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Effects of dietary supplementation with macroalgae on sperm quality and antioxidant system in Senegalese sole

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ABSTRACT

Within the efforts in the search for novel feed ingredients in aquaculture, macroalgae are being widely explored due to their diversity of bioactive compounds with considerable prophylactic and therapeutic potential. *Plocamium cartilagineum* and *Sargassum vulgare*, have the potential to positively impact fish health, mainly due to their antioxidant properties. The production of Senegalese sole (*Solea senegalensis*) still faces some constraints due to the low sperm quantity and quality of males from captivity, which is highly affected by oxidative stress. We hypothesized that the scavenger ability of *P. cartilagineum* and *S. vulgare* could ameliorate the reproductive performance of Senegalese sole males, once incorporated in the broodstock feeds. In this experiment, broodstock groups were fed with three different formulated experimental diets for 60 days: Control (Ctrl), a diet supplemented with 5% *P. cartilagineum* (*Pc*), and a diet supplemented with 5% *S. vulgare* (*Sv*). Fish were sampled twice to evaluate sperm quality using different techniques: sperm motility by Computer Assisted Sperm Analysis (CASA) software, and cell viability and Reactive Oxygen Species (ROS) by flow cytometer. At the final sampling, 6 fish per treatment were sacrificed and gonads were extracted to determine the relative expression of anti- and pro-apoptotic genes (*bcl2b, bcl-x, mcl1b, badb, bax, boka*), and genes involved in fish antioxidant system (*sod3, cat, gpx, hsp70*). Sex steroids were measured on blood plasma by ELISA. Specific growth rate (SGR) and feed efficiency were also evaluated at the end of the experiment. Senegalese sole breeders fed with *Pc* diet for 60 days showed enhanced sperm total motility and cell viability at the end of the experiment, when compared with the Ctrl diet. On the other hand, fish fed with *Sv* diet had lower spermatozoa intracellular ROS and upregulated *gpx* in the gonads. No differences were obtained in levels of sex steroids and gonadal apoptotic system after 60 days of feeding, however, the macroalgae species and the percentages of incorporation used proved to positively modulate different spermatozoa traits.

1. Introduction

The fulfilment of the nutritional requirements of breeders in aquaculture is a key factor in ensuring healthy broodstock, successful reproduction and, ultimately, healthy offspring (Fernández-Palacios et al., 2011). Fish meal (FM) and fish oil (FO) have traditionally been key ingredients in the production of aquaculture feeds due to their high-quality protein and lipid content, respectively (Turchini et al., 2009; Hodar et al., 2020). However, the sustainability and cost-effectiveness of these resources have led to partial or entire substitution of fish-based ingredients for suitable alternatives, the majority with insect (Musyoka et al., 2019; Basto et al., 2022) and plant-based ingredients (Boissy et al., 2011; Chakraborty et al., 2019). Nonetheless, there is some evidence that the total replacement of FM and FO by plant sources leads to poor feed efficiency and reduces growth and impair reproductive performance (Cardona et al., 2022).

A promising alternative for fish feeds is the inclusion of seaweeds in the diets. They have been used by the industry as gelling agents and stabilizers (Mohamed et al., 2012), but their proven immunostimulatory, antimicrobial, antibacterial and antioxidant effects in fish health (Naiel et al., 2021) opens the possibility of developing new functional feeds. Independently of their group, either brown, green or red, macroalgae are rich in proteins, dietary fibers, vitamins, minerals, polyunsaturated fatty-acids, phytochemicals, and antioxidants (Zhu et al., 2017). As reviewed by Naiel et al. (2021), there are several studies testing different macroalgae inclusion percentages in fish feeds, showing

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positive impacts on the health of species like European seabass (*Dicentrarchus labrax*) (Wassef et al., 2013; Peixoto et al., 2019), gilthead seabream (*Sparus aurata*) (Wassef et al., 2005), red seabream (*Pargus major*) (Sony et al., 2019), turbot (*Scophthalmus maximus*) (Wang et al., 2018), Atlantic salmon (*Salmo salar*) (Norambuena et al., 2015; Wan et al., 2016), rainbow trout (*Oncorhynchus mykiss*) (Araújo et al., 2015) or Nile tilapia (*Oreochromis niloticus*) (Ergün et al., 2008). Macroalgae are considered novel ingredients with added value in the formulation of fish feeds (Wan et al., 2018).

Nowadays, it is known that macroalgae are rich in pigments (phycobiliproteins and carotenoids), phenolic compounds (phlorotannins and bromophenols), nitrogen compounds (alkaloids), polysaccharides (agarans, carrageenans and alginate) and terpenoids (diterpenes and sesquiterpenes) (Torres et al., 2019). Different species of the genus *Sargassum* have been used in fish feeding trials (Zhu et al., 2017; Wang et al., 2019; Brontowiyono et al., 2022) and all proved to ameliorate the antioxidant defense system of fishes. According to the literature, the brown algae group, has the highest content of polysaccharides, constituting 10 to 47% of their dry weight (Tian et al., 2020). Within brown algae, the genus *Sargassum* was shown to contain several bioactive compounds, including sulfated polysaccharides (SPs) such as fucose, galactose, xylose, glucuronic acid and mannose, with proven scavenging activities on free radicals, which were already described to be present in high doses in *Sargassum vulgare* (Dore et al., 2013; Sanjeewa et al., 2018). In addition, this species is rich in phlorotannins, a polyphenol exclusive from brown algae known for its antioxidant activity (Chouh et al., 2022).

Plocamium cartilagineum belongs to the red algae group and is rich in organic acids (oxalic, ketoglutaric, pyruvic and acetic acids) and volatile compounds (bioactive substances with defense or attractant functions), including some carotenoids and terpenoids (Valentão et al., 2010). These biological agents, among others, contribute to its described antioxidant activity against reactive oxygen species (ROS) and nitrogen species (RNS) (Valentão et al., 2010) and to the promotion of osteogenic activity in fish (Carson et al., 2018).

Apart from the above-mentioned studies in fish species, and to the best of our knowledge, there are no other studies testing the impact of dietary macroalgae supplementation on fish reproductive performance. Based on the recent findings in terms of macroalgae bioactive compounds and their potent antioxidant activity, this lack of knowledge is important to address and deserves further research, since macroalgae have the potential to boost broodstock performance. For fish broodstock, an adequate nutrition is essential not only for their overall health, but also for the quality and quantity of eggs and sperm produced, which ultimately determine their reproductive capacity (Izquierdo et al., 2001).

Fish spermatozoa are prone to oxidative processes due to the reduction in their antioxidant status that occurs along with spermatogenesis (Cabrita et al., 2011a; Félix et al., 2021). In species like Senegalese sole, this problem is even more accentuated, because males born and reared in captivity (F1 males) have several reproductive impairments identified (Cabrita et al., 2006). The F1 Senegalese sole low sperm quality is normally associated with the susceptibility of spermatozoa to the action of reactive species (ROS and RNS) (Cabrita et al., 2014), which ultimately affects their fertilization rate (Beirão et al., 2015). Previous studies with juveniles of this species, which tested the inclusion of different species of green (Vizcaíno et al., 2019) and brown macroalgae (Moutinho et al., 2018) in the diets, revealed positive effects in the protection of the intestinal mucosa and nutrient utilization, respectively. However, those studies were performed on immature juveniles and focused on other physiological pathways of the fish. The objective of this study was to understand the potential reproductive benefits of supplementing broodstock diets with two species of macroalgae rich in antioxidant compounds, *Plocamium cartilagineum* and *Sargassum vulgare*. The effects of this inclusion have been monitored at several levels of the reproductive system of F1 Senegalese sole males, to determine the possibility of counteracting their reproductive dysfunctions using dietary supplementation with macroalgae.

2. Material and methods

2.1. Fish husbandry

For this experiment, a young broodstock of 63 Senegalese sole (2 years), with a mean body weight of 726 ± 145 g, was used. Fish were reared from larvae in-house and maintained at the research facilities of CCMAR, Ramalhete marine station. Once established, fish were individually identified with a pit tag and kept in 9 raceway tanks with 200 L capacity, with 7 fish per tank (5 males and 2 females) at a density of 6.4 ± 0.4 kg/m^2. Fish were maintained under a controlled temperature (20 ± 1 °C) and 14 h light:10 h dark photoperiod to ensure the optimal conditions for spermatogenesis. Fish were fed with standard diets for 3 months before the experiments started.

All experimental procedures were conducted in accordance with ARRIVE guidelines (directives 86/609/EU and 2010/63/EU of the European Parliament and Council) and Portuguese legislation for the use of laboratory animals (PORT 1005/92 from DGAV). All procedures were authorized under the ethic certification 005621.

2.2. Algae collection and feed formulation

For the algae feeds formulation, *Plocamium cartilagineum* was handpicked by a diver in the south-west of Portugal at Praia do Portinho (37°10′26.4'' N 8°54′36.7'' W) and *Sargassum vulgare* was acquired from a commercial source. The algae were washed with freshwater and air-dried in the shade at room temperature, according to Silva et al. (2024). Afterwards, algae were ground and delivered to SPAROS Lda (Olhão, Portugal) for feed formulation. The SPAROS commercial feed for this species was used as control diet (Ctrl), which contained 52% crude protein, 15% crude fat and 22 MJ/Kg feed gross energy considered to fulfil the nutritional requirements of the species (analytical composition can be consulted in Table S1 – supplementary material). The supplemented diets were formulated by substituting 5% of wheat meal from the Ctrl diet for the same percentage of dry macroalgae meal: 5% of *Plocamium cartilagineum* (*Pc*) and 5% of *Sargassum vulgare* (*Sv*). The formula of the three diets is detailed in Table 1. All diets were manufactured by temperature-controlled extrusion (pellet size: 5.0 mm) and throughout the trial feeds were stored frozen and protected from light.

2.3. Experimental design and samplings

The main objective of this experiment was to understand if

Table 1
Composition of the experimental diets in percentage of the different ingredients. Ctrl refers to the control diet, *Pc* to the *Plocamium cartilagineum* and *Sv* to the *Sargassum vulgare* supplemented diets.

Ingredients	Ctrl	*Pc*	*Sv*
Fishmeal LT70	15%	15%	15%
Soy protein concentrate	15%	15%	15%
Pea protein concentrate	10%	10%	10%
Wheat gluten	12%	12%	12%
Corn gluten meal	15%	15%	15%
Soybean meal	12%	12%	12%
Wheat meal	7.5%	2.5%	2.5%
Vitamin and mineral premix	1%	1%	1%
Monoammonium phosphate	1%	1%	1%
DL-Methionine	0.4%	0.4%	0.4%
Plocamium cartilagineum		5%	
Sargassum vulgare			5%
Fish oil	4.1%	4.1%	4.1%
Rapeseed oil	7%	7%	7%
Total	100%	100%	100%

incorporating macroalgae in fish diets could impact the F1 Senegalese sole male reproductive capacity. For that, fish from each treatment were fed twice a day for 60 days (1% of body weight) with the previously described formulated diets. The first sampling took place in the middle of the experiment (1 month of feeding) and the second at the end (2 months of feeding). In each sampling, after anesthesia with 200 ppm phenoxyethanol, sperm samples were collected by hand stripping, avoiding contamination with urine. Samples were kept on ice until further analysis.

At the final sampling, 5 to 6 males per treatment were randomly selected and euthanized. Blood samples were collected with a heparinized syringe for sex steroids determination, and gonads were extracted and directly plunged into liquid nitrogen, for further analysis of the gonadal antioxidant and apoptotic system. The feed efficiency ratio (FER) (weight gain per Kg of feed consumed) and specific growth rate (SGR) (percentage growth per day) were also determined at the end of the experiment, using the following formulas:

$$\mathrm{FER} = (\mathrm{FW} - \mathrm{IW})/\mathrm{Feed}\ (\mathrm{DW})$$

$$\mathrm{SGR}\ (\%) = [(\boldsymbol{ln}(\mathrm{FW}) - \boldsymbol{ln}(\mathrm{IW})]/\mathrm{N}\ \mathrm{x}\ 100$$

2.4. Sperm quality analysis

2.4.1. Spermatozoa motility analysis

Sperm motility was assessed using a Computer Assisted Sperm Analysis (CASA) (ISAS software, Proiser, Valencia, Spain), a phase-contrast microscope (Nikon E-200; Nikon, Tokyo, Japan) and an ISAS camera (25 fps). For each sample, 1 μL of sperm was activated on a Makler chamber (IVF, United States) with 10 μL of artificial seawater and total motility (TM, %) was registered immediately after (15 s post activation). Other parameters such as progressive motility (PM, %), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s) and linearity (LIN, %) were also recorded as a standard practice and can be consulted on Table S2 – supplementary material. For this analysis, a total of 9–10 samples per treatment were used for each sampling period.

2.4.2. Cell viability and reactive oxygen species (ROS)

For the analysis of cell viability, 1 μL of propidium iodide (PI, 1 mg/mL) was added to 1 μL of sperm previously diluted in 500 μL of 1% NaCl solution. After incubation for 5 min in the dark at 4 °C, fluorescent cells (PI positive) were counted using a flow cytometer (BD FACSCalibur™, BD Biosciences, CA) equipped with a 488 nm laser for PI detection with a 585/42 nm filter (FL2). Calibration was performed according to Félix et al. (2024). The levels of ROS in sperm were measured on the same equipment but calibrated for SYTOX® green detection with a 530/30 nm filter (FL1) and for DHE detection with a 585/40 nm filter (FL2). For this, sperm was stained with a combination of dihydroethidium (DHE) and SYTOX® green (Invitrogen™, ThermoFisher) in the following proportions: 0.5 μL of DHE (0.5 mM) added to 1 μL of sperm previously diluted in 500 μL of 1% NaCl solution. After 5 min of DHE incubation, 0.5 μL of SYTOX® green (1 μM) was added. The fluorescent cells were counted after 10 min of total incubation in a cold and dark environment. For data acquisition, the BD CellQuest Pro software (version 8.7, BD Biosciences, CA) was used and a total of 30.000 events were collected from each sample. In the end, the percentage of viable cells was calculated by subtracting the dead ones from the whole sperm population. For ROS determination, only the subpopulation of live cells producing ROS was considered. A total of 9–10 samples per treatment were used for each sampling.

2.5. Sex steroids determination

An enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemicals, Ann Arbor, Michigan, United States), was used to determine testosterone (T) and 11-ketotestosterone (11KT) levels in the blood plasma of males from each treatment, following the protocol from the manufacturer. A total of 12 samples per treatment were examined for each hormone measured.

2.6. Gene expression analysis: antioxidant and apoptotic systems

The gonads collected were used to measure the relative expression of anti- and pro-apoptotic genes (*bcl2b, bcl-x, mcl1b, badb, bax, boka*), and genes involved in the fish gonadal antioxidant system (*sod3, cat, gpx, hsp70*) by quantitative real-time PCR (qPCR), using *rRNA18S*, *ubq* and *ef1a1* as housekeeping genes (Table 2).

Total RNA was extracted from individual gonads using the NZYol reagent (NZYTech, Lisbon, Portugal) according to the manufacturer's instructions. The concentration of RNA was determined using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA). RNA samples were then submitted to an RQ1 RNase-Free DNase treatment (Promega, USA) and followed by a reverse transcription reaction with M-MLV Reverse Transcriptase (Invitrogen, Paisley, UK), using 1 μg of DNase-treated total RNA. For each gene, a species-specific set of primers (Table 2) was designed using the Primer3Plus software (Untergasser et al., 2012), with sequences from GenBank (www.ncbi.nlm.nih.gov), and target specificity was checked using Primer-Blast (NCBI).

Real-time qPCR was performed in a BioRad CFX96 Real-Time PCR System (Biorad, USA). The efficiency of the qPCR reaction assay for each gene was previously evaluated to ensure that it was close to 100%. The specificity of each qPCR reaction was verified by including a control without template, melt curve analysis and electrophoresis. All reactions were performed in triplicates in 96-well plates with 20 μL reaction volume containing: 10 μL of NZYSpeedy qPCR Green ROX master mix (NZYTech); 0.6 μL of specific FW and RV primers (10 μM), in Sigma water; and using 2 ng of cDNA in each reaction. Standard amplification parameters were as follows: 95 °C for 2 min, followed by 40 amplification cycles (95 °C for 5 s and 65 °C for 10 s), and a final dissociation reaction (from 60 to 95 °C with 0.5 °C increments) to assess amplification specificity. Relative gene expression was determined using the ΔΔCt method (Livak and Schmittgen, 2001) using the control group as reference sample. Briefly, the ratio between PCR efficiency and threshold cycle of a sample was compared with the control and expressed in comparison to the reference gene. Relative gene expression was normalized using the geometric mean of 3 housekeeping genes: *ubq*, a known reference gene for accurate normalization in qPCR studies with Senegalese sole (Infante et al., 2008), *ef1a1* and *rRNA18S*.

2.7. Statistical analysis

Data obtained as percentages were arcsine square-root transformed. All the statistical analysis was performed on SPSS software (IBM, USA). For data following the normality and variance homogeneity a one-way ANOVA, followed by the *post-hoc* Tukey test was applied. For the data that did not assume those principles, the respective non-parametric Kruskal-Wallis test was applied to search for differences between treatments. Significant differences were assumed when $p < 0.05$.

3. Results

3.1. Growth and feed efficiency

The results from feed efficiency rate (FER) and specific growth rate (SGR) did not reveal any difference between treatments (one-way ANOVA, $p < 0.05$) (Fig. 1). The average FER was 0.41 ± 0.17%, being the highest value observed on *Pc* treatment (0.58 ± 0.24) (Fig. 1A). The same happened for SGR, which average was 0.18 ± 0.13%, and the highest value was obtained for the *Pc* treatment (0.25 ± 0.14%) (Fig. 1B).

Table 2
Primers used to analyze gene expression by qPCR in 5′ to 3′ orientation. Fw, forward; Rv, reverse.

Gene name	Gene symbol	Primers		Genebank number
bclb2 apoptosis regulator b	*bcl2b*	fw	TAAAGAGAACGGGGGATGG	XM_044019317.1
		rv	AGAGGCACGAGGAAATAGAGC	
bcl-2-like protein 1	*bcl-x*	fw	AGCTGGTTTCCCGCATTGTA	XM_044025112.1
		rv	GTCTCCCGACATCTTCTCGC	
mcl1 apoptosis regulator	*mcl1b*	fw	TGACTTATCTTGTGTTCCCTTCC	XM_044033843.1
		rv	TGACTTCCCATTACCATTTGC	
bcl2 agonist of cell death b	*badb*	fw	GGAGGAAGCCACAAGAACAAT	XM_044022723.1
		rv	CATCACAACCAACAAAACCAC	
Apoptosis regulator bax-like	*bax*	fw	GACCCCAACCAGAAGAAGC	XM_044050841.1
		rv	CGACAGGCAAAGTAAAAGAGTG	
bcl2 apoptosis regulator boka	*boka*	fw	CCTTCGCTCCCTATGCTC	XM_044015561.1
		rv	CCAAACCGCTACTCAAACAG	
Superoxide dismutase 3	*sod3*	fw	AGTCGGAAGCAACACTGTTTGAAGGGATGT	XM_044028883.1
		rv	GCCAGCATCTCCACCCAGTCCTAGGTCA	
Glutathione peroxidase	*gpx*	fw	GAAGTATGTCCGTCCAGGGAATG	JX294727.1
		rv	TGAACTTTGGATCGGTCATGAGA	
Catalase	*cat*	fw	GACTCTTCTCCTACCCGGACACA	JX294726.1
		rv	TAGTTTGGAGCACCACCTTGGTT	
Heat shock cognate protein 70	*hsp70*	fw	CTGTATTTGATGCTAAGCGTTTG	XM_044033943.1
		rv	GACCTCTACTTTGGGTTTGGA	
Elongation factor 1 alpha, isoform 1	*ef1a1*	fw	CCGGTGTTGGTGAGTTCGAG	AB326302.1
		rv	AGCGCTCACTTCCTTGGTGA	
Ribosomal protein 18S	*18S*	fw	CGGAGGTTCGAAGACGATCA	AM882675.1
		rv	TGCCCTTCCGTCAATTCCTT	
Ubiquitin	*ubq*	fw	CTCAGACAGCTGGCCCAGAA	AB374981.1
		rv	CAGCCCTCGCGTCTACTTCA	

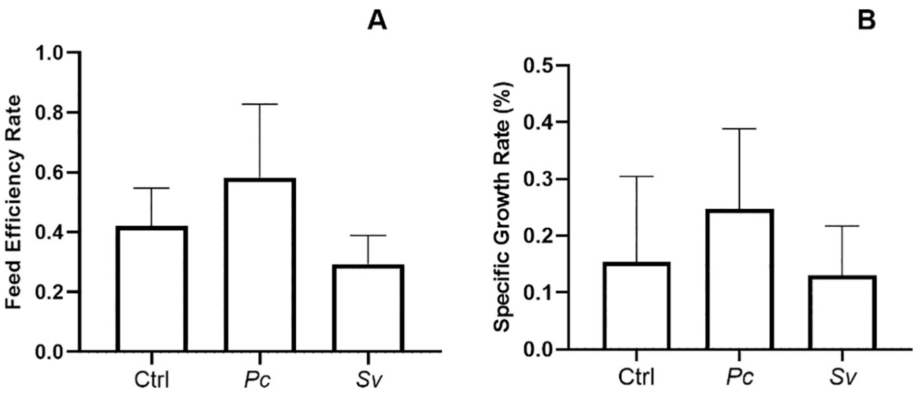

Fig. 1. Feed efficiency rate (A) and specific growth rate (B), of Senegalese sole fed with Control (Ctrl), *Plocamium cartilagineum* (*Pc*) and *Sargassum vulgare* (*Sv*) supplemented diets. Results are shown as mean ± SD (A: $n = 3$; B: $n = 11–13$,). No statistical differences were identified (one-way ANOVA, $p < 0.05$).

3.2. Sperm quality analysis

The sperm quality was evaluated through the analysis of motility parameters, cell viability and measurement of ROS after one and two months of feeding (one-way ANOVA, Tukey, $p < 0.05$) (Fig. 2). The spermatozoa motility at the first sampling, following one month of feeding, did not show significant differences (one-way ANOVA, $p < 0.05$) between treatments in any of the analyzed parameters (Fig. 2A and Table S2 – supplementary material). At the end of the assay, after two months of feeding with the different diets, TM was higher in fish fed with *Pc* diet (75 ± 12%), compared to the control (42 ± 28%) (Fig. 2D). Regarding viability there were no significant differences between treatments after one month of feeding (Fig. 2B) but, at the end of the feeding trial, the fish fed with *Pc* diet presented the highest spermatozoa viability (94 ± 3%) (Fig. 2E). The same was observed for ROS analysis, no differences were obtained on the first sampling (Fig. 2C). However, at the end of the trial, each treatment showed on average 96 ± 3% of live cells (data not shown) but fish fed *Sv* diet had significantly fewer live cells with ROS (56 ± 7%), compared with Pc (75 ± 3%) and Ctr (72 ± 4%) treatments (Fig. 2F).

3.3. Sex steroids

The results from testosterone (one-way ANOVA, $p < 0.05$) and 11-ketotestosterone (Kruskall-Wallis, $p < 0.05$) analysis in fish blood plasma did not reveal any significant differences between diets (Fig. 3) at the end of the feeding trial. The average level of testosterone was approximately 0.4 ± 0.1 ng/mL and 11-ketotestosterone was 1.9 ± 1.0 ng/mL.

3.4. Gonadal antioxidant and apoptotic systems

The analysis of the antioxidant system of Senegalese sole testis revealed that the expression of *gpx* was upregulated (Kruskal-Wallis, $p < 0.05$) on fish fed with the *Sv* diet (Fig. 4), while no differences were shown in the other (*sod3*, *cat* and *hsp70*) antioxidant biomarkers (one-way ANOVA, $p < 0.05$).

Regarding the expression of anti- and pro-apoptotic genes (*bcl2b*, *bcl-x*, *mcl1b*, *badb*, *bax*, *boka*), no significant differences were identified among fish fed with different diets (one-way ANOVA or Kruskal-Wallis, $p < 0.05$) (Fig. 5).

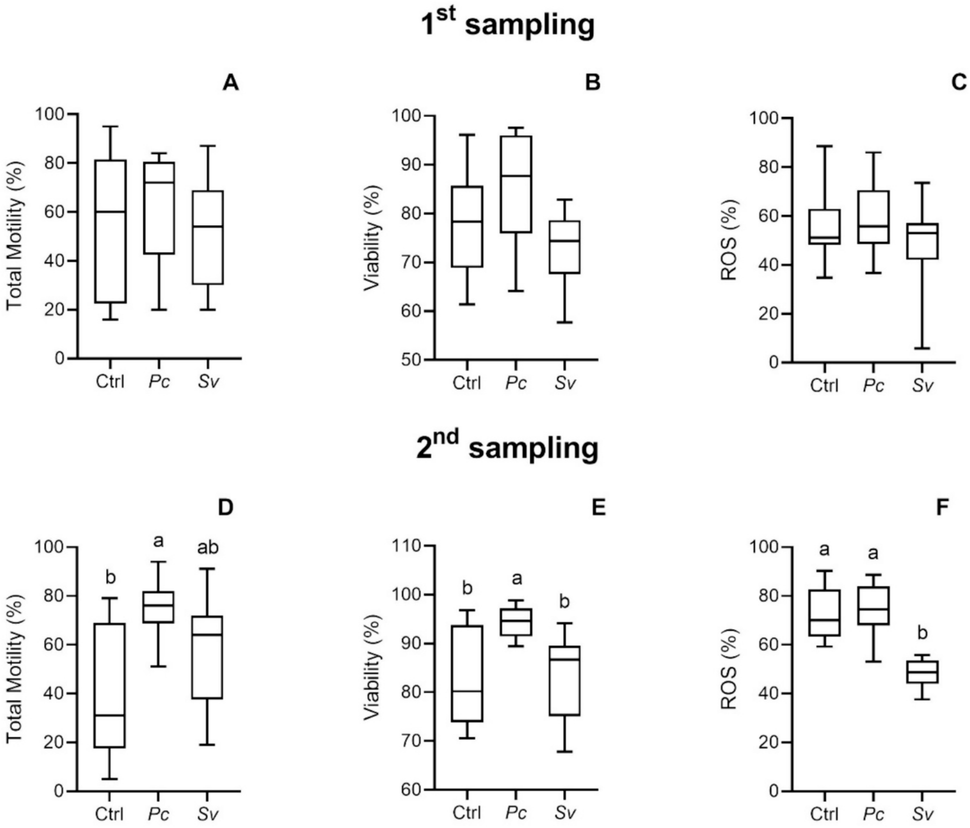

Fig. 2. Total motility (A, D), cell viability (B, E) and percentage of ROS determined in live cells (C, F) of Senegalese sole sperm at first and second sampling of the feeding trial. Fish were fed with control (Ctrl), *Plocamium cartilagineum* (*Pc*) and *Sargassum vulgare* (*Sv*) supplemented diets. Results are shown as mean ± SD (n = 9–10). Significant differences between treatments were identified with different letters (one-way ANOVA, Tukey, $p < 0.05$).

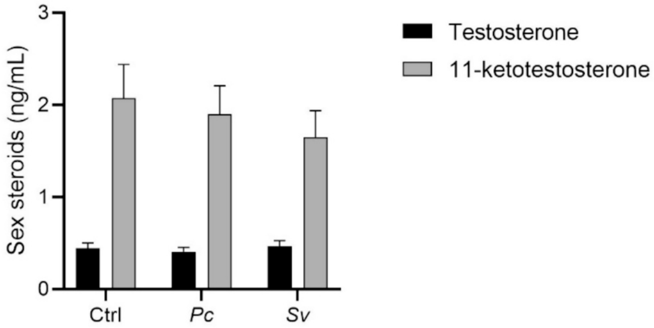

Fig. 3. Levels of testosterone and 11-ketotestosterone in blood plasma of Senegalese sole fed with control (Ctrl), *Plocamium cartilagineum* (*Pc*) and *Sargassum vulgare* (*Sv*) supplemented diets for 2 months. Results are represented as mean ± SE (n = 11–12). No differences were identified between treatments, within the same steroid hormone (one-way ANOVA, Tukey, $p < 0.05$).

4. Discussion

The inclusion of macroalgae in fish diets has been under study during the past decades, although this is the first report about their effects on fish reproductive performance, specifically in Senegalese sole males. In our experiment, two different feeds were formulated including 5% dry weight from two macroalgae, *Sargassum vulgare* (*Sv* diet) and *Plocamium cartilagineum* (*Pc* diet). Both seemed to modulate different reproductive traits without compromising either the FER or the SGR. In this experiment, fish had good acceptance of the diets supplemented with algae, and there was even a tendency for both parameters to be higher in fish fed with the *Pc* diet.

In different feeding trials performed on European seabass (Wassef et al., 2013) and gilthead seabream fry (Wassef et al., 2005), the SGR was also not affected by the algae supplemented diets, although there was a slight increase in weight gain in fish fed with 5% *Ulva sp.* and 10% *Pterocladia*, respectively. The fact that no differences were observed in those parameters in none of the above-mentioned studies, together with the positive results observed in other physiological parameters, namely on feed utilization, nutrient retention, stress response and survival rate, highlights the benefits of incorporating macroalgae in fish diets. Likewise, in a study that tested the inclusion of the microalgae *Schizochytrium sp.* in a plant-based diet for female rainbow trout, no differences were shown in growth and feed efficiency, but resulted in more viable eggs and higher fry survival rate, when compared to the eggs and progeny from fish fed with control diet (Cardona et al., 2022).

In the present study, there were no differences in FER and SGR and, at the end of the trial, spermatozoa viability was one of the sperm quality parameters enhanced in fish fed with *Pc* diet. Concerning reproductive effects, the only study in fish available in the literature is focused on the effects of microalgae diet inclusion in the female reproductive system of freshwater species. Besides the cited study, a *Spirulina* meal was also tested in the diet of yellow tail cichlid (*Pseudotropheus acei*), which showed an influence on the female's reproductive system by enhancing egg production and increasing the hatching rate (Güroy et al., 2012).

In the majority of feeding trials, results are not immediately visible, as time is required for differences to start revealing, especially when dealing with broodstock nutrition and their necessary time for gonadal maturation, which varies according to the species (Izquierdo et al., 2001). In our trial, after one month of feeding, no differences were observed between treatments in any of the quality parameters, which is in accordance with other feeding trials. In the previously mentioned study with European seabass, stress response parameters were only

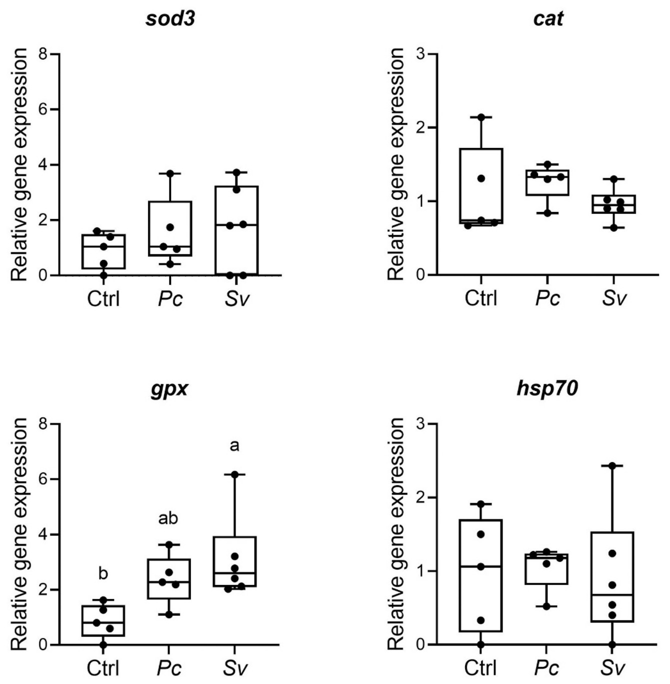

Fig. 4. Relative expression levels of genes of the antioxidant system of Senegalese sole testis, at the end of the feeding trial. Fish were fed with control (Ctrl), *Plocamium cartilagineum* (*Pc*) and *Sargassum vulgare* (*Sv*) supplemented diets for 2 months. Results are represented by interleaved boxes and whiskers with individual values (dots) for variance of data representation ($n = 5–6$). One-way ANOVA was applied to *superoxidase dismutase* (*sod3*) and *catalase (cat),* and Kruskal-Wallis to *glutathione peroxidase* (*gpx*) and *heat-shock protein* (*hsp70*) to identify statistical differences, which were considered when $p < 0.05$ and represented with different letters.

enhanced at the end of the trial (after 2 months) (Wassef et al., 2013). Nonetheless, at the end of our experiment, the sperm motility, cell viability, percentage of live cells with ROS, and expression of *gpx* in fish gonads displayed positive differences between treatments. Regarding sperm motility, the percentage of total motile cells (TM) showed a statistically significant increase between samplings. This parameter is considered one of the best indicators in terms of sperm quality and is directly linked with fertilization capacity and cell viability (Gallego and Asturiano, 2019). In fact, fish fed with *Pc* diet showed not only higher TM, but also a higher percentage of viable cells at the end of the experiment, which is in accordance with previous literature (Gallego and Asturiano, 2019). In an *in vitro* and *in vivo* experiment done with zebrafish, the extracts of the red algae *Plocamium lyngbyanum* stimulated osteogenic activity by enhancing cell proliferation (Carson et al., 2018). Unfortunately, the authors did not identify the bioactive compound responsible for that result, however, they compared its potential to the action of sulfated polysaccharides (fucoidans) naturally present in brown algae (Sony et al., 2019). The higher TM observed can be related to several factors, but the most common would be either a higher intracellular ATP content (Suquet et al., 2012), a higher percentage of mature spermatozoa or even a change in seminal plasma pH (Kowalski and Cejko, 2019) provoked by the *P. cartilagineum* supplementation. In fact, pyruvic acid is highly abundant in *P. cartilagineum* (Valentão et al., 2010) and is essential in many energy-supplying pathways utilized by fish spermatozoa, namely glycolysis, Krebs cycle and oxidative phosphorylation (Dzyuba and Cosson, 2014), which could explain the high TM observed on fish fed with *Pc* diet.

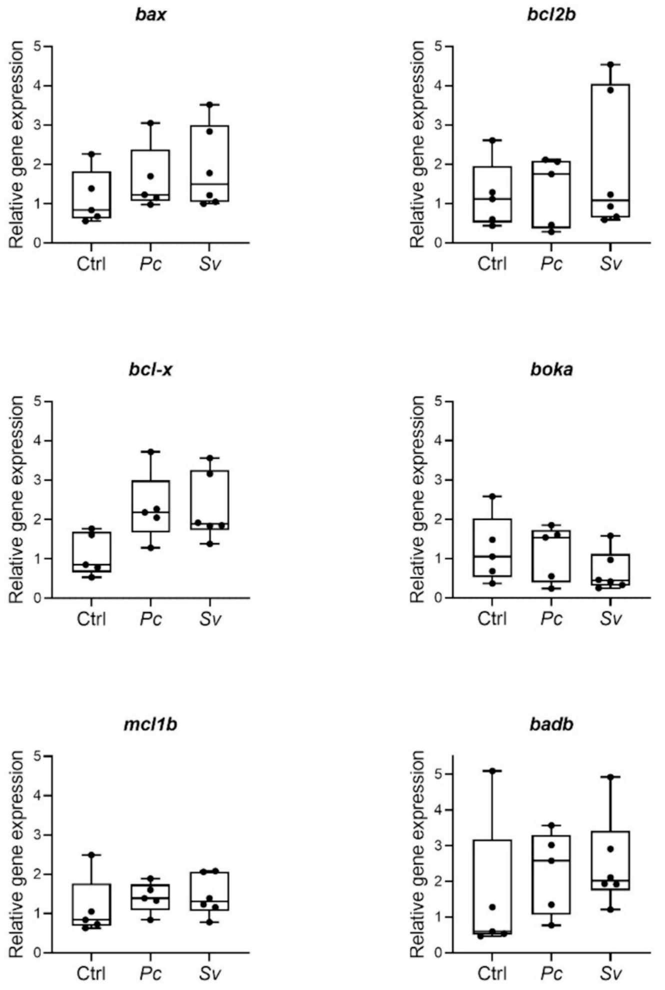

Fig. 5. Relative expression levels of anti- and pro-apoptotic genes in Senegalese sole testis. Fish were fed with control (Ctrl), *Plocamium cartilagineum* (*Pc*) and *Sargassum vulgare* (*Sv*) diets for 2 months. Results are represented by interleaved boxes and whiskers with individual values (dots) for variance of data representation ($n = 5–6$). One-way ANOVA was applied to *bax* and *bcl-x*, and Kruskal-Wallis to *bcl2b, boka, mcl1b* and *badb*. No differences ($p < 0.05$) were identified.

On the other hand, the fish fed with *Sv* diet had a higher percentage of live cells without ROS, which means that *S. vulgare* contain compounds which acted as a scavenger of the free radicals, as hypothesized. This output may result from the action of several phenolic compounds present in *S. vulgare*, such as the phenolic terpenoids, Isoflavones (flavonoids present in *Sargassum sp.*), bromophenols and phlorotannins (Cotas et al., 2020). This last group is exclusively present in brown algae, and its antioxidant potential was already considered to be 2 to 10 times higher than the antioxidant capacity of ascorbic acid (vitamin C) or tocopherol (vitamin E) (Shibata et al., 2009). Other abundant components of brown seaweeds are the polysaccharides, also known for their antioxidant properties (Tian et al., 2020). In an *in vitro* experiment with Vero cells, the polysaccharides isolated from the macroalgae *Sargassum fulvellum* showed a strong antioxidant effect by scavenging the intracellular ROS and improving cell viability (Wang et al., 2019). Within the same study, the polysaccharides also showed a strong protective effect against provoked oxidative stress *in vivo,* in zebrafish. Also, the apoptotic system of Vero Cells was modulated: the proapoptotic protein levels (BAX and caspase-3) were downregulated and antiapoptotic ones (BCL-XL and PARP) upregulated. In our experiment, no differences were observed in the relative expression of the anti- and proapoptotic genes analyzed. Nonetheless, regarding the gonadal antioxidant system, the *gpx* was upregulated on fish fed with *Sv* diet, a result that was in accordance with the decrease in cellular ROS from the same group of fish. Together, these results corroborate that the bioactive compounds present in the *S. vulgare* have strong antioxidant potential and its inclusion in the diets can confer extra protection to the reproductive system of Senegalese sole males. In turbot, a close flatfish species, similar results were obtained when 7.5 and 10% of *Sargassum horneri* meal were used, leading to higher catalase activity in blood serum (Wang et al., 2018). In accordance with what was observed in the turbot trial, in which no differences were obtained in SOD activity, in our study no differences were obtained in the relative expression of *sod3* in fish gonads.

The gonadal sex steroids, testosterone and 11-ketotestosterone, are important in controlling the different phases of spermatogenesis (Garcia-Lopez et al., 2006). The results from sex steroids analysis did not reveal differences between treatments, and the observed levels of T and 11KT were in accordance with previous studies performed on Senegalese sole F1 males (Cabrita et al., 2011b; Oliveira et al., 2020), which means that the introduction of 5% of macroalgae did not impact sex steroid levels of Senegalese sole.

Regarding the utilization of dietary macroalgae in Senegalese sole diets, literature is scarce (Moutinho et al., 2018; Vizcaíno et al., 2019), but in both studies different dietary macroalgae modulated different physiological effects, likewise in our feeding trial. The fact that different species improved different reproductive traits stimulates the research on using algae blends and studying their synergic effects. It is worth mentioning that the chemical composition of algae differs according to species, season, and collection site (Schmid et al., 2013; Naiel et al., 2021) and its effects on animal physiology will change accordingly. Thus, the results obtained from the present experiment intend to promote further research on macroalgae composition and its effects on the reproductive system of different fish species, once there are no doubts about the damage that excess ROS can cause in fish spermatozoa (Valcarce and Robles, 2018) and the potential of macroalgae to avoid and mitigate those oxidative processes.

5. Conclusions

The present study allowed to understand the effects of different dietary macroalgae supplementation on Senegalese sole sperm characteristics. The inclusion of the red algae *Plocamium cartilagineum* improved the performance of spermatozoa, through the total motility and cell viability. On the other hand, fish fed with brown macroalgae *Sargassum vulgare* supplementation had the genes involved in the gonadal antioxidant system upregulated and showed lower intracellular ROS. In face of the results, it is highly recommended to further explore the potential of macroalgae inclusion in fish feeds, namely in algae blends.

CRediT authorship contribution statement

F. Félix: Investigation, Methodology, Validation, Data curation, Formal analysis, Writing – original draft, Visualization. **N. Silva:** Investigation, Validation, Data curation, Formal analysis. **C.C.V. Oliveira:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision. **E. Cabrita:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **P.J. Gavaia:** Conceptualization, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Francisca Felix reports financial support was provided by Foundation for Science and Technology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741069.

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