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Diets supplemented with seaweed affect metabolic rate, innate immune, and antioxidant responses, but not individual growth rate in European seabass (*Dicentrarchus labrax*)

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Abstract This study investigated the effects of seaweed dietary supplementation on measures of fish performance including aerobic metabolism, digestive enzymes activity, innate immune status, oxidative damage, and growth rate using European seabass (*Dicentrarchus labrax*). Fish were fed for 49 days with three different diets: a control diet (CTRL), a *Gracilaria*-supplemented diet (GR7.5), and a mixed diet (Mix) composed of *Gracilaria*, *Fucus*, and *Ulva* genera representatives. All diets were isoenergetic (22 kJ g^{-1} adjusted for dry matter (DM)), isoproteic (47 %DM), and isolipidic (18 %DM) and tested in triplicate groups of 20 fish (initial body weight 25.5±4.1 g). Final results showed similar growth rates and digestive activities between diets. Maximum and standard metabolic rates and aerobic metabolic scope revealed comparable results for the three diets. In contrast, fish fed with GR7.5 exhibited elevated routine metabolic rate (190.7 mg O_2 kg^{-1} h^{-1}). Fish fed with the GR7.5 and Mix diets had lower alternative complement pathway (ACH50) (62.5 and 63 units mL^{-1} respectively) than CTRL (84 units mL^{-1}) GR7.5 increased lipid peroxidation and cholinesterase levels, as well as glutathione s-transferase activity. Mix diet increased glutathione reductase activity when compared to CTRL. Collectively, our findings suggest that dietary seaweed supplementation may alter seabass metabolic rate, innate immune, and antioxidant responses without compromising growth parameters.

Keywords Digestive enzymes · Growth rate · Innate immune response · Metabolic rate · Oxidative stress · Seaweeds

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Introduction

Marine-derived bioactives have high nutraceutical potential, encompassing anti-inflammatory, antioxidant, and pathogen inhibition proprieties (Okuzumi et al. 1993; Yan et al. 1999; Chandini et al. 2008; Kadam and Prabhasankar 2010; Lordan et al. 2011). Because many performance traits are modulated by specific nutrients (Atherton and Smith 2012), functional diets could affect the physiological, innate immunity, and antioxidant capacities of fish. Interestingly, various types of seaweed possess valuable nutritional compounds such as vitamins, minerals, proteins, polysaccharides, steroids, and dietary fibers (El-Said and El-Sikaily 2013). In addition, seaweed often contains important carotenoids, saturated and polyunsaturated fatty acids (De Almeida et al. 2011), and several phytochemicals advantageous for the control of diseases including hyperlipidemia, thrombosis, tumor, and obesity (Plaza et al. 2008).

There is an emerging interest in determining the role of dietary seaweed supplementation on antioxidant and immune

responses. Understanding the effects of seaweed supplementation is particularly important in aquaculture, because farming protocols often induce stress conditions that affect the immune system responses (Scapigliati et al. 2002), including an increase in reactive oxygen species (ROS) production. In the long term, this situation may compromise growth performance (Leal et al. 2011) and animal welfare and ultimately cause a reduction in revenues. Previous studies suggested that feed supplemented with seaweed may mitigate stress responses and improve vitality, illness resistance (Mohamed et al. 2012; Samad 2013; Araújo et al. 2015), and flesh quality of fish (Hamauzu and Yamanaka 1997; Valente et al. 2015), all parameters with direct commercial interest for the aquaculture sector (Luna-Acosta et al. 2011).

The determination of innate immune and oxidative stress responses can be used to monitor fish condition by analyzing lysozyme, peroxidase, and complement systems (i.e., non-specific protection systems) that defend fish against bacteria, fungi, and parasites (Sunyer and Tort 1995; Tort et al. 1996) and by measuring the oxidative stress enzymes activities that are involved in the clearance of ROS (Blier 2014).

However, maintaining a functional immune system is energetically and nutritionally costly (Lailvaux and Husak 2014), requiring energy and nutrients that could have been allocated for growth (Sheldon and Verhulst 1996). Indeed, upregulation of the immune system is associated with elevated energy and protein expenditures (Lochmiller and Deerenberg 2000). Newsholme and Newsholme (1989) compared the metabolic costs of elicited macrophages by infection to a maximally functioning heart, emphasizing the need of macrophages for large amounts of ATP. The fuel for these energetic demands derives from augmented processes such as lipolysis, proteolysis, and glycolysis (Lochmiller and Deerenberg 2000). Consequently, an enhanced immune system could be metabolically costly and result in allocation of energy away from somatic growth (Bashir-Tanoli and Tinsley 2014). If confirmed, the influence of this mechanism on somatic growth might counter any positive influence of seaweed on fish health and survival.

Two important physiological parameters to understand aerobic energy metabolism in ectothermic animals are standard metabolic rate (SMR) and maximum metabolic rate (MMR), measured as the lower and upper boundaries of oxygen consumption rates (Norin and Malte 2011; Roche et al. 2013). Subtracting SMR from MMR provides a limit measure for the total aerobic expanse of simultaneous metabolically demanding processes (Clark et al. 2013; Norin and Malte 2011), known as the aerobic metabolic scope (AMS). Many fish species show spontaneous bouts of activity, typically fueled by aerobic energy with metabolism fluctuating around an average level, termed the routine metabolic rate (RMR) (Wieser 1985). Previous studies have suggested that metabolic rates are coupled with immune responses in endothermic animals (Downs et al. 2013). For example, Ots et al. (2001) found that in wintering adult birds, even a non-pathogenic immune challenge affects basal metabolism (equivalent to SMR in ectothermic animals). The effects of diet on metabolism, and interactions with immune system variables, are largely unknown in fish, in particular in relation to aquaculture.

The objective of this study was to examine metabolic and immunological effects of diets supplemented with seaweed, using European seabass *Dicentrarchus labrax* as a model. Specifically, we tested the hypothesis that dietary seaweed supplementation enhances the innate immune and antioxidant responses of fish. Because the immune system is energetically expensive, such enhanced responses could be associated with elevated metabolic rates and therefore decreased growth performance.

Materials and methods

This experiment was carried out under the guidance of a Laboratory Animal Science-certified supervisor (1005/1092, DGV-Portugal, C category, FELASA), according to European Union directives (2010/63/UE).

Experimental diets

Gracilaria spp., *Ulva* spp., and *Fucus* spp. were reared in an integrated multi-trophic aquaculture (IMTA) system (Abreu et al. 2011) at ALGAPlus, Lda. (Ílhavo, Portugal). Crude seaweeds were dried and added as supplements (concentrations of 2.5 and 7.5 % adjusted for dry matter content (DM)) to the experimental diets. The experiment comprised three isoenergetic (22 kJ g^{-1} DM), isoproteic (47 %DM), and isolipidic (18 %DM) diets. All diets were formulated with the same basic ingredients detailed in Table 1. Diet formulation and proximate composition are presented in Table 2. Dietary protein and fat levels were adjusted in accordance with recommendations for seabass (FAO 2005–2015; Webster and Lim 2002). Dietary macronutrient balance after seaweed incorporation was achieved by reducing contents of soy protein and wheat meal.

All ingredients were finely grounded (hammer mill, 0.8-mm sieve), mixed, and then extruded (twin screw extruder, 2.0-mm pellet size, SPAROS, Portugal). Diets were finally dried at 45 °C for 12 h and stored at 4 °C until use.

Fish and rearing conditions

A total of 180 seabass (initial body weight: 25.5±4.1 g) were obtained from a fish farm (MARESA) in Spain and transported to University of Porto in Portugal. For the physiological and immunological sampling, 27 random seabass were marked with Passive Integrated Transponder (PIT) tags, and body mass was measured (to nearest 0.1 g) (Boel et al. 2014). Next, seabass were acclimated to a basal diet (CTRL) and the new holding facilities for 2 weeks and then randomly distributed in nine tanks of 80 L each (n=3 tanks $treatment^{-1}$).

Table 1 Proximate composition of the ingredients present in the experimental diets

Ingredients	Proximate composition				
	%DM	Ash (%DM)	Fat (%DM)	Protein (%DM)	Energy (DM)
Gracilaria	93.4	34.3	1.1	25.9	12.8
Ulva	85.7	34.8	1.5	23.2	12.1
Fucus	87.0	20.7	3.4	17.2	15.1
Wheat gluten	92.5		3.8	84.7	23.3
Corn gluten	90.3		4.0	69.6	23.3
Soy protein concentrate	92.0		1.3	67.9	19.6
Whole peas	86.2		3.4	23.3	18.3
Wheat meal	89.6		3.5	10.7	18.2
Fishmeal standard	87.1		12.7	76.8	22.2
Fishmeal SOLOR	90.2		8.4	73.4	20.2
Soy bean meal 48	95.5		2.0	48.6	18.2
Rapeseed meal	90.2		2.7	40.1	19.5

DM dry matter

The tanks were connected to a flow-through seawater system providing environmental conditions optimized for seabass (30 ‰ salinity, oxygen content ≥80 % air saturation (O_{2sat}), 20± 1 °C, ammonia ≤0.5 mg L^{-1}, and nitrites ≤3 mg L^{-1}).

The experimental diets were randomly assigned to the tanks establishing triplicate groups of fish per treatment that were hand-fed, twice a day (09:30 and 17:30 h) for 49 days until apparent visual satiety. Food intake was determined by weighing the daily amount of diet distributed to each tank (Araújo et al. 2015). Subsequently, the three tagged fish per tank (n=9 fish treatment^{-1}) were sampled for analyses of growth performance in addition to physiological and immunological variables as outlined below.

Respirometry

Three static respirometers (each 0.42 L) and a mixing pump were submerged in a 150-L opaque tank. The tank was in the same room as the dietary treatment tanks and filled with water (30‰ salinity) from the same source as used for the dietary groups. The tank was partly covered by polystyrene sheets to isolate experimental fish from outside stimuli. Air stones were used to maintain water O_{2sat} in the tank at a normoxic level (>95 % O_{2sat}), and water temperature was maintained at 20±0.1 °C using a temperature-controlling instrument (TMP-REG; Loligo Systems; Denmark). Water in the tank was recirculated through a loop consisting of a separate biological filter (TMC-IBERIA, Portugal) and a UV sterilizer (UV-10000; Tetra Pond, Germany) (Svendsen et al. 2015).

Measurements of MO_2 (mg O_2 kg^{-1} h^{-1}) were carried out every 7 min using intermittent flow respirometry, allowing long-term (>24 h) repeated measurements (Forstner 1983; Steffensen 1989). Each respirometer was fitted with two inlet and outlet ports and two water pumps as described previously (Svendsen et al. 2012). Oxygen partial pressure (kPa) was measured inside the respirometers at 1 Hz using galvanic oxygen sensors (Mini DO Probe; Loligo Systems; Denmark). Oxygen levels above 80 % O_{2sat} in the respirometers were secured using flush pumps that were activated intermittently and controlled by AutoResp software (Loligo Systems). Between flushings, the declining oxygen partial pressure (kPa) was recorded to calculate MO_2 using the equation:

$$MO_2 = \frac{KV\beta}{M} \quad (1)$$

where K is the linear rate of decline (kPa h^{-1}) in the oxygen content over time (h) in the respirometer, V is the volume of the respirometer (L) corrected for the volume of fish, β is the solubility of oxygen in the water (mg O_2 L^{-1} kPa^{-1}) (β=0.3683), and M is the body mass of the fish (kg). The coefficient of determination (r^2) associated with each MO_2 measurement was always >0.98, similar to previous studies (Schurmann and Steffensen 1997; Svendsen et al. 2013). Corrections of background respiration (i.e., microbial respiration) followed Rosewarne et al. (2015). Measurements of MO_2 were adjusted to a common body mass of 53 g (Claireaux et al. 2006; Herskin and Steffensen 1998) using a mass exponent of 0.77.

Seabass were fasted for 48 h to ensure a postabsorptive state prior to sampling (Axelsson 2002; Dupont-Prinet et al. 2010). Fish were transferred to respirometers to determine MMR and left undisturbed for 24 h for measurements of RMR, SMR, and AMS as described below. For each 24-h period, data collection involved a fish from each of the three dietary groups. Selection of respirometer for each fish was randomized. Promptly after the respirometry trials, seabass were euthanized by an anesthetic overdose of ethylene glycol monophenyl ether (Merck, Germany) for measurements of individual growth parameters as well as innate immune and antioxidant systems.

Table 2 Formulation and proximate composition of the experimental diets

	Dietary treatments		
	CTRL	GR7.5	Mix
Fish ingredients (%DM)			
Fishmeal standard	10.0	10.0	10.0
Fishmeal SOLOR	20.0	20.0	20.0
Soy protein concentrate (Soycomil)	11.8	10.0	10.3
Wheat gluten	4.0	4.0	4.0
Corn gluten	8.0	8.0	8.0
Soybean meal 48	12.0	12.0	12.0
Rapeseed meal	5.0	5.0	5.0
Wheat meal	9.0	3.3	3.0
Peas gelatinized (Aquatex 8071)	3.2	3.2	3.2
Fish oil - COPPENS	6.5	6.5	6.5
Soybean oil	4.0	4.0	4.0
Rapeseed oil	4.0	4.0	4.0
Vit & Min Premix PV01	1.0	1.0	1.0
Binder (Kieselguhr)	0.5	0.5	0.5
Antioxidant powder (Paramega)	0.2	0.2	0.2
MCP	0.5	0.5	0.5
L-Lysine	0.2	0.2	0.2
DL-Methionine	0.1	0.1	0.1
Gracilaria		7.5	2.5
Ulva			2.5
Fucus			2.5
Proximate composition			
Dry matter (%DM)	94.74	95.90	94.83
Ash (%DM)	8.59	10.76	10.55
Crude protein (%DM)	47.80	47.76	47.90
Crude fat (%DM)	19.09	19.57	19.28
Gross energy (kJ g^{-1} DM)	22.70	22.41	22.39

DM dry matter

Maximum metabolic rate, routine metabolic rate, standard metabolic rate, and aerobic metabolic scope

MMR was measured using a standard chase protocol (Cutts et al. 2002). Briefly, individual seabass were transferred to a circular trough and chased to exhaustion (Dupont-Prinet et al. 2010; Roche et al. 2013; Svendsen et al. 2014). Upon exhaustion, identified by no further response after 5 min of manual stimulation, seabass were transferred to the respirometer where MO_2 measurements started immediately. MMR was the highest of three consecutive MO_2 measurements (Svendsen et al. 2012).

Fish were left undisturbed in respirometers after the measurement of MMR. The mean value of MO_2 measurements collected during the last 4 h of the full 24-h respirometry period was used to estimate routine metabolic rate (RMR) (between 09:00 and 13:00 h the day after the fish was introduced in to the respirometer) following Killen et al. (2012a).

SMR in individual fish was estimated using two different methods: (1) SMR was estimated as the average of the lowest 10 MO_2 values collected over the 24-h periods (Schurmann and Steffensen 1997; Svendsen et al. 2014). This method to estimate SMR was employed because it provides measurements that are repeatable in individual fish (Norin and Malte 2011); (2) SMR was estimated as the lowest 10th percentile of all MO_2 values collected over the 24-h periods (Killen et al. 2012b).

Growth performance

Growth response parameters were evaluated by calculating weight gain (2), daily growth index (3), voluntary feed intake (4), and feed conversion ratio (5).

Weight gain (WG) was calculated as:

$$\mathrm{WG} = Wf - Wi \tag{2}$$

where *Wi* is the initial body weight at the start of the feeding trial and *Wf* is the final body weight at the end of the respirometry trial;

Daily growth index (DGI) was calculated as percentage of body weight increase per day (% BW day^{-1}):

$$\mathrm{DGI} = \frac{\mathrm{FBW}^{1/3} - \mathrm{IBW}^{1/3}}{t} \times 100 \tag{3}$$

where *t* is the feeding duration in days and FBW and IBW are the final and initial body weight, respectively;

Voluntary feed intake (VFI; % BW day^{-1}) was determined as:

$$\mathrm{VFI} = \frac{\dfrac{\text{Total feed intake}}{\text{Average body weight (ABW)}}}{t} \times 100 \tag{4}$$

where $\mathrm{ABW} = \frac{\mathrm{IBW} + \mathrm{FBW}}{2}$

Feed conversion ratio (FCR) was calculated as:

$$FCR = \frac{\text{Total feed intake}}{\text{Weight gain}} \times 100 \tag{5}$$

Biochemical analysis

Immediately after the respirometry trials, blood was collected and centrifuged and resulting plasma stored for posterior analysis, and the liver and intestines were sampled for enzymatic screening. All samples were immediately stored at −80 °C.

Fish innate immune status was accessed by plasma contents of lysozyme and peroxidase activity as well as alternative complement pathway response (ACH50). Lysozyme is a bacteriolytic protein widely used because of its capacity to cleave bacterial peptidoglycans (Guardiola et al. 2014). It was

measured by a turbidimetric assay based on *Micrococcus lysodeikticus* lysis and using hen egg white lysozyme (Sigma, Portugal) as standard (Valero et al. 2014).

Peroxidase levels were used as indicator of activation state of circulating leukocytes that are known to increase in response to infection or stress (Cuesta et al. 2006). Peroxidase levels were determined by 3,3′,5,5′-tetramethylbenzidine hydrochloride (Sigma, Portugal) reduction (Quade and Roth 1997).

Hemolytic activity of the alternative complement system was assayed via rabbit red blood cells agglutination as described by Sunyer and Tort (1995).

Antioxidant systems are divided by enzymatic, catalase, and peroxidases and non-enzymatic such as lipid peroxidation processes. To quantify oxidative damages, the liver was used and its protein content determined as described by Bradford (1976).

Lipid peroxidation was accessed by the presence of its by-product, thiobarbituric acid reactive substances (Ohkawa et al. 1979). Catalase (EC 1.11.1.6.) was determined by its action over peroxide hydrogen according to previous studies (Clairborne 1985). Glutathione s-transferase (EC 2.5.1.18) was evaluated by conjugation of glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (Habig et al. 1974). Glutathione peroxidase (EC 1.11.1.9.) and reductase (EC 1.8.1.7) were studied by oxidation of NADPH based on previous studies (Cribb et al. 1989; Mohandas et al. 1984). Total glutathione was evaluated by the formation of 5-thio-2-nitrobenzoic acid as detailed by Baker et al. (1990). Cholinesterase was measured using acetylcholine as substrate and assessed according to Ellman et al. (1961).

The intestines were homogenized for digestive enzymes extraction (Rungruangsak-Torrissen 2007) and the protein content analysis performed according to previous studies (Lowry et al. 1951). Amylase was examined by formation of maltose (Bernfeld 1951). Measurements of nitroaniline production allowed trypsin and chymotrypsin determination (Rungruangsak-Torrissen and Sundby 2000). Lipase assay was carried out using *p*-nitrophenyl for substrate as described by Winkler and Stuckmann (1979).

Statistical analysis

Statistical analyses followed the methods outlined by Zar (1999). Data were tested for normality and homogeneity of variances using Kolmogorov–Smirnov and Levene's test, respectively. Then, data were analyzed with one-way analysis of variance (ANOVA) to test for differences between dietary treatments. When this test showed significance, individual means were compared using Tukey's test. Significant differences were considered when $P<0.05$. When ANOVA assumptions were not fulfilled, data were submitted to the non-parametric tests (Kruskal–Wallis test), followed by Mann–Whitney test when needed. All values are reported as means ±S.E. unless noted otherwise. The tests were carried out using SigmaPlot 11.0 (Systat Software, USA).

Table 3 Standard metabolic rate (SMR), maximum metabolic rate (MMR), and aerobic metabolic scope (AMS) (mg O_2 kg^{-1} h^{-1}) in seabass reared at 20 °C and fed with three different diets

Diet	SMR[a]	SMR[b]	MMR	AMS
CTRL	117.61±4.88	122.62±15.41	420.01±19.29	302.40±0.53
GR7.5	124.98±6.00	134.28±17.56	455.06±21.71	330.08±14.98
Mix	112.76±5.43	120.37±14.01	432.41±22.04	319.65±16.36

$N=9$ for each diet. No statistical differences between diets ($P>0.05$). Values presented as mean±S.E. No differences in AMS were detected ($P>0.05$) regardless of the method used to calculate SMR

[a] Determined using the 10 lowest MO_2 values (i.e. Method 1).

[b] Determined using the 10 % lowest MO_2 values (i.e. Method 2).

Results

Metabolic rates

Metabolic rates were estimated after correcting MO_2 for background respiration and body mass. No statistical differences were found between dietary treatments for SMR (Table 3). Both supplemented diets (GR7.5 and Mix) revealed higher MMR values than the control diet (CTRL); however, differences were not significant ($P>0.05$) (Table 3). AMS was calculated by subtracting SMR from MMR and demonstrated no statistical difference ($P>0.05$) (Table 3) between diets, although mean values were higher for both supplemented diets. This result was consistent for both methods to estimate SMR (methods 1 and 2; see above).

RMR differed significantly between diets (Fig. 1). GR7.5 revealed higher RMR than CTRL ($P<0.05$) whereas mix was intermediate. This finding indicated that GR7.5 exhibited higher routine metabolic expenditures than CTRL.

Morphometric and growth data

Growth parameters showed no statistical differences between diets (Table 4) indicating no direct effect of seaweed supplementation on fish growth capacity.

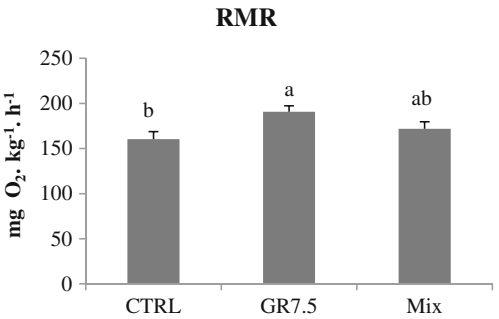

Fig. 1 Routine metabolic rate (RMR; mg O_2 kg^{-1} h^{-1}) in seabass reared at 20 °C and fed with three different diets. $N=9$ for each diet. *Different letters* indicate statistical differences ($P<0.05$) between diets. Values presented as mean±S.E.

Table 4 Growth parameters of seabass reared at 20 °C and fed with three different diets

Diets	WG (g)	FCR	DGI (%BW. day^{-1})	VFI (g kg^{-1} day^{-1})
CTRL	22.56±1.57	1.20±0.08	1.26±0.06	1.24±0.05
GR7.5	22.19±1.64	1.20±0.09	1.31±0.10	1.30±0.04
Mix	19.82±1.66	1.31±0.11	1.12±0.09	1.19±0.04

N=9 for each group. No statistical differences between diets (P>0.05). Values presented as mean±S.E. See Eqs. 2–5 for definitions of the growth parameters

Table 5 Digestive enzymes study of seabass reared at 20 °C and fed with three different diets

Diets	Amylase (mU mg^{-1}$_{protein}$)	Lipase (mU mg^{-1}$_{protein}$)	Chymotrypsin (mU mg^{-1}$_{protein}$)	Trypsin (mU mg^{-1}$_{protein}$)
CTRL	1786.69±186.35	113.71±18.68	4.00±0.52	16.20±3.74
GR7.5	1987.67±88.94	106.10±9.41	3.80±0.43	22.40±3.25
Mix	1831.03±150.22	107.23±13.30	4.64±0.55	15.81±1.89

N=9 for each dietary group. No statistical differences between diets (P>0.05). Values presented as mean±S.E. All data normalized by protein content

Digestive enzyme data

Digestive enzyme analysis provides an overall indicator of fish nutritive physiology. There was no effect of diets on seabass digestive enzymes (Table 5).

Immune parameter data

Plasma analysis showed no differences between dietary treatments for lysozyme and peroxidase contents (Table 6). Conversely, when testing for hemolytic capacity of the alternative pathway complement system (ACH50) (Fig. 2), clear differences were found between control diet and supplemented diets (GR7.5 and Mix) (P<0.05). This finding indicated that diet may influence how and how fast pathogens are opsonized and destroyed by complement proteins.

Oxidative stress data

Analysis of liver antioxidant systems revealed no significant differences (P<0.05) for catalase (CAT), total glutathione (GT), glutathione peroxidase (GPx), and oxidized glutathione (GSSG) (Table 7). Lipid peroxidation (LPO) revealed higher levels of lipid oxidation in GR7.5 and mix (P<0.05) (Fig. 3a).

Table 6 Innate immune parameters analyzed in plasma from seabass reared at 20 °C and fed with three different diets

Diets	Peroxidase (units mL^{-1})	Lysozyme (units mL^{-1})
CTRL	11.29±0.82	386.81±38.22
GR7.5	12.58±1.97	366.05±37.41
Mix	17.32±3.34	466.67±45.50

N=9 for each group. No statistical differences between diets (P<0.05). Values presented as mean±S.E.

Cholinesterase enzyme (ChE) followed the LPO pattern and presented differences between control and both supplemented diets, indicating higher circulating esterase content in GR7.5 and mix (Fig. 3b) (P<0.01). Considering cell oxidative state, a higher effort was expected by other antioxidant enzymes, which we found for glutathione s-transferase (GST) in GR7.5 (Fig. 3c) (P=0.01). Also, glutathione reductase (GR) showed higher levels for the mix diet (Fig. 3d) (P<0.05). Collectively, these findings indicated that *Gracilaria* inclusion level can be a key factor for the dietary effects on the oxidative stress condition.

Discussion

This study examined the use of seaweed-supplemented diets to improve fish growth performance and health. Specifically, we tested the hypothesis that enhancing the innate immune and antioxidant responses using seaweed could alter fish metabolism and therefore growth. Our data

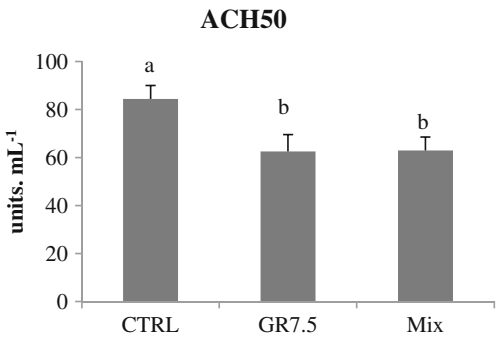

Fig. 2 Plasma alternative complement (ACH50; units mL^{-1}) content of seabass reared at 20 °C and fed with three different diets. Values presented as mean±S.E. *Different letters* indicate statistical differences (P<0.05) between diets

Table 7 Levels of catalase (Cat), total glutathione (GT), glutathione peroxidase (GPx), oxidized glutathione (GSSG), reduced glutathione (GSH), and reduced over oxidized glutathione ratio (GSH/GSSG) in seabass liver at 20 °C

Diets	Cat (μM min^{-1} $mg^{-1}_{protein}$)	GT (nM min^{-1} $mg^{-1}_{protein}$)	GPx (nM min^{-1} $mg^{-1}_{protein}$)	GSSG (nM min^{-1} $mg^{-1}_{protein}$)	GSH (nM min^{-1} $mg^{-1}_{protein}$)	GSH/GSSG (nM min^{-1} $mg^{-1}_{protein}$)
CTRL	72.62±13.99	15.16±4.72	0.54±0.13	7.19±2.13	7.08±0.55	1.09±0.10
GR7.5	77.09±10.82	17.06±8.55	0.63±0.19	6.19±2.17	10.88±2.25	1.70±0.20
Mix	72.86±8.09	11.75±2.26	0.41±0.15	5.68±2.46	6.55±0.56	1.61±0.40

N=9 for each group. No statistical differences between diets (P<0.05). Values presented as mean±S.E. Fish were fed with three different diets

revealed no effects of seaweed on fish growth and metabolism measured as SMR, MMR, and AMS. In contrast, we found evidence of altered innate immune and antioxidant responses in fish fed with the seaweed-supplemented diets. Collectively, the data suggest that seaweed supplementation can modulate innate immune and antioxidant responses without affecting fish growth and metabolism measured as SMR, MMR, and AMS.

SMR and MMR provide the lower and upper boundaries for aerobic energy metabolism. The present study found no influence of diets on SMR and MMR in seabass (Table 3). As a direct result, AMS also showed no dietary effect. Conversely, RMR was higher in fish fed with the GR7.5 diet suggesting that the routine activity level was directly influenced by ingestion of *Gracilaria*. Shao et al. (2013) found a similar result for mice fed with *Gracilaria eucheumoides*, where an anti-fatigue effect of this diet was evaluated. Their work indicates altered expression levels in genes responsible for energy metabolism and transport, as well as plasmatic markers of energetic metabolism, when mice were fed with increasing levels of *Gracilaria*. Elevated RMR may influence growth (Burton et al. 2011; Killen et al. 2011) because energy is allocated from storage to activity. This would suggest that seabass fed with the GR7.5 diet should be growing less, or feeding more, because they exhibited elevated RMR. However, we found no effects of diets on the growth and feed intake parameters (Table 4). These findings support the intriguing possibility that the GR7.5 diet allowed seabass to allocate resources to growth more efficiently and thereby compensated for the extra energy allocated to routine activity (i.e., RMR). While this hypothesis warrants further study, it is possible that the modulated innate immune and antioxidant responses associated with the GR7.5 diet allowed the fish to allocate resources differently and grow more efficiently.

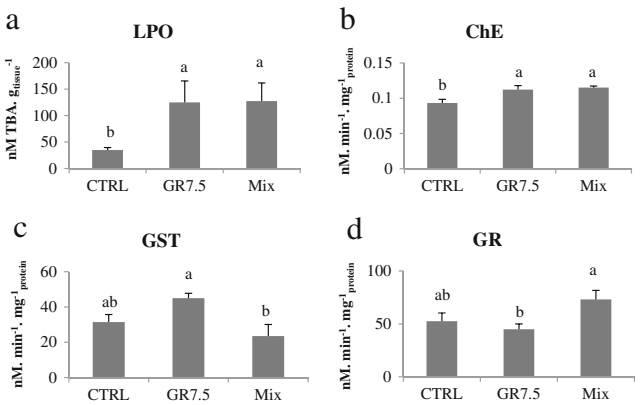

Fig. 3 Liver contents of lipid peroxidation (**a**), cholinesterase (**b**), glutathione *s*-transferase (**c**), and glutathione reductase (**d**) in seabass reared at 20 °C. Fish were fed with three different diets. *Different letters* indicate statistical differences (P<0.05) between diets. Values presented as mean±S.E.

Detrimental components in diets may influence digestive functions (Hartviksen et al. 2014; Krogdahl et al. 2010). The present study, however, found no significant differences between diets in terms of digestive enzymes (Table 5), which supports two possible conclusions. Firstly, the basic composition of the three diets may have met all the nutrient requirements of seabass (Dias et al. 2005). If so, seaweed represents no antinutritional effect over seabass digestive enzymes and we would expect to see no differences between diets. Secondly, the fact that we found no significant differences in digestive enzymes might be related to the non-absorptive state that fish were in, prior to testing, because fish were subjected to a 48-h fasting period (Eroldoğan et al. 2008; Hidalgo et al. 1999) for the respirometry trials. Thence, the lack of nutritional stimulation of the digestive enzymes production may have inhibited the expression of the possibly different responses between diets. Further studies are needed to examine the two possible conclusions in detail. Nevertheless, despite the fact that seabass is a piscivorous species (Le Boucher et al. 2013) that does not feed on seaweed in the wild, our data support the conclusion that seaweed dietary supplementation has no deleterious effect on this species digestion and growth.

Elevated metabolism may be associated with upregulated specific and non-specific immune mechanisms (Bashir-Tanoli and Tinsley 2014; Burton et al. 2011; Skinner et al. 2010). According to Råberg et al. (2002), variations in metabolic rates may be attributed to differential energetic costs associated with stimulating innate or adaptive immune defenses. Therefore, adaptive (and not just innate) immune markers should be analyzed in an attempt to correlate the results found for RMR in GR7.5 % diet, since no direct correlation was established with the innate parameters analyzed in this work. However, we

found that the plasma alternative complement (ACH50) was elevated in the CTRL group (Fig. 2), while this group did not exhibit elevated metabolism (i.e., SMR, MMR, and AMS). Elevated ACH50 in the CTRL group means higher hemolytic capacity (i.e., upregulated immune status) when compared with GR7.5, yet the CTRL diet caused a low RMR (Fig. 1). Using rainbow trout (*Oncorhynchus mykiss*) as model species, previous studies with *Gracilaria* dietary inclusion at 5 and 10 % levels showed an increase in ACH50 in the 5 % inclusion group, whereas in the 10 % inclusion group, a decrease was found, although both appeared higher than control (Araújo et al. 2015). These findings suggest that the immunostimulant properties of *Gracilaria* may decay above 5 % inclusion, which seems to be in accordance with the results found in the present work for 7.5 % inclusion. Results obtained by Valente et al. (2015) when using 5 and 10 % levels of *Ulva* supplementation in Nile tilapia (*Oreochromis niloticus*) showed an increased ACH50 in fish fed with 10 % *Ulva* supplementation and no differences between the CTRL and the 5 % *Ulva*-supplemented diet, which may explain the low ACH50 values found for mix diet in our work.

Oxidative stress biomarkers are widely used as a tool to determine toxicity in aquatic ecosystems (Almeida et al. 2012; Gravato et al. 2006; Rodrigues et al. 2013). In the present study, we aimed at identifying if seaweeds, known for their antioxidant abilities (Faten et al. 2009; Souza et al. 2012; Woo et al. 2013), may protect fish from the deleterious effects of ROS. Our data on lipid peroxidation (LPO) (Fig. 3a), when compared to CTRL, showed a remarkable increase in seabass fed with supplemented diets. These results suggest that seaweed supplementation increases lipid layer degradation. This conclusion is in accordance with a recent study (Woo et al. 2013) that revealed a dose-dependent inhibition in lipid accumulation in cells treated with *Gracilaria verrucosa* extracts. Moreover, ROS production increases with physical activity (Liu et al. 2000), due to high energy requirements of muscle cells (McClelland 2004). In this sense, the lipid peroxidation increase in fish fed with GR7.5 may be directly linked with the observed increase in RMR.

The role of esterase activity in lipid metabolism is not clear. Nonetheless, these enzymes are considered capable of hydrolyzing water-soluble carboxylic acids (Tocher 2003) and are precursors of lipases that hydrolyze lipids in the digestive tract (Bele et al. 2014). In this study, we analyzed cholinesterase activity (Fig. 3b), where a pattern similar to LPO (Fig. 3a) was found, with supplemented diets presenting higher levels of this enzyme. The results may suggest that seabass fed with CTRL diet suffer an esterase activity inhibition. While similar findings were reported recently (Andrade et al. 2013), the biological implications of the inhibition remain unclear.

Analyzing specific antioxidant enzymes, we performed an activity analysis of glutathione s-transferase (GST) (Fig. 3c), an enzyme responsible for removing reactive oxygen intermediates (Tocher 2003). According to previous studies (Leaver et al. 1993), this enzyme is also involved with prostaglandin biosynthesis and steroid isomerization, having an essential role in detoxification, as well as metabolism and transport. In our study, we found higher GST activity in seabass fed with *Gracilaria* when compared with mix diet (Fig. 3c). CTRL diet showed no differences from the supplemented diets. Considering that mix diet was supplemented with a lower level of *Gracilaria*, its combination with *Ulva* and *Fucus* inhibited GST activity, showing that this enzyme antioxidant potential may rely in *Gracilaria* and is sensitive to its supplementation level.

Glutathione reductase (GR) is the enzyme responsible for recovering the oxidized glutathione (GSSG), to its reduced form, inhibiting the exhaustion of the electron donor, reduced glutathione (GSH) (Srikanth et al. 2013; Tocher 2003). Its activity can therefore represent the antioxidant restoration potential. Total glutathione (GT) represents the state of the peptide glutathione, summing both reduced and oxidized forms. Its level is related to antioxidants depletion, considering the incomplete recovery of the GSSG (Eroglu et al. 2014; Owen and Butterfield 2010). In our data, no differences were found in GSSG, GSH, GT, and GSH/GSSG ratio (Table 7). The significant increase in GR observed in the mix diet (Fig. 3d) suggests higher capacity to mold the glutathione metabolism state in this dietary treatment. The differences found for GR (Fig. 3d) might also be related to the tendency for lower GSH concentrations in mix diet (Table 7), placing a greater demand in the reduction of GSSG to sustain adequate GSH/GSSG levels. Overall, the differences found in antioxidant enzyme activities in seabass fed with mix diet may be interpreted as the result of a synergetic effect between *Gracilaria*, *Ulva*, and *Fucus*. This hypothesis will remain unanswered due to the lack of research on the effect of *Ulva* and *Fucus* on the antioxidant system in fish.

In conclusion, seaweed supplementation in seabass feeds does not impair growth at 2.5 and 7.5 % supplementation levels. The present results have implications for aquaculture because dietary supplementation may be used to prevent economic losses related to fish disease and stress by improving survival. The use of crude seaweed for experimentation leaves a gap in the mechanistic understanding of the biological effects observed in seabass because the specific chemical compounds responsible for the observed effects remain unknown. Therefore, further studies would benefit from using specific seaweed extracts to further our understanding of the effects on fish metabolic rate, innate immunity, and antioxidant capacity.

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