antioxidant defences: Methionine: Oxidative stress biomarkers: *Sparus aurata*: White tea

Free radicals and reactive species, from oxygen or nitrogen (henceforth named ROS), are continuously generated in all aerobic biological systems under normal or stressful conditions. In order to avoid and/or repair the damage these compounds may cause in the tissues, organisms possess adequate protection systems that are both enzymatic and non-enzymatic in origin[1].

The most important of non-enzymatic defences in fish include numerous low-molecular weight antioxidants like reduced glutathione (GSH), and several vitamins such as α-tocopherol (vitamin E) or ascorbic acid (vitamin C)[1]. Key enzymatic antioxidant defences include enzymes such as superoxide dismutases (SOD), a group composed of different metalloenzymes[2,3], which catalyse the conversion of the superoxide anion dismutation to molecular oxygen and H_2O_2. H_2O_2 is subsequently detoxified by two types of enzymes, catalase (CAT) which metabolises H_2O_2 to molecular oxygen and water, and glutathione peroxidase (GPX) which detoxifies H_2O_2 into water, or organic peroxides into their corresponding stable alcohols, by oxidising the GSH into its oxidised form (GSSG). Finally, glutathione reductase (GR) maintains GSH/GSSG homeostasis, catalysing the reduction of GSSG into GSH[1].

Despite all these defence mechanisms, when an uncontrolled ROS production takes place or antioxidant defences are deficient, an oxidative stress situation arises, with a large number of biochemical and physiological consequences. The resulting oxidative damage then affects lipids, proteins, carbohydrates and DNA, with negative effects on cellular structures which affect the organism's welfare and may even lead to death[1].

Under normal physiological status, oxidative balance can be tilted in favour of an improved welfare by supplying in the diets nutrients that enhance the antioxidant system.

Abbreviations: CAT, catalase; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); G6PDH, glucose 6-phosphate dehydrogenase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidised glutathione; Met, 0·3 % methionine; OSI, oxidative stress index; ROS, reactive oxygen species; SOD, superoxide dismutase; Tea, 2·9 % white tea dry leaves; Tea + Met, 2·9 % of white tea dry leaves + 0·3 % methionine; tGSH, total glutathione.

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Except for traditional antioxidants such as some vitamins or minerals[4], little is known about alternative nutrients with antioxidant potential in the fish redox state. This is the case with amino acids like methionine or phytochemicals such as tea polyphenols whose potential application in fish antioxidant status improvement has been poorly studied[5–8].

Methionine is an essential sulphur amino acid which, besides being a component of proteins, is a precursor of cysteine, an indispensable compound for the synthesis of glutathione and taurine, molecules with important roles in oxidative defence mechanisms[9–11]. Additionally, the methionine oxidation/reduction cycle also acts in the natural scavenging of ROS via the methionine sulphoxide reductase system[11,12]. Although a few studies have been performed in rats[13,14], to our knowledge, research on the methionine effect in the oxidative status of fish is scarce[5,7].

Tea, a product obtained from *Camellia sinensis* leaves, is an important beverage consumed worldwide, which has been considered to possess powerful antioxidant properties due to its high content of polyphenols, especially catechins and flavonoids[15–17]. These compounds may directly or indirectly act as antioxidants by inducing antioxidant enzymes, inhibiting pro-oxidant enzymes or reacting with oxidant agents[16]. Composition of the different commercialised teas differs depending on leaf processing. In the case of white tea, the leaves are harvested before they are fully open and are not oxidised or rolled, but simply withered and dried by steaming. This simple process allows white tea to retain the maximum amount of original polyphenols, making it one of the teas with the highest antioxidant potential[18,19]. This makes white tea a candidate as an antioxidant additive to be used in fish feeding. However, although there have been many studies performed in rats[20–22], the evaluation of the effect of dietary tea in fish is not abundant[6,23,24], especially regarding its effects on the oxidative status of the animals[8,25].

The aim of the present study was to assess the influence of diets containing methionine, white tea or a mixture of both on the oxidative status of gilthead sea bream (*Sparus aurata*). For this purpose, the activity of key antioxidant enzymes, non-enzymatic defences and lipid oxidative damage were evaluated in the liver of sea bream. Additionally, possible modifications induced by the different dietary treatments in the hepatic SOD isoenzymatic pattern were also determined.

Materials and methods

Experimental diets

A control diet was formulated based on fish meal and fish oil as the main protein and lipid sources (containing 45 % protein and 18 % lipid, respectively). Following this, three other diets were formulated similar to the control diet but supplemented with 0·3 % methionine (Met diet), 2·9 % white tea dry leaves (Tea diet) and 2·9 % white tea dry leaves + 0·3 % methionine (Tea + Met diet). Dietary ingredients were thoroughly mixed and dry-pelleted in a laboratory pellet mill (California Pellet Mill) using a 3 mm die. The pellets were air-dried at 40°C for 24 h and stored in a refrigerator until use. Ingredient composition and proximate analyses of the experimental diets are shown in Table 1. Chemical analyses of the diets were performed following Association of Official Analytical Chemists methods[26].

Animals and experimental conditions

This experiment was directed by trained scientists (following FELASA category C recommendations) and was conducted according to the European Economic Community animal experimentation guidelines Directive of 24 November 1986 (86/609/EEC). The experiment was performed at the Marine Zoology Station, University of Porto. Gilthead sea bream juveniles (*S. aurata*) obtained from a commercial hatchery were randomly distributed into twelve groups of twenty fish each (35 g mean initial weight). Each group was maintained in a fibreglass tank of 100-litre capacity within a thermoregulated recirculation water system kept at a constant temperature of 22·0 ± 1·0°C, and supplied with filtered seawater (37 ‰) at a flow rate of 4·0 litres/min. Dissolved oxygen averaged 95 % of the saturation level. The photoperiod was the natural one for November and December. After 15 d of acclimatisation to the rearing conditions, the experimental diets were randomly assigned to triplicate groups of animals that were hand-fed to apparent visual satiation, twice a day, 6 d a week, during a

Table 1. Composition and proximate analysis of the experimental diets

	Diets			
	C	Met	Tea	Tea+Met
Ingredients (% dry weight)				
Fish meal*	57·3	57·3	57·3	57·3
Soluble fish protein concentrate†	1·0	1·0	1·0	1·0
Cod liver oil	10·9	10·9	10·9	10·9
Pre-gelatinised starch‡	23·4	23·0	23·4	23·0
White tea	–	–	2·9	2·9
Met	–	0·3	–	0·3
Mineral premix§	1·0	1·0	1·0	1·0
Vitamin premix‖	1·0	1·0	1·0	1·0
Choline chloride (50 %)	0·5	0·5	0·5	0·5
Agar	1·0	1·0	1·0	1·0
Binder¶	1·0	1·0	1·0	1·0
Cellulose	2·9	2·9	0·0	0·0
Proximate analyses (% dry weight)				
DM	92·4	90·3	91·5	90·3
CP	44·7	45·4	45·9	46·0
Crude lipid	18·2	18·7	18·5	18·3
Ash	10·1	10·0	10·3	10·4
Nitrogen-free extract**	27·0	25·9	25·3	25·3

C, control; CP, crude protein.
* Steam-Dried LT; Pesqueira Diamante (CP: 77·2 % DM; gross lipid: 8·5 % DM).
† Sopropêche (CP: 75·8 % DM; GL: 18·8 % DM).
‡ Cerestar (approximately 99 % amylopectin).
§ Minerals (mg/kg 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, 5·9 (g/kg diet); potassium chloride, 1·15 (g/kg diet); NaCl, 0·4 (g/kg diet).
‖ Vitamins (mg/kg diet): retinol, 18 000 (IU/kg diet); calciferol, 2000 (IU/kg diet); α-tocopherol, 35; menadion sodium bisulphate 10; thiamine, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0·02; biotin, 1·5; ascorbyl monophosphate, 50; inositol, 400.
¶ Aquacube.
** Nitrogen-free extract = 100 − (CP + crude lipid + ash).

30 d period. Food intake and mortality were recorded daily and fish in each tank were bulk-weighed at the beginning and at the end of the experimental period.

Sampling

Fish feeding was discontinued 24 h before sampling. Then, two animals per tank (six per treatment) were randomly sampled and killed with a sharp blow on the head. The liver was excised and immediately frozen in liquid N_2 and thereafter stored at −80°C until use.

Enzyme activity

The liver samples were homogenised in nine 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. The procedure was 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 until use. All enzyme assays were carried out at 25°C and the changes in absorbance were monitored to determine the enzyme activity using a microplate reader (ELx808; Bio-Tek Instruments). The optimal substrate and protein concentrations for the measurement of maximal activity for each enzyme were established by preliminary assays. The molar extinction coefficients used for H_2O_2 and NADPH were 0·039 and 6·22 cm/mM, respectively. The assay conditions were as follows:

Glucose 6-phosphate dehydrogenase (G6PDH; *EC* 1.1.1.49) activity was measured as previously described by Morales *et al.*[27], using a reaction mixture containing 50 mM-imidazole–HCl buffer (pH 7·4), 5 mM-$MgCl_2$, 2 mM-NADP and 1 mM-glucose-6-phosphate.

CAT (*EC* 1.11.1.6) activity was determined by measuring the decrease in H_2O_2 concentration at 240 nm according to Aebi[28]. The reaction mixture contained 50 mM-potassium phosphate buffer (pH 7·0) and 10 mM-H_2O_2 freshly added.

SOD (*EC* 1.15.1.1) activity was measured by the ferricytochrome *c* method using xanthine/xanthine oxidase as the source of superoxide radicals. The reaction was monitored at 550 nm according to McCord & Fridovich[29]. The reaction mixture consisted of 50 mM-potassium phosphate buffer (pH 7·8), 0·1 mM-EDTA, 0·1 mM-xanthine, 0·012 mM-cytochrome *c* and 0·025 IU/ml xanthine oxidase. Activity is reported in units of SOD/mg of protein. Here, one unit of activity is defined as the amount of enzyme necessary to produce a 50 % inhibition of the ferricytochrome *c* reduction rate.

GPX (*EC* 1.11.1.9) activity was measured following the method of Flohé & Günzler[30]. The GSSG generated by GPX was reduced by GR, and NADPH oxidation was monitored at 340 nm. The reaction mixture consisted of 50 mM-potassium phosphate buffer (pH 7·1), 1 mM-EDTA, 3·9 mM-GSH, 3·9 mM-sodium azide, 1 IU/ml GR, 0·2 mM-NADPH and 0·05 mM-H_2O_2.

GR (*EC* 1.6.4.2) activity was assayed as described by Morales *et al.*[31], measuring the oxidation of NADPH at 340 nm. The reaction mixture consisted of 0·1 M-sodium phosphate buffer (pH 7·5), 1 mM-EDTA, 0·63 mM-NADPH and 0·16 mM-GSSG.

Soluble protein concentration was determined using the method of Bradford[32], with bovine serum albumin used as a standard.

Except for SOD, the units of expression of which were indicated earlier, enzyme activity is expressed as units (CAT) or milliunits (G6PDH, GPX and GR) per mg of soluble protein. Here, one unit of enzyme activity is defined as the amount of enzyme required to transform 1 μmol of substrate/min under the aforementioned assay conditions.

Superoxide dismutase isoforms separation

Assays were performed as described previously by Pérez-Jiménez *et al.*[33]. Briefly, non-denaturing PAGE was performed on 10 % acrylamide minigels (MiniProtean II; Bio-Rad Laboratories) and carried out at 4°C. SOD isoenzymes were detected in the gels immediately after the completion of electrophoresis by the photochemical nitroblue tetrazolium staining method. The various types of SOD were differentiated by performing the activity stains in gels previously incubated for 30 min at 25°C in 50 mM-potassium phosphate buffer (pH 7·8), in the absence and the presence of 25 mM-KCN. CuZn–SOD are inhibited by KCN, whereas Mn-SOD are resistant to this inhibitor[34]. The different isoenzymatic bands of SOD were quantified by a densitometric analysis using the software Fujifilm Multigauge version 3.0 for Windows (Fuji Film Co., Ltd). The results for each band were expressed as arbitrary intensity units.

Total glutathione, oxidised glutathione and oxidative stress index

A portion of the liver was homogenised in nine volumes of ice-cold solution containing 1·3 % 5-sulphosalicylic acid (w/v) and 10 mM-HCl. The procedure was carefully performed always on ice in order to avoid the oxidation of glutathione (GSH). Homogenates were centrifuged at 14 000 ***g*** for 10 min at 4°C and the resulting supernatants were analysed immediately.

Total glutathione (tGSH) and GSSG were measured following the method described by Griffith[35] and Vandeputte *et al.*[36] with some modifications. Both tGSH and GSSG analyses were measured at 25°C and the changes in absorbance as consequence of the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were monitored at 405 nm using a microplate reader (ELx808; Bio-Tek Instruments). The optimal substrate and protein concentrations for the measurement of maximal activity for each enzyme were established by preliminary assays.

tGSH was determined using a reaction mixture containing 133 mM-phosphate buffer with 5·8 mM-EDTA at pH 7·4, 0·71 mM-DTNB, 0·24 mM-NADPH and 1·2 IU/ml GR.

GSSG was measured using an aliquot from the solution obtained after 60 min of incubation of 100 μl of sample with 2 μl vinylpyridine and 6 μl 1·5 M-triethanolamine. The reaction mixture contained 122 mM-phosphate buffer with

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5·4 mM-EDTA at pH 7·4, 0·71 mM-DTNB, 0·24 mM-NADPH and 1·2 IU/ml GR.

The results were calculated using standard curves of GSH and GSSG for tGSH and GSSG, respectively. GSH level was calculated by subtracting GSSG from tGSH values. The results are expressed as nmol/g of tissue.

Oxidative stress index (OSI) was calculated as

$$OSI = 100 \times (2 \times GSSG/tGSH).$$

Lipid peroxidation

Lipid peroxidation was determined in the liver. The concentration of thiobarbituric acid-reacting substances was determined according to Buege & Aust[37]. An aliquot of supernatant from the homogenate (100 μl) was mixed with 500 μl of a previously prepared solution containing 15 % (w/v) TCA, 0·375 % (w/v) thiobarbituric acid, 80 % (v/v) HCl 0·25 M and 0·01 % (w/v) butylated hydroxytoluene. The mixture was heated to 100°C for 15 min and after cooling to room temperature, centrifuged at 1500 ***g*** for 10 min. Absorbance in the supernatant was measured at 535 nm and compared with a blank. Concentration was expressed as nmol malondialdehyde/g of tissue, calculated from a calibration curve.

Statistical analysis

Results are presented as means with their standard errors. All statistical analyses were carried out using the SPSS version 18.0 for Windows software package (SPSS, Inc.). After data normalisation, the effect of the assayed parameters was analysed using one-way ANOVA. When F values were significant ($P<0·05$), means were compared using Tukey's honestly significant difference test[38].

Results

Feed efficiency, growth and mortality

No mortality occurred during the trial. Values for final body weight of fish and feed efficiency ranged from 49·9 to 53·4 g and from 0·63 to 0·70 g, respectively, and were not significantly different with regard to the control group.

Glucose 6-phosphate dehydrogenase and antioxidant enzyme activities

Specific activities of antioxidant enzymes and G6PDH in sea bream liver are shown in Table 2. Dietary tea supplementation induced higher CAT activity, while SOD activity was higher in fish fed the methionine-supplemented diets. Lower GPX activity was observed in the Tea + Met group compared to the Met group. Although a tendency to lower values of liver G6PDH activity was observed in fish fed the Tea and Tea + Met diets, differences among the diets were not significant. GR activity was not affected by dietary composition.

Superoxide dismutase isoenzymatic profile

Fig. 1 shows the hepatic SOD isoenzymatic profiles in the experimental groups. In all groups, three SOD isoenzymes were detected. They were named as CuZn-SOD I, CuZn-SOD II and Mn-SOD in the order of increasing mobility. Intensity of CuZn-SOD I isoenzyme was five times higher than that of CuZn-SOD II. Diet composition only influenced the intensity of the Mn-SOD isoenzyme which was significantly higher in the experimental groups than in the control group. However, group fed the tea-supplemented diet had significantly higher Mn-SOD isoenzyme than the Met group and the effect of diet supplementation on the Mn-SOD isoenzyme was additive, as activity was further increased in the group fed the Tea + Met diet.

Non-enzymatic antioxidant defences

Liver glutathione levels are presented in Table 3. tGSH and GSH levels in the experimental groups were not different from those of the control group; however, both tGSH and GSH levels were significantly higher in the Tea + Met group than in the other experimental groups and GSSG was significantly lower in the Tea group than in the other groups.

Oxidative damage markers

Data on oxidative damage biomarker for lipids and OSI are presented in Table 3. Neither lipid peroxidation levels nor OSI index values were affected by dietary composition.

Table 2. Specific activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR) and glucose 6-phosphate dehydrogenase (G6PDH) in the liver of sea bream fed the different diets

(Mean values with their standard errors (n 6))

	CAT (U/mg protein)		SOD (U/mg protein)		GPX (mU/mg protein)		GR (mU/mg protein)		G6PDH (mU/mg protein)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Diets										
Control	189·6^{a,b}	13·7	159·4^{a}	9·7	121·0^{a,b}	9·6	7·78	0·32	163·8	7·7
Met	169·6^{a}	5·4	187·9^{b}	4·4	130·5^{b}	4·6	9·99	0·62	160·2	3·7
Tea	245·2^{c}	14·4	169·8^{a,b}	6·1	112·5^{a,b}	8·9	8·82	0·42	142·3	6·4
Tea+Met	218·9^{b,c}	12·4	181·3^{b}	6·3	96·3^{a}	5·7	8·75	0·77	142·8	7·8

a,b,c Mean values within the same column with unlike superscript letters were significantly different ($P<0·05$).

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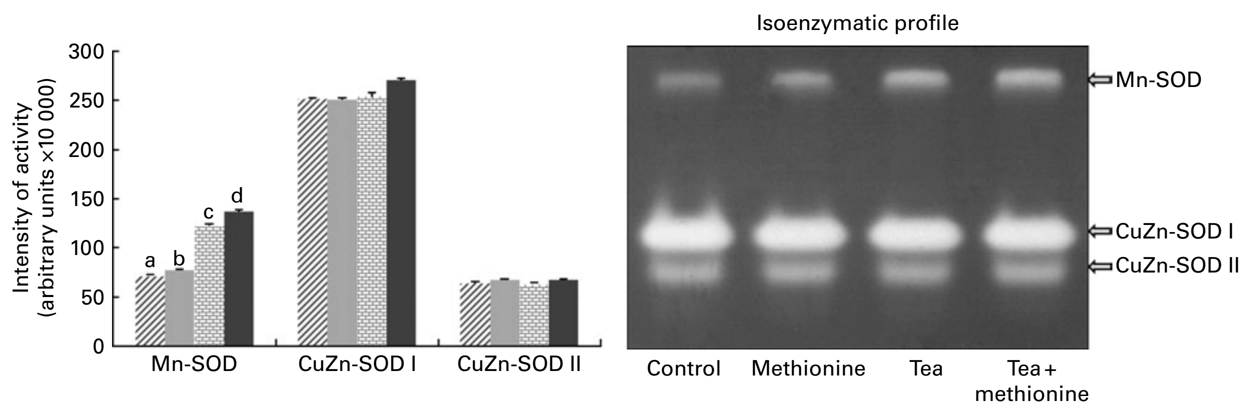

Fig. 1. Superoxide dismutase (SOD) isoenzymatic profile in the liver of sea bream fed on different dietary treatments. Crude extracts (35 µg protein) were loaded onto non-denaturing polyacrylamide gels, and staining of gels was performed by the nitroblue tetrazolium photochemical reduction method. Determination of Mn-SOD was performed by incubating gels with 25 mM-potassium cyanide to inhibit CuZn-SOD activity. Graphics show densitometric analyses of the intensity of activity for Mn-SOD, CuZn-SOD I and CuZn-SOD II. Values are means with their standard errors represented by vertical bars (*n* 2). Data are expressed as arbitrary intensity units of each band. [a,b,c,d] Mean values with unlike letters indicate the influence ($P<0.05$) of diet composition for each determined parameter. ▨, Control; ■, methionine; ▦, tea; ■, tea+methionine.

Discussion

Free radicals and ROS and reactive nitrogen species are continuously generated during the life span of an organism. Consequently, adequate protection systems, both enzymatic and non-enzymatic, are necessary to avoid and/or repair the damage these compounds may cause in the tissues. This is particularly relevant in aquaculture, since the oxidative damage of fish tissues besides affecting animal welfare, is directly associated with the quality and palatability of the final product for the consumer[39–41].

One way of improving the animal's oxidative status is by incorporating in feeds specific components that stimulate the antioxidant defences. Within these supplements, methionine and tea have been evaluated as potential agents for enhancing the defence capacity against free-radical attack in several animal species[7,8,11,13,16,17]. Methionine, besides being a natural scavenging agent of ROS via the methionine sulphoxide reductase system[11,12], is a precursor of cysteine, which is indispensable for glutathione and taurine synthesis, two molecules with an important role in the oxidative defence[9–11]. In the present study, dietary methionine supplementation did not affect liver glutathione levels or CAT and GPX activities, but increased SOD activity. Similarly, Walton *et al.*[42] also did not observe any effect of dietary methionine in liver GSH levels of *Oncorhynchus mykiss*. However, Keembiyehetty & Gatlin[5] observed lower tGSH levels (both reduced and oxidised form) in *Morone chrysops* × *Morone saxatilis* fed on a methionine-deficient diet. According to Li *et al.*[7], methionine deficiency may reduce or even exhaust the main non-enzymatic antioxidant defences such as ascorbic acid, vitamin E or glutathione with consequent negative repercussions in the normal physiological state of the animals. Thus, it may be concluded that dietary methionine supplementation above the requirement level for the species does not seem necessary for maintaining normal hepatic glutathione levels in sea bream juveniles. In this study, dietary methionine supplementation also did not induce significant modifications in the lipid oxidative damage biomarkers, while in *M. chrysops* × *M. saxatilis* a tendency for liver thiobarbituric acid-reacting substances content to decrease was observed in fish fed on increased levels of dietary methionine[7].

In other animals, the important antioxidant capacity of tea polyphenols was already described in several studies[15–17,19]. There are, however, few studies in fish on the effect of dietary tea in antioxidant enzymes. Thawonsuwan *et al.*[8] observed

Table 3. Total glutathione (tGSH), oxidised glutathione (GSSG), reduced glutathione (GSH), oxidative stress index (OSI) and oxidative damage biomarker for lipids in the liver of sea bream fed on different dietary treatments

(Mean values with their standard errors (*n* 6))

	tGSH (nmol/g tissue)		GSSG (nmol/g tissue)		GSH (nmol/g tissue)		OSI (%)		Lipid peroxidation (nmol MDA/g tissue)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Diets										
Control	3082·8[a,b]	128·4	17·6[b]	0·4	3065·1[a,b]	128·4	1·15	0·05	11·02	1·26
Met	2814·9[a]	65·0	14·9[a,b]	0·7	2800·0[a]	65·6	1·07	0·07	10·54	0·46
Tea	2725·4[a]	47·3	12·5[a]	0·9	2713·0[a]	46·9	0·91	0·06	9·45	0·58
Tea+Met	3471·6[b]	120·4	18·1[b]	1·3	3453·5[b]	120·0	1·04	0·07	9·37	0·98

MDA, malondialdehyde.
[a,b] Mean values within the same column with unlike superscript letters were significantly different ($P<0.05$).

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that liver SOD activity of *O. mykiss* fed on diets containing epigallocatechin gallate, a tea catechin, was not different from that of the control, although plasma SOD activity was lower in fish fed epigallocatechin gallate-supplemented diets than in the control group. Accordingly, in the present study, both SOD and GPX activities in liver were unaffected, but CAT activity was enhanced by dietary tea supplementation.

Although four groups of SOD isoenzymes, characterised according to the metal content (Fe, Ni, Mn and Cu–Zn), have been identified in living organisms[2,3], only Mn (Mn-SOD) and Cu–Zn (CuZn-SOD) SOD isoenzymes have been observed in fish[31,33,43–48]. All SOD isoenzymes catalyse the same reaction but they are structurally different and differ in cellular location. CuZn-SOD is found in cytosolic and extracellular fractions[49–54], whereas Mn-SOD is mostly mitochondrial[50,55]. Typically, Mn-SOD represents 5–20 % of total SOD activity in fish[43,56], although there are exceptions, with values reaching 48 % or even 94 % in *Argentina silus* and *O. mykiss*, respectively[43]. In the present study, Mn-SOD ranged from 18·5 to 28·8 % of total activity which is within the normal activity values found in fish. Also, although the activity of CuZn-SOD isoenzymes was unaffected by diet composition, Mn-SOD isoenzyme activity was 75 % higher in the groups fed diets incorporating tea than in the control.

Different studies in mammals and fish observed that CuZn-SOD and Mn-SOD activities were regulated by dietary Cu, Zn and/or Mn content[44–47,57–60]. For example, dietary deficiencies of Cu and Mn in humans decreased CuZn-SOD and Mn-SOD activities, inducing peroxidative damage and mitochondrial dysfunction[57]. In different tissues of rats, Mn-SOD activity increased with the increase of dietary Mn levels[59,60]. In fish, Hidalgo *et al.*[46] observed that Zn-deficiency caused inactivation of two CuZn-SOD isoenzymes in *O. mykiss*. In the same species, Knox *et al.*[44] observed that Mn-SOD activity was sensitive to the availability of Mn in the diet, reducing its activity and even CuZn-SOD activity, when fish were fed on low dietary Mn levels. A similar response of Mn-SOD was observed in *Salmo salar* fed Mn-deficient diets[45]. Recently, Lin *et al.*[47] also found that high levels of dietary Mn increased liver Mn-SOD activity in *Oreochromis niloticus* × *Oreochromis aureus*.

Similarly, the increased Mn-SOD activity observed in sea bream fed the tea-supplemented diets could be related to a higher bioavailability of this metal in these diets. Indeed, tea is a rich source of minerals such as K, Ca, Mg, Al, Mn, Fe and others[61–64]. Tea Mn content depends on several factors such as leaf processing and especially the tea origin, ranging from 390 to 1224 mg/kg for tea leaves without processing, depending on whether the tea was from Africa or Asia[63]. Hence, tea is also an important source of dietary Mn, essential to be incorporated in several metalloenzymes, including SOD. To the best of our knowledge, this is the first study providing evidence that tea inclusion in fish diets could be an important source of Mn with metabolic repercussions on antioxidant mechanisms.

G6PDH plays a fundamental role in NADPH production, which is required in the regeneration of GSSG to GSH by GR. In this study, liver G6PDH activity was not affected by diet composition although a trend for a decrease in activity was noticed in sea bream fed on Tea and Tea + Met diets. This is consistent with the lack of dietary effects on GR activity and on GSH levels. Thus, the slightly lower values of G6PDH observed in the Tea and Tea + Met groups might be related to the putative effects of tea in lipid metabolism rather than in ROS metabolism. Indeed, studies performed both in mammals and fish indicate that tea polyphenols inhibited the activity of lipid digestion enzymes, thus reducing intestinal absorption and serum TAG and cholesterol levels and increased lipolytic activity and β-oxidation and decreased lipogenesis pathways by reducing the activity of the enzymes involved, including G6PDH activity[8,19,24,65,66].

Accordingly, with the overall response of the antioxidant mechanisms measured in this study, the oxidative damage biomarkers measured in the present study were also not affected by diet composition. Thawonsuwan *et al.*[8] showed in *O. mykiss* that dietary epigallocatechin gallate supplementation decreased liver lipid hydroperoxide content, an indication of lipid damage. Similarly, Ishihara *et al.*[6] observed a suppression of lipid oxidation, a deterioration of flesh colour and of microbial growth in fish body during round iced storage in *Seriola quinqueradiata* fed a diet supplemented with green tea.

In conclusion, the results of the present study showed that dietary methionine supplementation increased hepatic SOD enzyme activity, while white tea induced higher hepatic CAT activity and of Mn-SOD isoenzyme synthesis probably due to the increased content and/or bioavailability of Mn in white tea. Methionine and white tea had no synergistic effects on the antioxidant response of sea bream juveniles.

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