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ORIGINAL RESEARCH

Effect of dietary seaweed supplementation on growth performance, antioxidant and immune responses in European seabass (*Dicentrarchus labrax*) subjected to rearing temperature and salinity oscillations

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Abstract The current study evaluated the effects of dietary seaweed supplementation in European seabass juveniles (*Dicentrarchus labrax*) subjected to rearing temperature and salinity oscillations, simulating natural variations in pond aquaculture conditions. Two experimental diets where formulated: a control diet (CTRL) with no supplementation and one supplemented with 7.5% seaweed mix (SW 2.5% *Fucus* sp., 2.5% *Gracilaria* sp. and 2.5% *Ulva* sp.). Seabass from both dietary groups (40.7 g initial body weight) was subjected to either combined salinity and temperature oscillations, or to fixed conditions. Growth performance, innate immune, and oxidative stress responses were evaluated. Results showed that seaweed supplementation had no significant effect on the analyzed parameters. However, environmental oscillations revealed significant effects on growth performance and oxidative stress response. Fish subjected to salinity and temperature oscillations had a significantly lower weight gain and daily growth index than those subjected to fixed conditions, regardless of dietary treatment. Total glutathione, oxidized glutathione, and catalase increased in fish subjected to oscillatory conditions. Lysozyme and peroxidase were not influenced by either diet or environmental conditions. In conclusion, this particular dietary seaweed mix supplementation did not mitigate the negative effects of environmental oscillations on growth performance and innate immune responses in European seabass.

Keywords Seaweeds · Dietary supplementation · Immunostimulants · European seabass · Environmental stressors · Immune and oxidative stress response

Introduction

In intensive aquaculture, fish are exposed to several stress factors of biological, chemical, and/or physical origin, that can affect fish well-being and immune status (Colombo et al. 1990). Sub-optimal biotic and abiotic

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conditions are common causes for growth inhibition and mass mortalities (Sakai 1999). For instance, Vadstein et al. (1993) suggested that the majority of problems associated with fish production are caused by bacteria commonly present in hatcheries and emphasizes the importance of fish homeostasis. In addition, Björnsson and Ólafsdóttir (2006) showed a negative influence of ammonia and nitrogen high concentrations in cod rearing. It is important to highlight that fish have great adaptation strategies to counteract unfavorable environmental conditions. Hence, these constrictions will more often lead to decreases in their metabolic rates and reduction of feed intake than necessarily disease or death (Barnabe 2003).

In fish, feed intake and the kinetics of digestive processes are limited by temperature (Arana 1997). On a cellular level, temperature variations can affect protein and lipid conformation, as well as genetic modulation, altering transcription, translation, or path activation (Sengupta and Garrity 2013). Therefore, temperature influence over molecular kinetics modulates the occurrence of enzyme–subtract complexes, affecting numerous biochemical activities (Cornish-Bowden 1979). This kind of alterations induces an initial stress phase, increasing the formation of reactive oxygen species (ROS) and energy demands, potentially causing damage in proteins, lipids and DNA (Lushchak and Bagnyukova 2006). Similarly, salinity influences multiple aspects related to fish metabolism. Euryhaline fish have developed specific biochemical and physiological machinery that enables them to perceive and adapt to a wide range of salinity oscillations. When facing severe environmental conditions oscillations, compensation mechanisms are activated to relieve osmotic stress. Examples of some compensation mechanisms include alterations on fish standard metabolic rates (Lambert and Dutil 1997), feed intake, and feed conversion efficiency (Imsland et al. 2007). These adaptive strategies are a liability for aquaculture profitability, since they can cause immunosuppression, growth depression, and higher susceptibility to diseases. To solve this, alternative strategies of environmental-friendly techniques are constantly being explored. These alternatives include chemical therapy, vaccination (Bagni et al. 2000; Sakai 1999), or the application of dietary immunostimulants.

The use of dietary immunostimulants has been studied as a viable complement or alternative to traditional methods and has become widely accepted by fish farmers (Bricknell and Dalmo 2005). Immunostimulants, used as dietary supplements can improve fish immunity, increasing resistance during periods of great stress or more resilience during periods of deteriorating water conditions (Bagni et al. 2000). In vivo experiments with bacterial challenges recognized immunostimulants as antiparasitic, growth enhancers, and antibody production promoters (Bricknell and Dalmo 2005). Immunostimulatory substances, like glucans, have been reported to improve fish non-specific defense mechanisms (Jeney and Jeney 2002) and oral administration of yeast glucans increased protection against *Vibrio* spp. in Atlantic Salmon (Raa et al. 1992). Increased lysozyme activity was detected in seabass fed high levels of α-tocopherol (Obach et al. 1993) and enhanced phagocytic activity was reported in seabass fed diets supplemented with levamisole and glucans (Jeney et al. 1994).

Seaweeds have been recognized as an important resource for aquaculture. They are a source of bioactive compounds, such as laminarin, fucoidan, and β-glucans that act as immunostimulants in several fish species (Mustafa and Nakagawa 1995; Peixoto et al. 2016a, b, c).

The use of dietary seaweed supplementation has revealed significant effects on growth, feed utilization, stress response, physiological condition, and carcass quality of cultured fish. Supplementation with 5% *Ulva* spp. increased resistance to infection by *Pasteurela piscicida* in red seabream (Satoh 1987) and *Ulva* spp. and *Chondrus crispus* extracts have shown to increase respiratory burst and immune system stimulation in turbot and Atlantic salmon phagocytes (Castro et al. 2004). Besides immunocompetency, a positive correlation has been reported between seaweeds phenolic content and antioxidant capacity through lipid peroxidation inhibition (Heo et al. 2005). Extracts of red and brown seaweeds may also be effective therapeutic and prophylactic treatments against *Pseudomonas* spp. infection (Thanigaivel et al. 2015) and a 2.5% dietary supplementation of *Gracilaria* spp. led to improved antioxidant and innate system responses in European seabass (Peixoto et al. 2016a). In addition, according to Xu and Hirata (1990), the use of *Ulva* sp. as a feed additive for black seabream (*Acanthopagrus schlegeli*) and red seabream (*Pagrus major*) has been shown to be beneficial on growth and color. To date, studies on the dietary *Fucus* sp. supplementation in fish feed are very scarce. Most recently, however, Peixoto et al. (2016a, b, c) showed that *Fucus* sp. supplementation of up to 7.5% in practical diets for European seabass had no impact on growth performance.

To reduce economic losses caused by immunosuppression in intensive aquaculture, it is necessary to develop strategies that account for environmental stressors for farmed fish. Therefore, the aim of this study

was to evaluate dietary seaweed supplementation on growth performance, antioxidant, and immune responses in seabass subjected to environmental stressors.

Materials and methods

All procedures were supervised by an accredited expert in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations), according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

Experimental diets

Two experimental diets were formulated according to the species requirements (isoproteic, 48%; isolipidic, 19%; isoenergetic, 23 kJ g^{-1}) (Table 1): a basal diet (CTRL) and a supplemented diet (SW), with an added seaweed mix at 7.5% level. The seaweed mix was supplied by ALGA+® and contained a mixture of dried and minced *Gracilaria* sp., *Ulva* sp., and *Fucus* sp. at a 1:1:1 ratio. Proximal composition of seaweeds is presented in Table 2.

Table 1 Feed formulation and proximate composition of the experimental diets

	Dietary treatments	
	CTRL	SW
Feed ingredients (% DM)		
Fishmeal standard	10.0	10.0
Fishmeal SOLOR	20.0	20.0
Soy protein concentrate (Soycomil)	11.8	10.3
Wheat gluten	4.0	4.0
Corn gluten	8.0	8.0
Soybean meal 48	12.0	12.0
Rapeseed meal	5.0	5.0
Wheat meal	9.0	3.0
Peas gelatinized (Aquatex 8071)	3.2	3.2
Fish oil: COPPENS	6.5	6.5
Soybean oil	4.0	4.0
Rapeseed oil	4.0	4.0
Vit & Min Premix PV01	1.0	1.0
Binder (Kieselghur)	0.5	0.5
Antioxidant powder (Paramega)	0.2	0.2
MCP	0.5	0.5
L-Lysine	0.2	0.2
DL-methionine	0.1	0.1
Gracilaria sp.	–	2.5
Ulva sp.	–	2.5
Fucus sp.	–	2.5
Proximate composition (%DM)		
Dry matter	94.7	94.8
Ash	8.6	10.6
Crude protein	47.8	47.9
Crude fat	19.1	19.3
Gross energy (kJ g^{-1} DM)	22.7	22.4

Table 2 Proximate composition (% DM) of the seaweeds used in this trial

	Gracilaria spp.	*Fucus* spp.	*Ulva* spp.
Dry matter	93.4	87.0	85.7
Crude protein	25.9	17.2	23.2
Ash	34.3	20.7	34.8
Crude fat	1.1	3.4	1.5
Gross energy (kJ g^{-1} DM)	12.8	15.1	12.1

Fish and experimental facilities

This trial was conducted in ICBAS (Instituto de Ciências Biomédicas Abel Salazar) at the Aquatic Engineering and Production Systems facilities. The experimental systems consisted of fiberglass tanks (80 L) connected to a single water recirculation system (TMC® System 5000P Marine). Six tanks were used for oscillatory conditions and in parallel, and six tanks were kept under constant conditions and used as control/fixed group. Both systems (fixed and oscillatory) were identical in design. The water flow rate was set to 4 L min^{-1}, with continuous aeration. Temperature and salinity were monitored twice a day. Oxygen, pH, ammonia, and nitrates were monitored and kept within optimal levels. Photoperiod was set to 12:12 h light:dark and water was sterilized by ultraviolet radiation.

The values for temperature and salinity oscillations were selected from a 3-year data log period to represent the most representative combination of temperature/salinity annually observed in a Portuguese seabass farm (Materaqua Lda, Ílhavo, Portugal) (Fig. 1). Variations in temperature were achieved using thermostat heaters (Trixie®—200 W) and water chillers (TECO® TR60). Variations in salinity were obtained by sea salt addition or replacing salt water with dechlorinated fresh water. Groups subjected to temperature and salinity oscillations were compared with groups subjected to fixed rearing conditions (temperature 25 °C, salinity 30 ppt).

European seabass juveniles were provided by IPMA (Olhão, Portugal) and kept in quarantine for 2 weeks. Then, fish with an average weight of 40.7 g were randomly distributed into the tanks (11 fish/tank). The experimental diets, CTRL and SW, were randomly attributed to each tank, assuring three replicates in the oscillatory group and three in the fixed group for each diet. Fish were hand fed, twice a day, until apparent visual satiation and feed consumption was recorded daily.

Sampling

After 63 days, fish were anesthetized with ethylene glycol monobutyl ether (0.25 mL L^{-1}), and sacrificed by decapitation. Weight was recorded for the entire lot. Blood and liver were sampled from two fish/tank. Blood was centrifuged for plasma collection and livers were immediately frozen in liquid nitrogen. All samples were then stored at − 80 °C until further analysis.

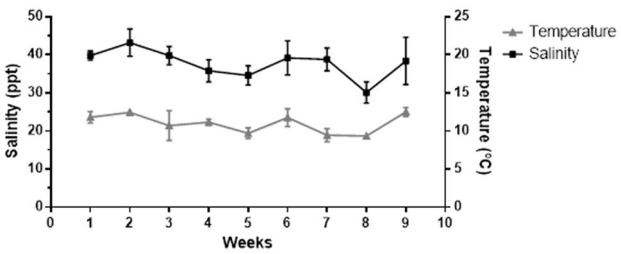

Fig. 1 Water salinity and temperature oscillations during the experimental period. Values chosen as the most representative of the natural variations in the aquaculture farm (Materaqua) over a 3-year period

Chemical analysis

Proximate composition of the diets was determined according to AOAC (2006) methods: dry matter by oven-drying at 103 °C for 16 h, ash by combustion in a muffle furnace (Nabertherm L9/11/B170, Bremen, Germany; 550 °C for 6 h), crude protein (N × 6.25) using a Leco nitrogen analyser (Model FP-528, Leco Corporation, St. Joseph, USA), lipid content by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Königswinter, Germany; 40–60 °C), and gross energy in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany).

Humoral immune parameters

Peroxidase levels were measured according to the oxidation of TMB (3,3′,5,5′-tetramethylbenzidine) in the presence of H_2O_2 (Quade and Roth 1997). Final unit was presented as enzymatic units (EU), with one EU defined as producing an absorbance change of 1 optic density (OD). Lysozyme concentration in the plasma was determined by turbidimetric assay (Ellis 1990), measuring *Micrococcus lysodeikticus* lysis. Results are presented as enzymatic units, where 1 EU = 0.001 absorbance units per minute.

Oxidative stress

Livers were homogenized using K-phosphate buffer (pH 7.4, 0.1 M). Protein levels required for standardization of antioxidant parameters were quantified using Bradford method (Bradford 1976).

Lipid peroxidation (LPO) determination was based on malondialdehyde (MDA) level, measured as the amount of thiobarbituric acid reactive substances (TBARS) as a result of fatty acids oxidation (Ohkawa et al. 1979). Spectrophotometric readings were performed at 535 nm.

Catalase activity (CAT) was determined by reaction with H_2O_2 (Claiborne 1985). Glutathione-*S*-transferase (GST) activity was analyzed according to Habig et al. (1974) based on the quantification of the conjugate GSH-CDNB (1-chloro-2, 4-dinitrobenzene). Glutathione peroxidase (GPx) was quantified following the method described by Mohandas et al. (1984), measuring the formation of $NADP^+$. Glutathione reductase (GR) was determined based on NADPH oxidation measured at 340 nm (Cribb et al. 1989). Total and oxidized glutathione (TG and GSSG) were measured by the concomitant reaction of the GSH with 5,5′-dithiobis-(2-nitrobenzoic acid) (Baker et al. 1990), read at and absorbance of 412 nm. For GSSG evaluation, 2-vinylpyridine was used to trap the GSH present in the sample (Griffith 1980). Finally, GSH content was calculated by the difference between GSSG and TG levels.

Statistical analysis

Data were checked for normality (Shapiro/Wilk Test) and homogeneity of variances (Levene's test) and data transformation was applied when normality was not achieved. Two-way ANOVA was carried out using IBM SPSS Statistics 23. Tukey's HSD test was used for pairwise comparisons between treatments. A confidence level of 95% was considered in all statistical analysis.

Results

During the current trial, no mortalities were registered. Weight gain was significantly lower ($p < 0.05$) in fish subjected to oscillatory conditions (14.6 g and 22.6 g) when compared to fish subjected to the fixed condition (33.3 g and 40.1 g) (Table 3). The same pattern was observed for daily growth index (0.7 and 1.0 versus 1.47 and 1.6) (Table 3). Growth performance was not affected by dietary SW supplementation ($p > 0.05$) (Table 3).

Humoral immune responses (lysozyme and peroxidase activities) were not affected by dietary SW supplementation or rearing conditions ($p > 0.05$) (Fig. 2). The oxidative stress analyses showed that lipid peroxidation (LPO), glutathione peroxidase (GPx), glutathione *S*-transferase (GST), and reduced glutathione (GR) did not vary, regardless of the dietary and rearing conditions ($p > 0.05$; Figs. 3 and 4). Total glutathione

Table 3 Growth performance and feed utilization of seabass fed the experimental diets and subjected to oscillatory or fixed conditions

	Oscillatory		Fixed		*p* value		
	CTRL	SW	CTRL	SW	*C*	*D*	*C* × *D*
Initial body weight (g)	26.5 ± 2.8	37.8 ± 1.7	42.9 ± 1.6	42.4 ± 0.4	0.193	0.546	0.459
Final body weight (g)	43.6 ± 1.2	59.3 ± 2.9	80.2 ± 5.7	85.7 ± 7.3	0.114	0.310	0.603
Weight gain (g)[A]	14.6 ± 4.1[b]	22.6 ± 6.1[b]	33.3 ± 12.4[a]	40.1 ± 9.5[a]	0.003	0.342	0.655
Daily growth index[B]	0.7 ± 0.2[b]	1.0 ± 0.2[b]	1.47 ± 0.2[a]	1.6 ± 0.1[a]	0.004	0.489	0.655
Feed conversion ratio[C]	1.18 ± 0.1	1.73 ± 0.04	1.14 ± 0.00	1.24 ± 0.24	0.050	0.409	0.942
Feed intake[D] (g kg ABW day^{-1})	118.6 ± 4.2	158.5 ± 28.1	157.5 ± 9.9	156.4 ± 11.9	0.184	0.342	0.202

Corresponding *p* values to rearing condition (*C*) and diet (*D*) factors, and interaction *C* × *D* are presented for each parameter. Values presented as mean ± standard deviation. Different superscript letters indicate significant differences ($p < 0.05$)

[A]Weight gain (g) = FBW − IBW, where FBW and IBW are the final and initial average body weights (g)

[B]Daily growth index (DGI) = 100 × [(FBW)$^{1/3}$ − (IBW)$^{1/3}$] × trial duration in days

[C]Feed conversion ratio (FCR) = feed intake (g)/weight gain (g)

[D]ABW = (IBW + FBW)/2; day = feeding trial duration

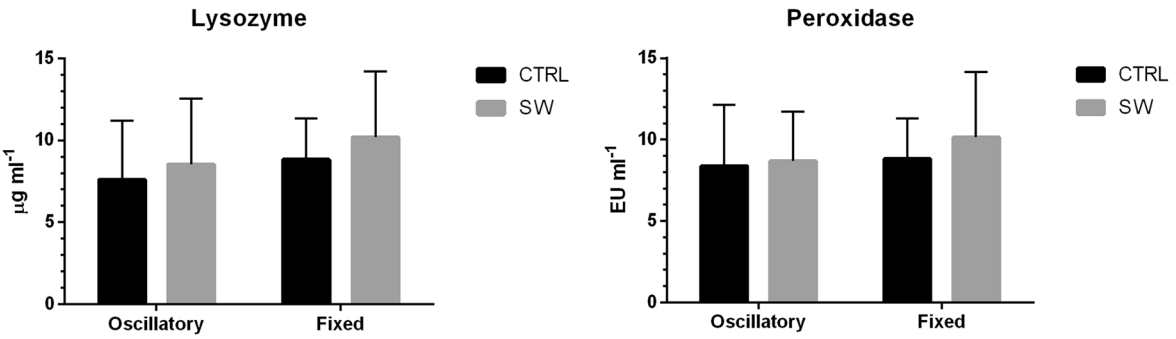

Fig. 2 Lysozyme and peroxidase activities determined in seabass fed the experimental diets and subjected to oscillatory or fixed conditions. Results presented as mean ± standard deviation

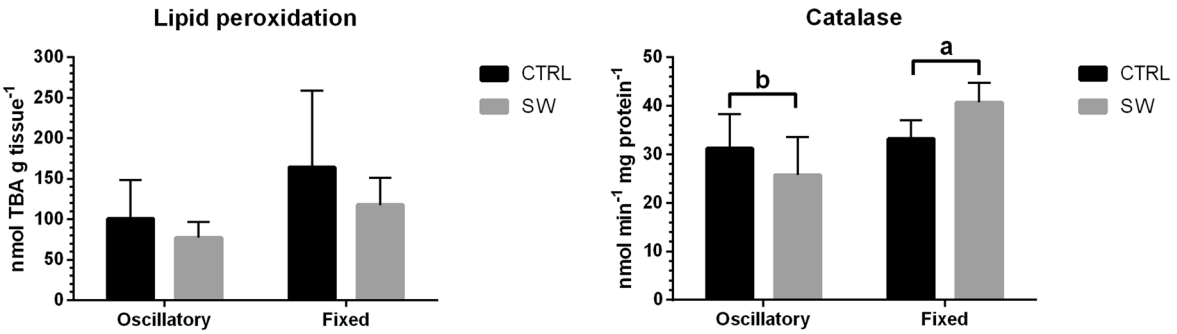

Fig. 3 Lipid peroxidation and catalase activity determined in the liver of seabass fed the experimental diets and subjected to oscillatory or fixed conditions. Results presented as mean ± standard deviation

(TG), oxidized glutathione (GSSG), and catalase (CAT) were significantly affected by rearing conditions; temperature and salinity oscillations led to a significant increase in TG and GSSG and to a significant decrease in CAT ($p < 0.05$, Fig. 4), with no influence of dietary SW supplementation ($p < 0.05$; Fig. 3).

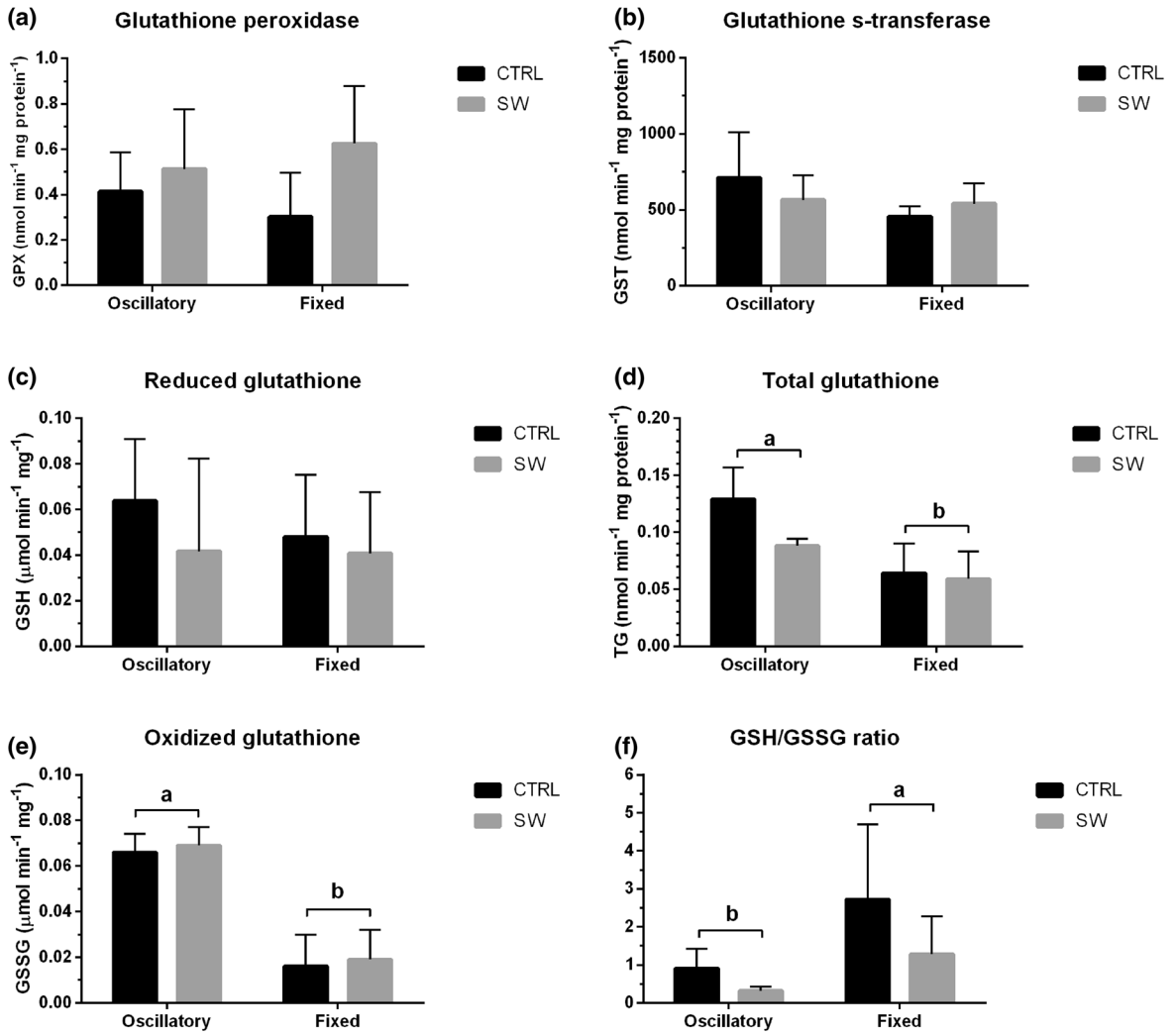

Fig. 4 Glutathione peroxidase (**a**), glutathione *S*-transferase (**b**), reduced glutathione (**c**), total glutathione (**d**) and oxidized glutathione (**e**) activities and GSH/GSSG ratio (**f**) determined in the liver of seabass fed the experimental diets and subjected to oscillatory or fixed conditions. In graphs **a**–**c**, absence of letters indicates non-significant differences and no interaction between conditions and diets. In graphs **d**–**f**, two-way ANOVA showed significant differences between conditions but not between diets, as indicated by letters

Discussion

Seaweeds have been previously studied as potential immunostimulants for many fish species including European seabass (Peixoto et al. 2016b), Nile tilapia (Güroy et al. 2007), rainbow trout (Güroy et al. 2013), and red sea bream (Mustafa and Nakagawa 1995). However, their effect as immunostimulants under environmental oscillations is yet to be accessed.

In this study, seabass growth was not influenced by dietary supplementation. This is consistent with results observed by Peixoto et al. (2016b) and Bagni et al. (2005), where no growth improvement was detected in European seabass fed diets supplemented with immunostimulants (algal extract with alginic acid and a yeast extract with β-glucans) at the same level of supplementation. Similarly, 5% *Ulva* sp. showed no effects on the growth performance of Nile tilapia (Güroy et al. 2007), while in European seabass, 10% inclusion of *U. rigida* or *Gracilaria bursa-pastoris* did not compromise growth (Valente et al. 2006). In addition, for striped mullet (Wassef et al. 2001) and Nile tilapia (Azaza et al. 2008), 10–25% inclusion with *Ulva* sp. had no effects on the

growth performance of these species. On the other hand, Mustafa and Nakagawa (1995) showed that 5% inclusion of a seaweed mix (*Ascophyllum nodosum*, *Porphyra yezeoensis,* and *U. pertusa*) increased body weight and feed utilization of red seabream fingerlings.

Temperature and salinity combined effect has been reported as the main parameter affecting seabass growth (Conides and Glamuzina 2006). In this study, dietary seaweed supplementation had no effect on seabass growth performance, while the oscillatory environmental factors were the most accountable for the reduced growth performance. Both temperature and salinity played an important role in the outcome of the current study. Nevertheless, low temperatures possibly played a stronger role, as it is one of the most important environmental factor affecting the biochemical and physiological processes of aquatic organisms (Reynolds and Casterlin, 1979). Fish subjected to oscillatory conditions showed a lower growth performance in terms of daily growth index and weight gain when compared to those reared at fixed conditions. This is in agreement with Bagni et al. (2005) that associated growth suppression in seabass fed a combination of yeast β-glucan and alginic acid to sub-optimal water temperature.

The compounds associated with the immunostimulant properties of seaweeds still require more clarification. Nonetheless, polysaccharides, such as those present in seaweeds, have been linked to enhancements of fish innate immune system (Kim et al. 2012). Lysozyme and peroxidase are key components of fish innate immunity, acting against pathogens by disrupting their cell walls, either directly or through oxidative radicals action (Nayak 2010). In another study, the inclusion *G. lemaneiformis* showed to increased lysozyme activity in white-spotted spinefoot, associating it with the polysaccharides, vitamin C, and β-carotenes present in seaweeds (Xu et al. 2011). However, in the present trial, lysozyme and peroxidase activities of seabass were not affected by dietary seaweed supplementation nor by the environmental conditions. Similarly, previous studies report no effects in lysozyme and peroxidase activities for Nile tilapia fed diets supplemented with 5% and 10% *Ulva* spp. (Valente et al. 2006) and in European seabass fed diets with algal extract (Lauridsen and Buchmann 2010). On the other hand, an increase in lysozyme concentration was reported in European seabass when fed a seaweed supplemented diet (Peixoto et al. 2016c) and when fed a combination of dietary glucans and vitamins (Bagni et al. 2000), contradicting present results. Given this, we hypothesize that the beneficial effects of seaweeds on the innate immune response may be highly influenced by biotic and abiotic factors such as species, age, weight, rearing water conditions, and/or seaweed composition.

Environmental factors such as rearing temperature and salinity have been described to influence cellular antioxidant balance. Vinagre et al. (2012) showed that both LPO and CAT increased when European seabass was reared at temperatures outside the optimal range (24 °C). Similar results were presented by Madeira et al. (2013), where multiple estuarine fish species (including seabass) were collected and subjected to a temperature increase of 1 °C/h, starting at 24 °C. In this study, LPO, CAT, and GST simultaneously increased with temperature rise. In our work, no differences were found in these parameters. It is possible that temperature oscillation masks consistent reactions from these parameters. However, GSSG in this work was significantly higher in the oscillatory treatments. Eroglu et al. (2015) showed that fish exposed to heavy metals had reduced GSH/GSSG levels, due to the consumption of GSH and formation of GSSG. Leggatt et al. (2007) showed that besides metal toxicity, temperature alone could influence GSH levels, with fish being acclimatized within the range of comfort temperatures showed increased GSH transcription. Altogether, GSH appears to reduce in hazardous conditions, either by conception (promoting GSSG increase) or/and by reducing its formation.

Results from present study indicate that dietary seaweed supplementation had no influence on these oxidative stress biomarkers, which has also been observed by Peixoto et al. (2016b). However, the differences observed are tightly associated with temperature and salinity oscillations which is well described by several works (Loro et al. 2012; Madeira et al. 2013; Vinagre et al. 2012). In this work, TG levels were higher in fish subjected to the oscillatory condition. Similarly, Eroglu et al. (2015) observed an increase of oxidized glutathione (GSSG) leading to higher TG concentrations and lower GSH/GSSG ratio. Therefore, it appears plausible to consider that the oscillatory environmental conditions acted as ROS inducing event. TG levels behave as a potential antioxidant reserve that reduces in prolonged exposure to stress (Eroglu et al. 2015). In more detail, TG levels seem to oscillate as compensatory effect of GSH depletion, due to antioxidant activities (Guyonnet et al. 1999; Tan et al. 1998). According to Vinagre et al. (2012), CAT activities in juvenile seabass are highly sensitive to environmental temperature, particularly those outside the optimal range of the species, which is in accordance with our results.

This work showed that seaweed supplementation in European Seabass juveniles does not mitigate the effects of environmental oscillations. Therefore, dietary supplementation trials should focus on determining the bioactive compounds present in seaweeds and their association with specific responses. In addition, antioxidant responses in this work were altered solely in the glutathione's system suggesting a similarity to antioxidant responses in toxicity trials. However, antioxidant results were affected by the high deviation between trials suggesting a greater number of samples should be considered for these parameters.

Overall the differences observed in our study were mainly associated with the oscillating conditions and not the dietary seaweed supplementation, nor were interactions between factors detected for the analyzed parameters.

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