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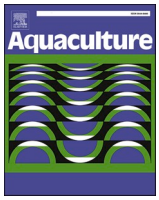

Testis transcriptomic analyses reveal the effects of an algae feed on sperm quality in Senegalese sole during the breeding season

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ABSTRACT

The usage of dietary algae antioxidants to improve fish reproduction is under-explored, especially in terms of the male reproductive system. In this experiment, 6 % of a blended meal of *Phaeodactylum tricornutum* and *Gracilaria gracilis* was incorporated in Senegalese sole broodstock feed, to evaluate the effects on sperm quality of F1 males throughout the breeding season. For that, two groups of breeders were fed during 6 months with the control and algae diets (6 % of control wheat meal replaced with 6 % algae blend). Every 2 weeks, fish were sampled for sperm quality evaluation, which included spermatozoa motility (CASA system), lipid peroxidation (MDA quantification), cell viability, reactive oxygen species (ROS), apoptotic status (flow cytometer), and DNA fragmentation (Comet assay). On a final sampling, 6 fish per group were sacrificed to dissect gonadal tissue, extract RNA and perform an RNA sequencing (RNA-seq) for each treatment. Sperm quality variability was high during the breeding season, including within the same month, irrespective of the diet. Cell viability was approximately 80 % during the whole experiment. Nonetheless, in specific sampling points, algae-fed fish showed higher spermatozoa protection against oxidative processes: in the 1st sampling live cells without ROS (%) were 3 times higher than in control group; on the last two samplings, spermatozoa showed half of MDA content; and on the 3rd sampling had less DNA fragmentation. No differences were found regarding apoptotic status. At the end of the reproductive season, gonadal transcriptomic analysis revealed that algae-fed fish were lacking stimuli for sperm production, both in terms of quantity and quality. This fish group seemed to have lipid metabolism and antioxidant capacity enhanced by the diet but, at the same time, were facing a compensatory mechanism due to an unknown algae compound that might be disrupting DNA replication and spermatogenesis. Altogether, this study suggests that algae blends can be used in broodstock feeds for Senegalese sole, however further research is needed to understand how to use only the desirable bioactive compounds and thus obtain higher and consistent sperm quality throughout the breeding season.

1. Introduction

In the last decades, significant advances have been made regarding physiological aspects related to captivity of Senegalese sole (*Solea senegalensis*), contributing to the welfare and production efficiency of this species. Despite these research efforts, there are still several topics to address that compromise the sustainability of its intensive production, as happens for other species. The reproductive impairment of Senegalese sole (Riesco et al., 2019), and the search for alternative feed ingredients (Oliva-Teles et al., 2022) are two main topics being investigated. Senegalese sole males born and raised in captivity (F1 males) do not spawn naturally and typically display lower gamete quality when compared with wild individuals (Chauvigné et al., 2016), which makes reproduction dependent on artificial reproductive techniques and quite often on the establishment of wild broodstocks, implying captures from the wild. Besides, the long-term viability of intensive aquaculture is compromised by the increasing prices and shortage supply of fish meal and fish oil (FM and FO), making the aquafeed industry focused on searching for sustainable ingredients for fish feeds (FAO, 2022). Broodstock nutrition is an important aspect when dealing with reproduction because it influences not only fish health, but also gamete and progeny quality (Fernández-Palacios et al., 2011). There is a lack of knowledge about the effects of FM and FO substitution on the reproductive performance of breeders (Izquierdo et al., 2015) due to the time

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necessary to evaluate those effects on consecutive spawning seasons and progeny. Thus, the preferable approach has been to supplement functional ingredients in diets and evaluate the immediate effects on a specific trait (Hernandez de Dios et al., 2022).

Dietary lipids are a major constituent of membranes, which makes them essential for fish growth, health, and several bodily functions (Hodar et al., 2020). In reproduction, fatty acids play a pivotal role in egg and sperm quality and offspring development (Cardona et al., 2022), being responsible for their anti-inflammatory response and antioxidant properties (Salas-Huetos et al., 2019). Since the discovery of microalgae as an optimal source of fatty acids, they have been used in aquatic feeds with success (Zhang et al., 2019). Either as an algae supplement or as an alternative feed ingredient for FM or FO, species such as *Chlorella* sp., *Spirulina* sp., *Phaeodactylum* sp., *Nannochloropsis* sp., *Tetraselmis* sp., *Schizochytrium* sp., *Tisochrysis* sp., and *Scenedesmus* have already proven positive effects in fish species like zebrafish (*Danio rerio*) (Carneiro et al., 2020), rainbow trout (*Oncorhynchus mykiss*) (Cardona et al., 2022), yellow tail cichlid (*Pseudotropheus acei*) (Güroy et al., 2012), longfin yellowtail (*Seriola rivoliana*) (Kissinger et al., 2016), red seabream (*Pagrus major*) (Seong et al., 2021), gilthead seabream (*Sparus aurata*) (Carvalho et al., 2020), European seabass (*Dicentrarchus labrax*) (Batista et al., 2020), Senegalese sole (Vizcaino et al., 2018; Peixoto et al., 2021) and common sole (*Solea solea*) (Shawky et al., 2021). Although there are many studies, the effects of algae-supplemented diets in fish reproduction have been a neglected topic of research, since the majority focus on growth performance and immunology, such as the studies mentioned before. Diatoms, like *Phaeodactylum tricornutum*, are known for their wide diversity of fatty acids and have been used in the food industry as a natural source of polyunsaturated fatty acids (PUFA), antioxidants and pigments (Daboussi et al., 2014). The inclusion of *P. tricornutum* biomass in fish diets demonstrated to increase the immunologic activity of gilthead seabream (Cerezuela et al., 2012; Reis et al., 2021), lipid digestibility and pigmentation of Atlantic salmon (*Salmo salar*) (Sørensen et al., 2016, 2023), and larval growth performance of Senegalese sole (Barreto et al., 2021). In terms of culture conditions, *P. tricornutum* showed to be a dominant species, tolerant to high pH, with quick growth, inclusive under low light and in a range of culture media, and with proven ability for being a commercially viable species for large-scale cultivation (Butler et al., 2020). Altogether, this diatom seems to be a sustainable and suitable supplement or feed ingredient for fish feeds.

Seaweeds are another type of marine product that has been studied as a supplement or alternative ingredient, due to their high nutritional quality and potential availability (Norambuena et al., 2015). Among other macroalgae, the genus *Gracilaria* (Gracilariales, Rhodophyta) is rich in biologically active phytochemicals, including carotenoids, terpenoids, xanthophylls, chlorophylls, phycobilins, sterols, PUFA, polysaccharides, vitamins, among others (Torres et al., 2019). Besides those, *Gracilaria gracilis* presents a high content of arachidonic acid (n-6 PUFA), prostaglandins, proteins, carbohydrates, phenols, and high levels of antibiotic, antioxidant and radical scavenging activity (Francavilla et al., 2013). Recently, European seabass fed with an 8 % *Gracilaria gracilis* supplemented diet showed higher immunocompetence against fish pathogens (Ferreira et al., 2022). Macroalgae supplementation also led to good results in Senegalese sole juveniles fed with *Ulva* sp. supplemented diets, which showed higher resistance of the intestinal mucosa (Vizcaíno et al., 2019), better nutrients utilization and fish growth (Moutinho et al., 2018). Recent research from our group with macroalgae included in Senegalese sole diets concluded that the different algae used (*Plocamium cartilagineum* and *Sargassum vulgare*) modulated distinct sperm and reproductive traits (Félix et al., 2024b). The Senegalese sole industry cannot reproduce naturally their F1 broodstock in captivity. The poor sperm quality, low volume and high sperm quality variability are some of the reasons for the presented reproductive impairment (Beirão et al., 2011). For spermatozoa proliferation and differentiation, lipids are of utmost importance due to their role in cell membranes and association of antioxidative response (Izquierdo et al., 2001). For the present study, based on the presented algae literature and previous results, we hypothesized that a blend of algae with different lipid and antioxidant capacity profiles (the microalgae *Phaeodactilum tricornutum* and the macroalgae *Gracilaria gracilis*), could result in a synergic effect and ameliorate the overall reproductive performance and gamete quality of F1 Senegalese sole males, throughout the reproductive season.

2. Material and methods

2.1. Ethical statement

The present study was performed according to the ARRIVE guidelines, with 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) of the Portuguese direction for veterinary and food services (DGAV), which previously approved experimental procedures with germ cells (ref. 003289). The CCMAR infrastructures are certified to conduct experiments with live animals (license with ref. 009238), and all technicians and researchers hold a FELASA B or C category certification, approved by DGAV.

2.2. Feed formulation

Two semi-moist feeds for Senegalese sole broodstock were formulated and produced by SPAROS Lda (Olhão, Portugal). The SPAROS commercial feed for sole broodstock (REPRO Sole) was used as the control diet, which contained all the protein, fat and gross energy considered essential for the species. The algae diet was formulated by substituting 6 % control wheat meal for the same percentage of macro and microalgae dried biomass (from commercial source): 3 % *Gracilaria gracilis* + 3 % *Phaeodactylum tricornutum*. All other ingredients were constant among the two diets. The formula of the diets is detailed in Table 1. All powder ingredients were mixed according to the target formulation in a double-helix mixer (model RM90, MAINCA Spain) and ground (below 200 μm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Subsequently, the oils were added to the mixtures, humidified with 25 % water and made into 6 mm pellets by a low-shear and low-temperature extrusion process (ITALPLAST, Italy). Semi-moist feeds were packed in sealed plastic bags and shipped to the *Ramalhete* research station, where they were stored frozen at −20 °C until use. Samples of diets were taken for analytical characterization (Table 2).

2.3. Experimental design and samplings

The objective of this study was to understand whether a broodstock diet with a blend of micro and macroalgae as a functional ingredient could improve the sperm quality of first-generation (F1) Senegalese sole males. For that, an established broodstock of F1 breeders, with approximately 1141 ± 353 g of body weight, was kept at the *Ramalhete* research station (Faro, Portugal) in 4 fibber-glass tanks of 5.89 m^3, each containing 23 animals. Fish were distributed with a sex ratio of 2:1 (male:female), and kept under natural photoperiod oscillations, in a semi-open water system, with temperatures varying between 14.6 and 23.6 °C along the experiment (February – July). The feeding started in February with two tanks being fed once a day with a control diet (control) and the other two with an algae diet (algae), which composition was described in the previous section. From April to June, all tanks were sampled for sperm collection every two weeks (total samplings: 5), allowing fish to recover from the stress caused by handling.

After anesthesia (300 ppm phenoxyethanol), the genital pore was cleaned with PBS (phosphate-buffered saline solution) and paper to avoid sperm contamination with urine or anesthesia. Samples were kept in a styrofoam box with ice, at approximately 10 °C, and immediately

Table 1
Formulation of the experimental feeds (control and algae), as percentages (%) of ingredients.

Ingredients, %	Control	Algae
Fishmeal [1]		
Fish protein hydrolysate [2]		
Squid meal [3]		
Krill meal [4]		
Wheat gluten [5]	70.50	70.50
Microalgae (*Phaeodactylum tricornutum*) [6]		3.00
Macroalgae (*Gracilaria gracilis*) [7]		3.00
Wheat meal [8]	6.00	
Vitamin and mineral premix [9]		
Other additives [10]	14.20	14.20
Soy lecithin [11]		
Fish oil [12]		
Fungal oil (*Mortierella alpina*)	9.30	9.30

[1] Fishmeal LT70: 71 % crude protein (CP), 9 % crude fat (CF), Pelagia AS, Norway.
[2] CPSP 90: 82 % CP 9 % CF, Sopropêche, France.
[3] Squid meal without guts: 83 % CP, 4 % CF, Sopropêche, France.
[4] Krill meal: 52 % CP, 22 % CF, Aker Biomarine, Norway.
[5] VITAL: 81 % CP, 4.3 % CF, Roquette, France.
[6] Dry algae biomass (*Phaeodactylum tricornutum)*: 35 % CP, 11 % CF, Allmicroalgae SA, Portugal.
[7] Dry seaweed (*Gracilaria gracilis*): 21 % CP, 0.8 % CF, ALGAplus, Portugal.
[8] Wheat meal: 10.2 % CP; 1.2 % CF, MOLISUR, Spain.
[9] PREMIX Lda, Portugal: Vitamins (IU or mg kg-1 diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamine, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; betaine, 500 mg. Minerals (g or mg kg-1 diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middling's.
[10] SPAROS proprietary blend of nutritional (amino acids, vitamins, minerals), sensory (carotenoids) and technological additives (binders).
[11] LECICO GmbH, Germany.
[12] Sopropêche, France

taken to the laboratory for sperm quality analysis. In a final sampling, one month after the 5th sampling, 3 male fish per tank were randomly sacrificed with a lethal dose of anesthesia (1000 ppm phenoxyethanol) and testis were immediately dissected and frozen in liquid nitrogen. Testis samples were stored at −80 °C for RNA extraction and further RNA-Seq quantification.

2.4. Sperm motility analysis

Spermatozoa motility from the collected samples was assessed using Computer Assisted Sperm Analysis software (ISAS, Proiser, Valencia, Spain). An upright phase-contrast microscope (Nikon E-200; Nikon, Tokyo, Japan) with an ISAS camera (25 fps), was used to search for differences in the gamete quality of fish fed with control ($n = 15–17$) and algae (n = 15–20) diets. To activate the spermatozoa, 0.5 μL of sperm was mixed with 10 μL of artificial seawater in a Makler chamber, under a 10× negative phase contrast objective. Different parameters of motility were recorded 15 s post-activation: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s) and linearity (LIN, %).

2.5. Lipid peroxidation

The quantification of malondialdehyde (MDA) was used to evaluate the lipid peroxidation of sperm samples at each sampling point of fish fed with control ($n = 16–21$) and algae ($n = 11–21$) diets. The procedure was performed according to previous studies from our group (Riesco et al., 2017), using a commercial kit (kit BIOXYTECH LPO-586™, OxisResearch) and following the manufacturer's protocol. In the end, the absorbance was measured at 586 nm in a microplate reader (Synergy 4, Biotek Instruments. Inc.), and MDA concentration (nM/million spermatozoa) was determined from a standard curve calculation.

Table 2
Analytical composition of the control and algae feeds.

	Control	Algae
Moisture, %	26.1 ± 0.6	26.8 ± 0.9
Crude protein, % feed	45.9 ± 0.7	46.3 ± 0.3
Crude fat, % feed	10.9 ± 1.1	11.2 ± 0.7
Ash, % feed	7.3 ± 0.3	7.8 ± 0.2
Gross energy, kJ/kg feed	16.8 ± 0.1	16.9 ± 0.1
Amino acids		
Arginine, % feed	2.7	2.7
Histidine, % feed	1.0	1.0
Isoleucine, % feed	1.7	1.7
Leucine, % feed	3.0	3.0
Lysine, % feed	2.5	2.5
Threonine, % feed	1.6	1.6
Tryptophan, % feed		
Valine, % feed	1.9	1.9
Methionine, % feed	1.0	1.0
Cysteine, % feed	0.4	0.4
Phenylalanine, % feed	1.8	1.7
Tyrosine, % feed	1.3	1.3
Aspartic acid, % feed	3.3	3.3
Glutamic acid, % feed	7.4	7.2
Alanine, % feed	2.4	2.4
Glycine, % feed	2.9	2.9
Proline, % feed	2.7	2.7
Serine, % feed	1.8	1.8
Minerals		
Phosphorus, % feed	1.2	1.2
Calcium, % feed	1.1	1.1
Sodium, % feed	0.5	0.4
Magnesium, % feed	0.1	0.1
Potassium, % feed	0.5	0.5
Copper, mg/kg feed	15.7	15.6
Iron, mg/kg feed	160.3	159.4
Iodine, mg/kg feed	4.8	5.2
Manganese, mg/kg feed	24.5	24.7
Selenium, mg/kg feed	1.3	1.5
Zinc, mg/kg feed	123.0	142.6
Fatty acids		
C14, % feed	0.5	0.5
C16, % feed	1.6	1.6
C18, % feed	0.4	0.4
C18:1n9, % feed	2.1	2.1
LNA (C1 8:2n6), % feed	0.6	0.6
ALA (C18:3n3), % feed	0.2	0.2
ARA, % feed	0.4	0.4
EPA, % feed	1.1	1.1
DHA, % feed	1.1	1.1
EPA + DHA, % feed	2.17	2.17

2.6. Flow cytometry analysis: Cell viability, ROS and apoptotic status

Cellular viability, Reactive Oxygen species (ROS) and apoptotic status analysis were performed on a flow cytometer according to the protocols developed by our group for Senegalese sole sperm samples. The flow cytometer (BD FACSCalibur™, BD Biosciences, CA) was set with different lasers and filters according to the technique performed: 488 nm laser +585/42 nm filter for cell viability; 488 nm laser +530/30 and 585/40 nm filters for ROS quantification; 488 nm laser +530/30 nm filter +670 nm long pass filter for early-apoptosis (caspases 3/7) detection.

Cell viability and ROS detection were performed following the protocol described by Félix et al. (2024a), adapted for fresh sperm samples. For cell viability analysis, 1 μL of sperm was stained with 1 μL propidium iodide (PI, 1 mg/mL) (Sigma-Aldrich) in 500 μL of 1 % NaCl solution, and the fluorescence emitted was measured after 5 min of incubation (control: $n = 12–22$; algae: $n = 16–17$). For ROS quantification sperm

was stained with dihydroethidium (DHE) and SYTOX® green (Invitrogen™, ThermoFisher) in the following proportions: 1 μL sperm and 0.5 μL of DHE (0.5 mM) in 500 μL of 1 % NaCl solution. After 5 min of DHE incubation, 0.5 μL of SYTOX® green (1 μM) was added and, after 10 min of total incubation time, the emitted fluorescence was measured (control: $n = 15–21$; algae: n = 15–17). The apoptotic status assay was performed using the commercial kit Muse™ Caspase-3/7 (Millipore), following the manufacturer's instructions and adapting it for Senegalese sole sperm. For that, 1 μL of sperm was stained with 150 μL 7-amino actinomycin D (7-AAD) solution (1:3000 in Buffer) in 50 μL of NaCl 1 %. After 30 min of incubation, 5 μL of Caspase 3/7 solution (1:8 in PBS) was added and after 1 h of total incubation time, the emitted fluorescence was measured (control: n = 15–22; algae: n = 15–17).

For each technique, 30.000 events were collected from each sample, using the BD CellQuest Pro software (version 8.7, BD Biosciences, CA). In terms of results, cell viability was presented as the percentage of viable cells in the sperm population; the subpopulation of live cells producing ROS was selected for the analysis of ROS production; both percentages of live cells and live cells in early apoptosis were considered for the representation of apoptosis detection.

2.7. DNA fragmentation

Spermatozoa DNA fragmentation was assessed by Comet assay following the protocol described by Riesco et al. (2017) for Senegalese sole. After staining each sample (control: $n = 13$, algae: $n = 16$) with 10 μL of diluted PI (20 μM), the observation of the comets was performed in an upright fluorescence microscope (Zeiss Axioscope 5), with 40× A-plan 0.65 objective and red channel filter cube (Excitation Bandpass 560/40, Beam splitter 585, Emission Bandpass 630/75). Images were captured with a digital camera Axiocam 202 mono through the acquisition software Zen PRO 3.1. At least 100 cells per slide were analyzed using imaging Komet v6.0 software (Andor Technology, Ltd.). The results were presented as a percentage of tail DNA (tDNA), which relates to both the amount and size of the DNA fragments.

2.8. Statistical analysis

The obtained percentage data was arcsine square-root transformed, and statistical analysis was performed using SPSS software (IBM). Data that assumed the principles of normality and homogeneity of variance (Shapiro-Wilk and Levene tests, respectively) was analyzed with a two-way ANOVA and post hoc Tukey test or with the student-*t*-test, depending on the number of fixed factors. Significant differences were considered when $p < 0.05$. In the case of motility and lipid peroxidation analysis, data did not assume the above-mentioned principles, and the respective Kruskal-Wallis ($p < 0.01$) and U-Mann-Whitney ($p < 0.05$) non-parametric tests were applied. Linear regression and Pearson's correlation were applied for the validation of RNA-seq results.

2.9. RNA sequencing of gonadal tissue

2.9.1. RNA extraction

The gonads collected at the final sampling were homogenized in 1 mL of TRI Reagent® (Sigma-Aldrich, Switzerland) using a tissue-lyzer Star-Beater (VWR). Total RNA was extracted following the instructions of the manufacturer and the obtained pellet was resuspended in RNase-free distilled water (Sigma-Aldrich, Switzerland). Total RNA concentrations were measured with a Nanodrop One-C spectrophotometer (Thermo Fisher Scientific, Washington, USA), adjusted to 1 μg/μL and sent to ADM Biopolis (Valencia, Spain) for RNA sequencing. Total RNA quality and integrity were checked using a 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA), and only samples with an RNA integrity number (RIN) > 8 were considered for sequencing. The extracted RNA was also used in the validation of RNA-seq results.

2.9.2. Library preparation and sequencing

A total of 12 libraries were prepared using a TruSeq™ RNA Library Preparation Kit v2 of Illumina (Illumina, USA). The RNA-seq libraries were paired-end (PE) sequenced (2 × 75 bp) with dual indexing on an Illumina NovaSeq 6000 System, with poly-A selection, according to the manufacturer's protocol (TruSeq Stranded mRNA Reference Guide # 1000000040498 v00). From the 12 sequenced samples, an average of 68 million reads/sample were obtained, i.e., approximately 1.6 billion PE reads in total.

2.9.3. Mapping and statistical analyses

Quality control (QC) analysis of raw reads was performed using FastQC v0.11.9 (Andrews, 2010). Reads were processed using Fastp v0.22.0 (Chen et al., 2018) to remove adapters, filter-out low-quality and short reads (cut-off = 69 bp), reads with too many Ns, and perform base correction in overlapped regions. The calculations of Q20, Q30, GC-content, and sequence duplication levels of the clean data were also done with Fastp. To ensure the quality of trimmed reads, FastQC was used for inspection before subsequent analyses. The trimmed FASTQ libraries were mapped against the reference genome of *Solea senegalensis* (RefSeq accession: GCF_019176455.1 from the NCBI database, https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_019176455.1/), using the splice-aware STAR aligner v2.7.10 mapping software (Dobin et al., 2012) with the following settings: overhang – 72 bp, length (bases) of the SA pre-indexing string – 13, minimum intron length – 20, minimum alignment score normalized to read length – 0.4, minimum matched bases normalized to read length – 0.4 and output BAM files sorted by coordinate. Mapped and unmapped reads were extracted from the BAM files using SAMTools v1.9 (Li et al., 2009). Unmapped reads were inspected by BLAST to assess their origin. Alignment QC was performed using Qualimap v2.3 (Okonechnikov et al., 2015). To improve annotation, a reference-guided transcriptome assembly was performed using Stringtie v2.1.1 (Pertea et al., 2015). Transfrags were assembled individually for each sample and merged to generate a non-redundant transcriptome, which was subsequently compared to the reference annotation file (GTF) using gffcompare v0.11.2 (Pertea and Pertea, 2020). Read counts for each assembled gene and transcript were estimated from the coverage values calculated by Stringtie using an in-built Python script and reverse strandedness counts. All results from the previous steps were merged with MultiQC v1.21 (Ewels et al., 2016) (report provided in supplementary material - File S1). All analyses were performed using the CCMAR's high-performance computing (HPC) facility, CETA. Differential expression analysis (DEA) was done by importing the genes and transcripts' count matrices into the R package DESeq2 v1.42.1 (Love et al., 2014) from Bioconductor. Genes with low expression were removed (kept if >10 counts in ≥4 samples), normalization was performed according to sequencing depth and RNA composition, and variance stabilizing transformation (VST) was applied for visualization. Surrogate variables were estimated using the R package sva v3.50.0, employing the method "be", and included in the differential expression model. The threshold for differentially expressed genes (DEG) and transcripts (DET), calculated using Wald's test, was an adjusted *p*-value (Benjamini-Hochberg correction) < 0.05 and a log2| fold-change| (LFC) > 1.0 for DET and > 0.6 for DEG, after Bayesian shrinkage (Zhu et al., 2018). Visualization of differential expression results was achieved with the package EnhancedVolcano v.1.20 (Blighe et al., 2024).

2.9.4. Annotation and functional enrichment analyses

Annotation of unknown genes and potential novel transcripts was performed by homology search against *Danio rerio* (cut-off threshold of *E*-value <0.01) using the HMMER v3.3 nhmmer tool (Wheeler and Eddy, 2013) and blastx (against the non-redundant protein sequences nr v5 database) via the OmicsBox software v3.2.4 (BioBam Bioinformatics S. L., Valencia, Spain) CloudBlast. OmicsBox was also used for Gene Ontology (GO) annotation on the Biological Process (BP), Molecular

Function (MF) and Cellular Component (CC) categories, using the default settings through Blast2GO (Götz et al., 2008). Prediction of candidate open reading frames (ORF) and potential peptides of at least 70 amino acids was achieved using TransDecoder v5.7.0 (https://github.com/TransDecoder/TransDecoder). Homology search using the predicted peptides' FASTA sequences was performed with Pannzer2 (Törönen et al., 2018) (cut-off threshold of positive predictive value (PPV) > 0.5) and STRINGDB v12.0 (Szklarczyk et al., 2023). Functional enrichment analysis of differentially expressed transcripts (DET) was performed with the PANGEA - PAthway, Network and Gene-set Enrichment Analysis tool, using the *Danio rerio* orthologs gene symbols, against the GO, KEGG and REACTOME knowledgebases (Benjamini-Hochberg *p*-value adjusted <0.1). Visualization of enrichment results was achieved with the enrichplot R package (Yu et al., 2024).

2.9.5. Validation of RNA-seq

The validation of RNA-seq was done by performing a real-time quantitative PCR (RT-qPCR) in 13 relevant genes (*ncoa1, akap12b, rtn4a, rxfp3a.3a2, cyp17a2, b4galt1l, adamts5a, mcm4, bend5, gins, card14, mier, tmem63c*). Complementary DNA (cDNA) was synthesized from 1 μg RNA using the cDNA synthesis kit (Thermo Scientific Maxima First Strand cDNA Synthesis Kit, Thermo Scientific, Porto Salvo, Portugal) according to the manufacturer' instructions. Primer efficiency was evaluated by serial dilutions, where primers less than 85 % of efficiency were discarded (Table S1 - supplementary material). The qPCR was performed in a Bio-Rad CFX96TM Thermocycler (Bio-Rad, Amadora, Portugal) in 96-well plates in duplicate. Reactions were displayed in 20 μL containing 10 μL of SsoFast EvaGreen Supermix (Bio-Rad, Amadora, Portugal), 2 μL of primers (0.5 mM) and 5 μL of cDNA at the validated dilution. A negative control was introduced containing no cDNA. The amplification conditions were as follows: an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 57 °C for 5 s and a melting curve with a 0.5 °C increase (65–95 °C) for 2–5 s. The relative expression of mRNAs was quantified by the 2− ΔΔCT method using a geometric average of two housekeeping genes, ubiquitin (*ubq*) and beta-actin (*b-actin*), as a reference (Livak and Schmittgen, 2001).

3. Results

3.1. Water temperature and male sperm fluency profiles

The minimum daily average in water temperature was registered at the first sampling (min = 16.9 °C, max = 19.6 °C, average = 18.3 °C) and the highest was registered at the final sampling (min = 22.7 °C, max = 24.5 °C, average = 23.6 °C) (Fig. 1). The highest daily thermic amplitude was recorded at the 2nd sampling (3.2 °C) and the minimum at the 3rd sampling (1.0 °C). The number of fluent males oscillated throughout the experiment in both fish groups (control and algae), with a high percentage of fluent males from the control group in the 2nd sampling (100 %) and from the algae group in the first sampling (91 %) (Fig. 1).

3.2. Sperm quality parameters and biochemical status

Spermatozoa motility showed significant (Kruskal-Wallis, $p < 0.01$) oscillations along the experiment (from 1st to 5th sampling) in all parameters analyzed (Fig. 2). The 1st sampling displayed the lowest motility values: a low percentage of TM and the lowest percentage of PM, VCL, VSL, VAP and LIN. The best spermatozoa motility was recorded on the 3rd sampling, which had the highest values in all parameters. In the 3rd sampling (U-Mann-Whitney, $p < 0.05$), the group of fish fed with the control diet had significantly higher VAP (153.0 ± 13.1 μm/s) compared with the algae diet group (140.6 ± 17.7 μm/s).

The cell viability results revealed that sampling (1st to 5th) significantly influenced (two-way ANOVA, $p = 0.000$; Tukey test, $p < 0.05$) the percentage of viable spermatozoa (Fig. 3A). Sperm viability was high throughout the whole experiment, although it reached a significant peak at the 3rd (average: 86.0 ± 5.5 %) and 4th (average: 86.1 ± 8.3 %) samplings and decreased on the final sampling (average: 76.2 ± 9.0 %). Fish fed with control diet had a significantly higher percentage of viable cells at the 1st (82.4 ± 3.1 %) and 4th (88.7 ± 5.8 %) samplings, compared with the algae diet (77.7 ± 4.9 % and 82.7 ± 9.9 %, respectively) (Student-*t*-test, $p < 0.05$) (Fig. 3A).

The spermatozoa lipid peroxidation was significantly affected (Kruskal-Wallis, $p < 0.01$) by the sampling date (1st to 5th). Sperm lipid peroxidation varied along the experiment, with the lowest quantification of MDA at the 1st and 4th samplings, and the highest at the 2nd and 3rd samplings (Fig. 3B). At the end of the experiment, fish fed with algae diet showed significantly lower content of MDA on spermatozoa (57.4 ± 38.9 nM/Mspz), when compared with the control group (144.6 ± 90.4 nM/Mspz) (U-Mann-Whitney, $p < 0.05$).

The Comet assay results showed that sampling (1st to 5th) significantly impacted (two-way ANOVA, $p = 0.000$; Tukey test, $p < 0.05$) the percentage of DNA fragmentation in Senegalese sole spermatozoa (Fig. 3C). The variability observed in the percentage of tail DNA was high: the 1st and 3rd samplings showed the lowest values of DNA fragmentation (average: 29.8 ± 16.3 %) and the last sampling the highest (average: 62.4 ± 14.7 %). In the 3rd sampling, the fish fed with

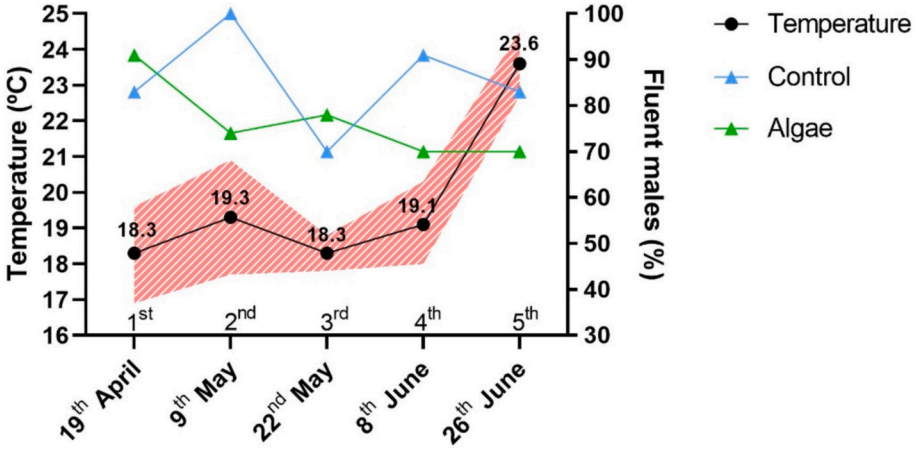

Fig. 1. Profiles of water temperature (left Y axis; black line) and Senegalese sole fluent males (right Y axis) fed with control (blue line) and algae (green line) diets throughout the feeding experiment. Water temperature (°C) values are represented per sampling point as daily average (black dots) and daily thermic amplitude (red area). The percentage (%) of fluent males is represented per sampling point with blue triangles referring to the fish fed the control diet and green triangles for the fish fed the algae diet. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

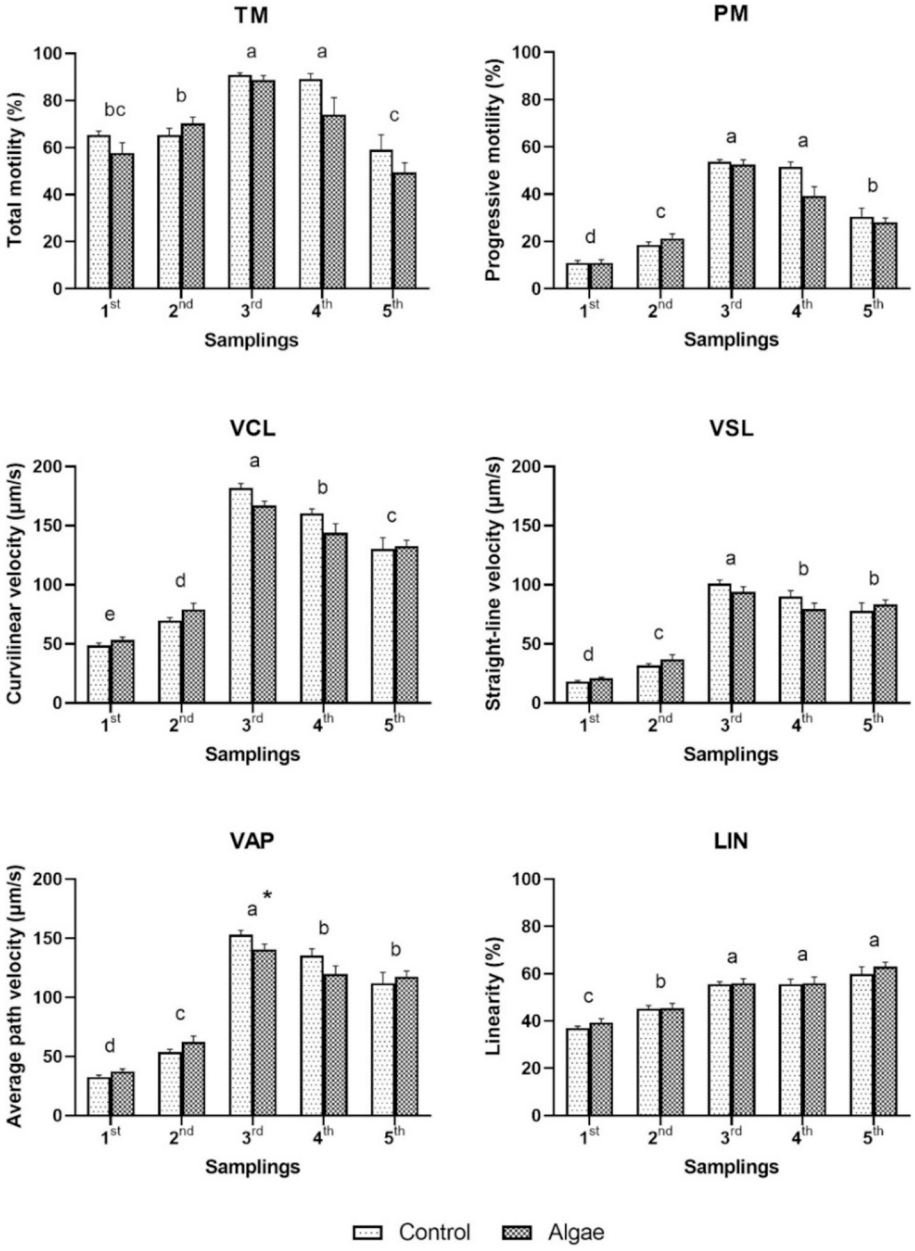

Fig. 2. Sperm motility parameters of Senegalese sole fed with control ($n = 15–17$) and algae (n = 15–20) diets, obtained for each sampling point (1st -19th April; 2nd - 9th May; 3rd - 22nd May; 4th – 8th June; 5th – 26th June). Total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s) and linearity (LIN, %) were registered at 15 s post-activation. Data are represented per treatment and sampling point as mean ± SE. Statistical differences (Kruskal-Wallis, $p < 0.01$) between samplings are represented with different letters, and between treatments (U-Mann-Whitney, $p < 0.05$) with an asterisk (*).

algae diet had less DNA fragmentation (27.2 ± 15.8 %) compared to the fish from the control group (32.3 ± 16.8 %) (Student-*t*-test, $p < 0.05$).

The apoptotic status analysis revealed that samplings (1st to 3rd) had a significant effect (two-way ANOVA, $p = 0.000$; Tukey test, $p < 0.05$) on the percentage of live cells (Fig. 4A), but also on early apoptotic and dead cells (Fig. 4B, D), irrespective of the fish diets. The 2nd sampling displayed better sperm quality: live cells were significantly higher at the 2nd and 3rd samplings (Fig. 4A). At each sampling, no differences were detected (Student-*t*-test, $p < 0.05$) between fish fed with different diets.

ROS analysis showed a significant influence (two-way ANOVA, $p = 0.000$; Tukey test, $p < 0.05$) of sampling (1st to 5th) in the percentage of live cells, live with ROS, dead and dead with ROS (Fig. 5). Moreover, the obtained percentages of live cells ($p = 0.006$) and live cells with ROS ($p = 0.009$) depended on the significant interaction between sampling x diet. Using the percentage of live cells as a reference, sperm quality reached its peak in the 3rd and 4th samplings, with significantly higher values in comparison with the remaining sampling points (Fig. 5A). Once again, the 1st sampling demonstrated the worst results: the percentage of live cells was low and live cells with ROS were high (Fig. 5A, B). At the 1st sampling, fish fed with the algae diet had better sperm quality compared with the control group: the percentage of live cells was significantly higher (algae: 21.93 ± 5.50 %; control: 7.67 ± 3.65 %) (Fig. 5A) and, although not significant, had less live cells with ROS and less dead cells with ROS (Fig. 5B, D).

3.3. RNA-seq data preprocessing and differential expression analysis

Raw reads' trimming and quality filtering resulted in an average of

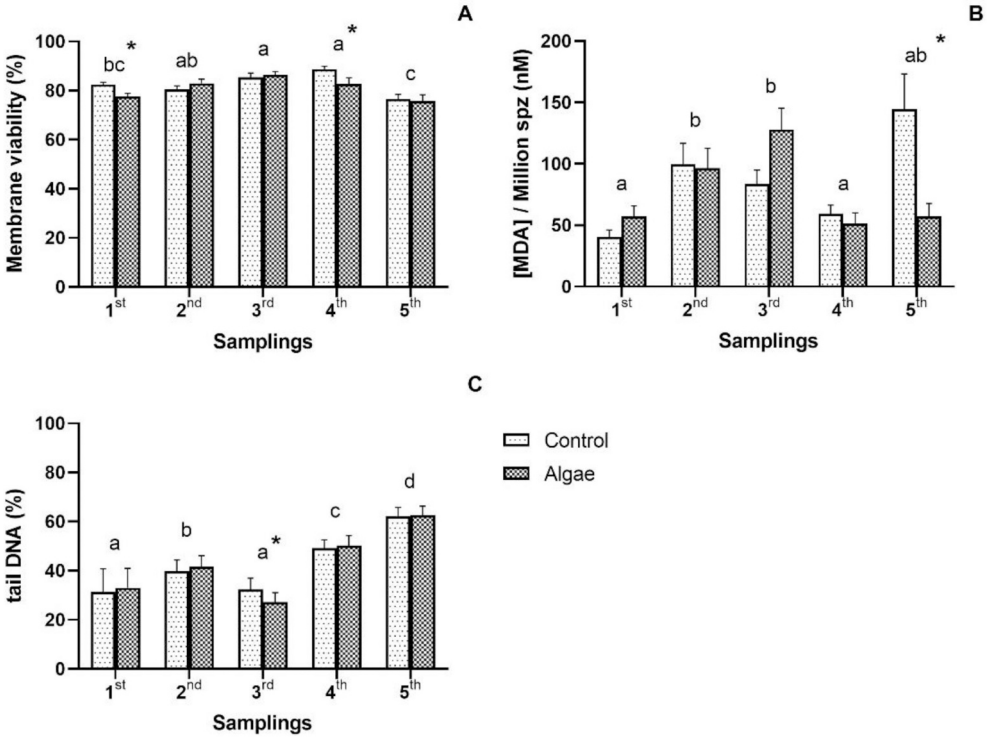

Fig. 3. Senegalese sole spermatozoa A) membrane viability (control = 12–22, algae = 16–17), B) MDA content (control = 16–21, algae = 11–21) and C) DNA fragmentation (control = 13, algae = 17) from fish fed with control and algae diets in each sampling point (1st -19th April; 2nd - 9th May; 3rd - 22nd May; 4th – 8th June; 5th – 26th June). Data are represented per treatment and sampling point as mean ± SE. The statistical differences of A and C (two-way ANOVA, Tukey test, $p < 0.05$) and B (Kruskal-Wallis, $p < 0.01$) between samplings are represented with different letters, and between treatments (Student-*t*-test or U-Mann-Whitney, respectively, $p < 0.05$) with an asterisk (*).

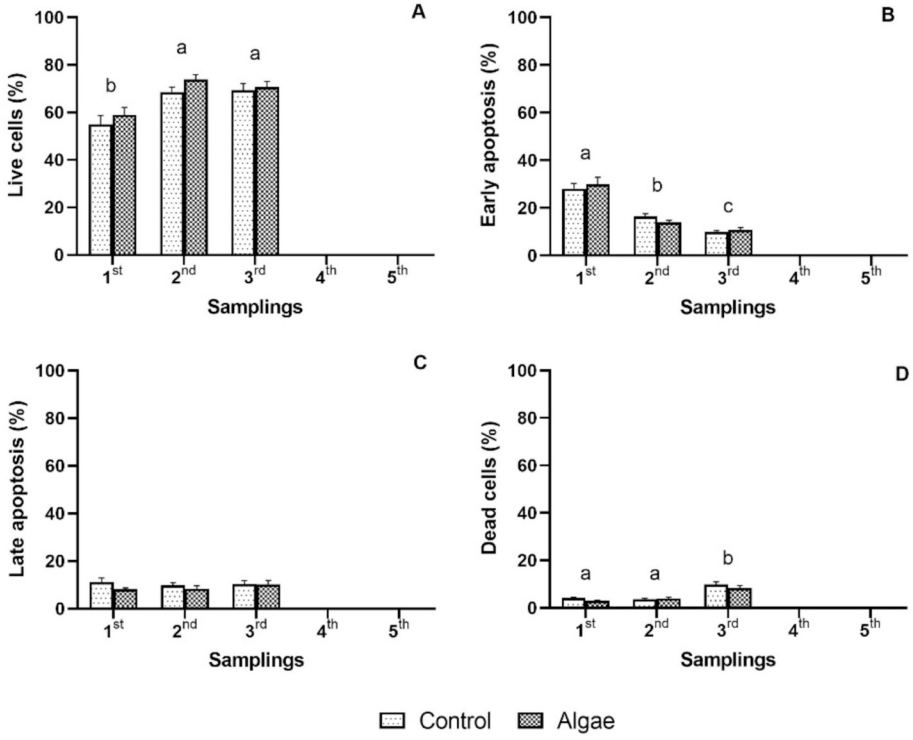

Fig. 4. Senegalese sole spermatozoa apoptotic status (caspases-3/7 detection). The percentages of A) live cells, B) early apoptotic cells, C) late apoptotic cells, and D) dead cells from fish fed with control (n = 14–23) and algae (n = 15–16) diets in each sampling point (1st -19th April; 2nd - 9th May; 3rd - 22nd May; 4th – 8th June; 5th – 26th June; n/d, non-detected) are given. It was not possible to perform the spermatozoa apoptotic status on the last two samplings (4th and 5th). Data are represented per treatment and sampling point as mean ± SE. The statistical differences of A, B, D (two-way ANOVA, Tukey test, $p < 0.05$) between samplings are represented with different letters.

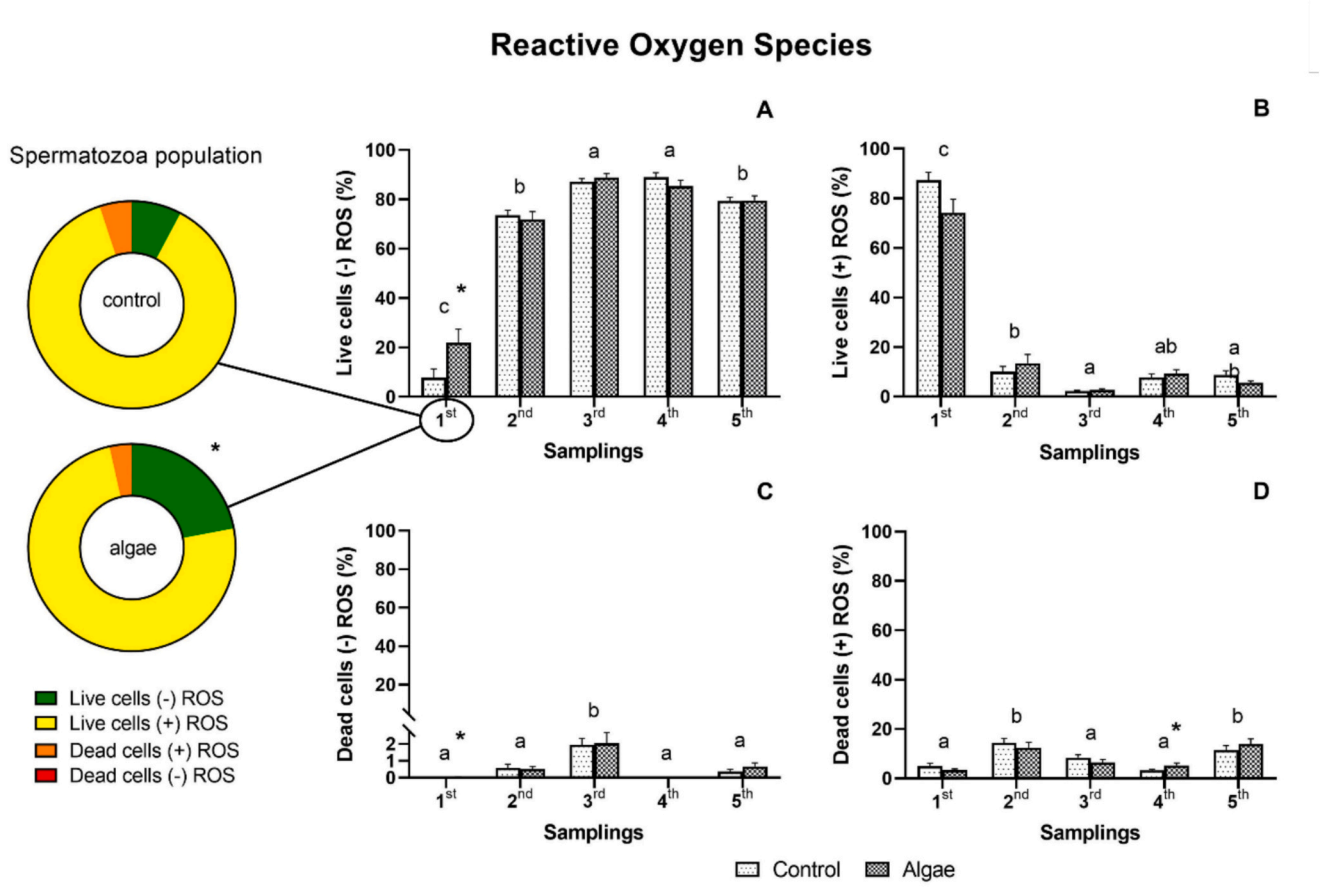

Fig. 5. Spermatozoa reactive oxygen species (ROS) of Senegalese sole fed with control (n = 15–21) and algae (n = 15–18) diets in each sampling point (1st -19th April; 2nd - 9th May; 3rd - 22nd May; 4th – 8th June; 5th – 26th June). Percentages of cells registered as live (A), live with ROS (B), dead (C), and dead with ROS (D). Data are represented per treatment and sampling dates as mean ± SE. Statistical differences (two-way ANOVA, Tukey test, $p < 0.05$) between samplings are represented with different letters, and between treatments (Student-*t*-test, $p < 0.05$) with an asterisk (*). A graphical highlight of the spermatozoa population at the 1st sampling is shown on the left side.

1.91 % discarded reads per sample, mainly due to short size (cut-off of 69 bp) and/or low quality (Fig. S1 – supplementary material). Two bases were trimmed at the 5′ end due to low phred score (> 20). Reads that passed the filter ranged between 76.7 and 178.1 million per sample (Fig. S1A), with a maximum length size of 73 bp and a GC content of 47.43 %. Regarding the mapping, more than 88 % of the trimmed reads were mapped to a unique location in the reference genome, and approximately 9 % were mapped to too many *loci*. Unmapped reads correspond mainly to ribosomal RNA. From the mapped reads, a mean of 11.61 % mapped to no features (i.e., unannotated regions of the genome) (Fig. S1B). Alignment quality control revealed that a mean of 73 % of reads mapped to exonic regions.

A reference-guided transcriptome assembly was performed to improve genome annotation, resulting in 92,259 consensus transcripts assembled, 9129 genes with at least one potentially novel transcript (multi-exon with at least one junction match) assembled and 4653 potentially novel transcription regions (i.e., no overlap with any reference gene/transcript). Summary statistics of the comparison between the assembled transcriptome and the reference genome are displayed in Table 3.

Gene and transcript count matrices were then imported into R for differential expression analysis (DEA) and low expression features were removed, resulting in two datasets with 36,333 and 69,972 assembled genes and transcripts, respectively. DEA retrieved 49 DEG (padj <0.05, LFC > 0.6) and 378 DET (assembled IDs) (padj <0.05, LFC > 1) among control and algae groups (supplementary material – Table S2). The distribution of DET was not homogeneous, as approximately 84 % were downregulated in the algae group (Fig. 6).

Table 3
Summary statistics of gffcompare.

Data summary		
Query mRNAs	92,259 in 30,586 *loci* (86,288 multi-exon transcripts)	
Reference mRNAs	48,619 in 28,766 *loci* (44,169 multi-exon)	
Matching intron chains	44,169	
Matching transcripts	48,388	
Matching *loci*	28,553	
Missed exons	0/283696	(0.0%)
Novel exons	39,284/361572	(10.9 %)
Missed introns	109/253311	(0.0%)
Novel introns	25,833/298039	(8.7 %)
Missed *loci*	0/28766	(0.0%)
Novel *loci*	4065/30586	(13.3 %)
Accuracy estimation		
	Sensitivity	Precision
Base level	100.0	73.0
Exon level	97.6	77.7
Intron level	99.9	84.9
Intron chain level	100.0	51.2
Transcript level	99.5	52.4
Locus level	99.3	86.1

Comparison between the experimental transcriptome assembled with Stringtie and the *Solea senegalensis* reference genome.

After DET annotation, no ORFs were found for 40 assembled transcripts, which were excluded from further analyses. A consensus

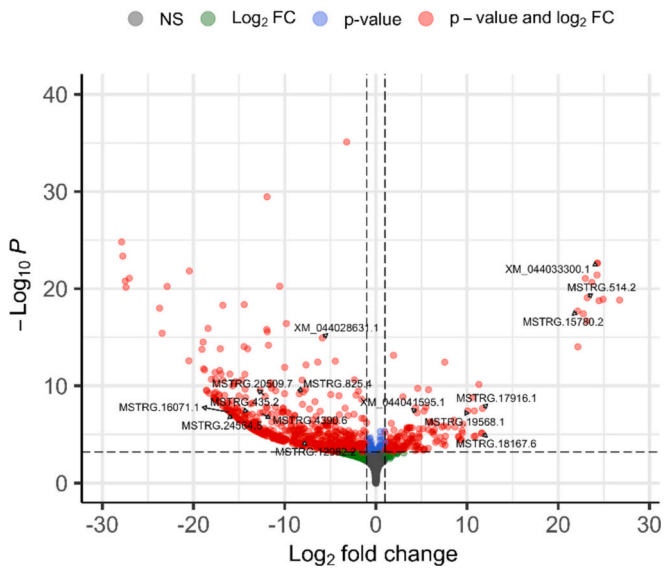

Fig. 6. Volcano plot of differentially expressed transcripts (DET) in the gonads of Senegalese sole fed with algae diet against the control group. Red points on the right side represent up-regulated DET, on left side represent down-regulated DET; remaining colors represent transcripts with no significant change in expression level between groups (padj < 0.05 and absolute LFC < 1). Key DET for reproduction and antioxidant systems are identified with Stringtie's accession number. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

annotation from the three databases used was attributed for the remaining transcripts, and among these, three transcripts corresponding to potentially novel transcription regions were found (MSTRG.13600.2, MSTRG.50.2, MSTRG.12906.3), while other three mapped to transcript variants corresponding to miscellaneous RNA types (misc_RNA), i.e., a type of non-coding RNA that cannot be classified. From the 338 annotated DET, 28 were detected exclusively in fish fed with algae diet, and 35 had more than one transcript corresponding to the same reference gene, which might correspond to different isoforms or splice variants. Some key DETs, that demonstrated to be implicated in reproduction-related processes and antioxidant system, were identified and highlighted in the volcano plot with Stringtie's accession number (Fig. 6).

Functional enrichment analysis based on GO, KEGG and REACTOME, revealed several genes involved in 23 significantly over-represented terms (padj < 0.1) (supplementary material – Table S3), such as metabolic processes, DNA replication, mRNA splicing, germ cells differentiation, and biosynthesis of lipids and steroid hormones. Some genes were identified to participate in more than one pathway: *gart* and *atic* in metabolic processes and de novo purine biosynthesis; *mcm4*, *gins2* and *gins4* in DNA replication and repair. A node graph was generated to represent the most relevant terms and associated genes (Fig. 7).

3.4. Validation of RNA-seq

The RT-qPCR performed on 13 relevant genes (*ncoa1*, *akap12b*, *rtn4a*, *rxfp3a.3a2*, *cyp17a2*, *b4galt1l*, *adamts5a*, *mcm4*, *bend5*, *gins*, *card14*, *mier*, *tmem63c*) validated the data obtained in the RNA-seq analysis (Fig. 8). The comparison between the results of gene expression in both techniques presented the same tendency with a linear regression of $r^2 = 0.84$ and a strong Pearson's correlation $r = 0.92$ (Fig. S2 – supplementary material).

4. Discussion

4.1. Sperm quality parameters and biochemical status

This study tested the inclusion of 6 % dried algae blend in Senegalese sole broodstock diet as functional ingredients: 3 % of *Phaeodactylum tricornutum* and 3 % of *Gracilaria gracillis*, to see if it could ameliorate the sperm quality and reproductive performance of F1 males throughout the breeding season. Overall, F1 broodstock displayed the typical wild males' sperm quality variation along the reproductive season, irrespective of the experimental diets, which has been documented as one of the reproductive problems in Senegalese sole (Beirão et al., 2011). Besides the description of annual oscillations in sperm quality, the present study also showed oscillations within each month of the reproductive season, with the best results at the peak of the spawning season.

Although the higher percentages of sperm motility parameters were observed in the middle of the reproductive season (3rd experiment), spermatozoa TM was not impacted by the experimental diet and varied throughout the experiment. Such variation was previously demonstrated by our group in wild Senegalese sole, in which sperm traits and production throughout the year were higher in April and May (Cabrita et al., 2006) when water temperatures were similar to our study. Different results were obtained in a study performed on Nile tilapia (*Oreochromis niloticus*) broodstock, which tested the inclusion of the diatom *Cyclotella* sp. in diets, for 30 days, and the males fed microalgae-supplemented diet increased sperm motility and concentration (Hassaan, 2022). However, the sperm motility assessment was done without CASA software, which compromises the reliability of the data (Gallego and Asturiano, 2018). A previous study with macroalgae diets in Senegalese sole young breeders, demonstrated that fish fed with 5 % *Plocamium cartilagineum* had TM enhanced after one month of trial (Félix et al., 2024b). At least in terms of spermatozoa motility, it seems that the algae blend used in the present study did not ameliorate any parameter. Literature about the effects of algae-supplemented diets on the reproductive performance and gamete quality of fish is limited. The majority focused on the effects of microalgae in the female reproductive system of freshwater species, such as *Schizochytrium* sp. on rainbow trout (Cardona et al., 2022), *Chlorella* sp. on zebrafish (Carneiro et al., 2020), *Cyclotella* sp. on Nile tilapia (Hassaan, 2022), and *Spirulina* sp. on yellow tail cichlid (Güroy et al., 2012) and three-spot gourami (*Trichopodus trichopterus*) (Khanzadeh et al., 2015). All these studies showed positive results: the microalgae diets have improved fish growth, enhanced the gonadosomatic index (GSI), stimulated egg production, increased egg viability, hatching and larval survival rates. In male breeders, the few reports about the positive impact of algae-supplemented diets on the antioxidant and reproductive systems are found in mammals, mainly in lambs (Assar et al., 2023), rabbits (El-Ratel, 2020) and rats (Kong et al., 2019).

At the beginning of the reproductive season (1st sampling), sperm had the lowest motility parameters (progressive motility, velocities and linearity), which according to our results is associated with higher intracellular ROS and an overall lower sperm quality. These results are very common to observe at the beginning of the reproductive season in Senegalese sole and other species, such as Atlantic cod (*Gadus morhua*) (Butts et al., 2010), rainbow trout (Büyükhatipoglu and Holtz, 1984) and zebrafish (Diogo et al., 2019). Although fish fed with control diet had higher spermatozoa viability on 1st and 4th samplings, viability was around 80 % during the whole breeding season, irrespective of the diet, which was in accordance with previous results for this species (Beirão et al., 2011), inclusive in studies with macroalgae diets (Félix et al., 2024b).

The algae diet had a positive impact in terms of oxidation processes, which is crucial to avoid sperm damage. In terms of ROS, at the 1st sampling, the fish fed with algae diet had a higher percentage of live cells compared to the control group. Similarly, in the above-mentioned study performed in the same species, the breeders fed with a 5 %

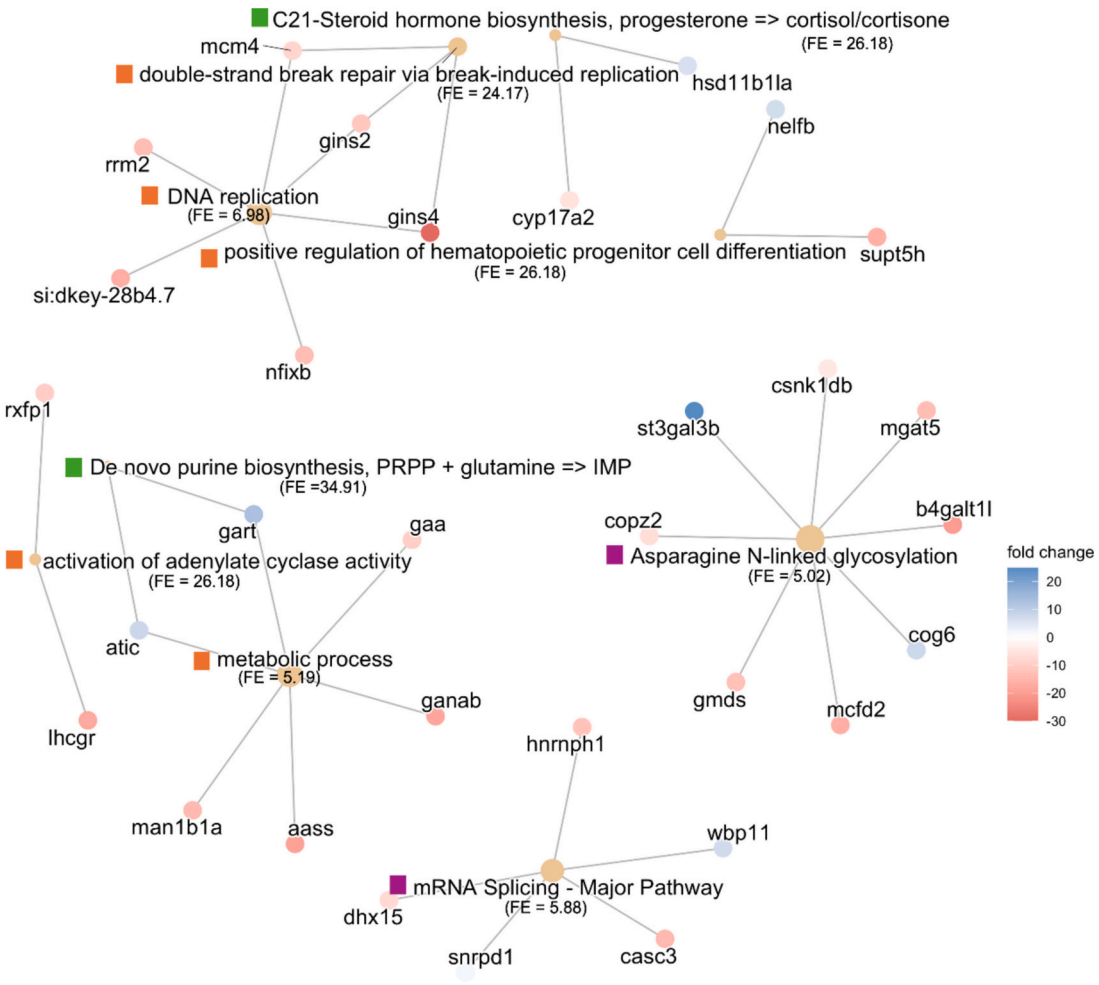

Fig. 7. Functional enrichment analysis of differentially expressed transcripts (DET) in the gonads of Senegalese sole fed with algae diet against the control group. Analysis was done on the basis of GO (orange), KEGG (green) and REACTOME (purple) knowledgebases (Benjamini-Hochberg *p*adj < 0.1). Each cluster represents a specific pathway with the corresponding fold enrichment (FE) indicated; gene nodes are represented with the respective fold change colour (blue: upregulated, red: downregulated), as described in the legend. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

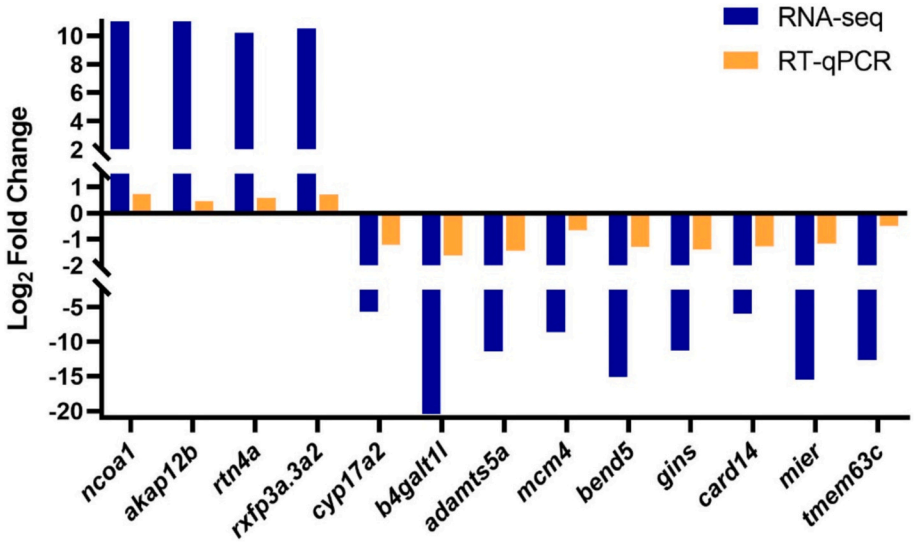

Fig. 8. Validation of RNA-seq results through RT-qPCR performed in 13 target genes. Results were given in terms of their relative expression (control vs algae).

S. vulgare diet had lower sperm ROS after one month of trial (Félix et al., 2024b). These results demonstrated the antioxidant capacity of the algae supplementation in the diet, even at the beginning of the reproductive season (bad quality at 1st sampling). Regarding the spermatozoa MDA content, it decreased drastically at the 4th sampling for both fish groups. However, fish fed with algae maintained the low levels to the next sampling, while fish fed the control diet continued displaying an irregular pattern, increasing MDA content on the final sampling. Differently from our experiment, in a study with Senegalese sole larvae fed with 5 % *P. tricornutum* supplemented diet, better growth performance was obtained, but no effects were observed on the whole larvae biochemical and enzymatic markers of oxidative stress (Barreto et al., 2021). However, in other fish species challenged with pathogenic bacteria, fish fed macroalgae-supplemented diets had lower lipid peroxidation: 5 % *Sargassum horneri* diet decreased the MDA concentration in the blood serum of turbot (*Scophthalmus maximus*) juveniles (Wang et al., 2018); also 5 % *Gracilaria* sp. diet resulted in the lowest hepatic lipid peroxidation in meagre (*Argyrosomus regius*) (Peixoto et al., 2016) and European seabass (Peixoto et al., 2019). For the discussion of antioxidant protection between different experiments, it is important to keep in mind that the antioxidant compounds present in the algae may change according to several intrinsic (type, age, reproductive stage) and extrinsic factors (depth, salinity, nutrients, irradiance) (Valentão et al., 2010). However, despite the variability above described, the diatom *P. tricornutum* is known for its high fatty acids content (Daboussi et al., 2014), and has proven that its inclusion in fish feeds increases the whole body omega-3 PUFA content (Norambuena et al., 2015), which could explain the positive results obtained in terms of lipid peroxidation processes. Moreover, both *P. tricornutum* and *G. gracilis* are rich in carotenoids, which are pointed to be essential for fish reproduction (Carneiro et al., 2020) since marine animals accumulate them in their gonads (Maoka, 2011).

In accordance with the oxidation process results, the algae-fed group presented lower spermatozoa DNA fragmentation in comparison to the control group in the 3rd sampling. Nonetheless, DNA fragmentation increased considerably in the last two samplings, which may be related to the DNA sensitivity to the increasing water temperature during the breeding season (Beirão et al., 2011). According to the literature, an increase of 4 °C in water temperature is enough to impact rainbow trout spermatogenesis (Brionne et al., 2023) and, in our experiment, fish experienced a 4.5 °C shift in water temperature in June and a low daily thermic amplitude, which explains the higher DNA fragmentation in both fish groups at the end of the experiment. In terms of apoptotic status, no differences were obtained between fish fed with the experimental diets.

Generally, it is accepted that lower percentages of algae inclusion led to better results (Norambuena et al., 2015; Younis et al., 2018). Indeed, positive results in terms of growth, immune response and antioxidant protection were achieved with low inclusion percentages of *G. gracilis* in European seabass (2.5 % dried algae) (Passos et al., 2021b) and gilthead seabream (5 % dried algae) (Passos et al., 2021a). In our study, a low percentage (6 %) of micro and macroalgae was blended and tested in feeds to understand if distinct phytochemicals and antioxidant compounds could act synergically and ameliorate the spermatozoa characteristics of Senegalese sole, as already described in other fish species (Cerezuela et al., 2012; Batista et al., 2020; Ferreira et al., 2022). It is worth mentioning that the type of algae used for the blend can strongly influence the results, more than the percentage of algae inclusion (Cabral et al., 2011). In a recent study with European seabass and gilthead seabream, no effects from the microalgae blend (*Tisochrysis lutea, Tetraselmis suecica*) used were observed, neither in terms of growth nor in physiological status or gut health (Randazzo et al., 2023). Similarly, the sperm quality results obtained in our study suggest that the algae blend used may not be the most adequate for improving Senegalese sole sperm quality. However, it does not mean that those algae could not be suitable ingredients or additives for broodstock feeds, since several positive results were observed at some point in the experiment.

4.2. Gonadal transcriptomics analysis

At the end of the breeding season, an RNA-seq was performed in fish gonads to understand if the algae diet had prolonged protective effects in the gonads, reflected at the transcriptional level. From our enrichment analysis, several genes involved in reproductive processes, DNA replication and repair, apoptosis, autophagy, immune system, cytoskeletal rearrangements, cell communication processes and lipid metabolism were found to be overexpressed in the algae-fed group. Among the overexpressed genes, four transcripts' variants of myosin heavy chain *myh14*, a gene belonging to the actin-dependent molecular motors family (Hasan et al., 2021), were shown to be exclusively expressed in the gonads of the algae group. In a similar study during the spawning season of Atlantic cod, different *myh* isoforms were found to be downregulated in the muscle, contributing to muscle wasting during the reproductive period, which is a normal process, since fish redirect the energy into reproductive development and performance (Nagasawa et al., 2016). Also, in zebrafish, different *myh14* paralogs were found to be highly expressed during embryonic development (Hasan et al., 2021). In our case, overexpression of *myh14* in gonadal tissue may be associated with spermatogenesis, since it has been reported that non-muscle myosin interacts with cytoskeletal actin, playing an important role in cell morphology and motility (Golomb et al., 2004), more specifically in spermatids differentiation and shaping (Sun et al., 2011).

The *synaptojanin1* (*synj1*) gene is involved in the cytoskeletal rearrangement processes in zebrafish (Van Epps et al., 2004) and was found to be upregulated in the algae-fed group. Besides that, *synj1* participates in the inositol phosphate metabolism (Yang et al., 2021), involved in nutrient response and bioenergetic homeostasis signaling pathways (Tu-Sekine and Kim, 2022). Moreover, some inositol phosphatases are lipid-based and normally associated with various subcellular membranes (Tu-Sekine and Kim, 2022). The microalgae *Phaeodactylum tricornutum* present in the algae diet could be contributing to higher availability of lipids and thus modulating transcriptomic changes in lipid synthesis, reflecting a changing tendency for lipid composition, metabolism, and homeostasis, as described for chicken (Yang et al., 2021). In fact, the *st3gal3b* gene, previously described to be involved in lipid metabolism in swamp eel (*Monopterus albus*) (Liu et al., 2023), was found to be exclusively expressed in the algae group of our study.

The transcriptomic analysis also revealed that the majority of genes associated with reproductive processes, such as spermatogenesis (*mier1*, *cyp17a2*, *lhcgr*, *cd9a*), DNA replication and repairment (*gart*, *usp47*, *spg20a* and *gins* isoforms) and cell proliferation and differentiation (*dusp26*, *mcm4, bend5, fhl1b*) were downregulated in the algae diet group (Guzmán et al., 2014; Lakisic et al., 2016; Varga et al., 2020; Greaves et al., 2022; Han et al., 2022). An experiment with Nile tilapia proved that a deficiency of *cyp17a2,* involved in the steroidogenic function and production of sex steroids, compromised meiosis and led to a reduction of other germ cell-related gene expression in male fish. In addition, after gene expression recovery, those fish showed lower sperm motility and fertility (Yang et al., 2022). Also, *lhcgr* gene is involved in the progression of spermatogenesis in Senegalese sole (Chauvigné et al., 2014) and its downregulation in the present study suggests constraints in spermatogenesis development. This information, together with the three downregulated transcripts of *cd9a*, which is associated with the number of eggs and fertilization rate in zebrafish (Greaves et al., 2022), could explain the lower male fluency found in the algae-fed fish compared with the control group.

On the other hand, other reproductive-associated genes like *supt5h*, *akap12b, ncoa1, rxfp3.3a2*, and *rtn4a*, were overexpressed in the algae-fed group. The *supt5h* was described to be especially important for late spermatogenesis (sperm maturation) in mice (Margolin et al., 2014), and *nelfb* is described to be crucial for proper spermatogenesis and cell differentiation (Kaye et al., 2024). Also, the *akap12,* expressed

exclusively in the algae-fed group, had previously reported expression in male gonads of humans and mice (Su et al., 2004) and in rams' sperm, being associated with fertility and sperm motility (Urena et al., 2022). The fact that some genes with described similar functions were simultaneously up and downregulated, could reflect a masked ongoing compensation mechanism and explain the non-obvious biochemical results obtained in terms of sperm quality. A study with different *Gracilaria* species showed that extracts from *G. edulis* had spermicidal capacity, by disrupting the plasma membranes and inactivating 100 % of the sperm samples (Almeida et al., 2011), which was also reported for a different green seaweed (*Halimeda gracilis*) and human sperm (Prakash et al., 2014). From our cellular quality results, we can infer that this is not true for *Gracilaria gracilis* and Senegalese sole sperm, although, we do not know if there is some bioactive compound compromising proper spermatogenesis.

The *rtn4a* was shown to be crucial for early embryonic development in zebrafish (Pinzon-Olejua et al., 2014), but more important for our study, is its protective capacity against several cell death stimuli (Teng and Tang, 2013). In an in vitro study with SH-SY5Y cells exposed to several apoptotic-like stimuli, the *rtn4a* gene was important in fighting the increasing levels of intracellular ROS, especially after apoptosis and autophagic events. In their study, the increasing levels of the pro-apoptotic protein Bax and Caspase-3 activity did not compromise the survival of RTN-expressing cells, but masked the protective effects from *rtn4* expression (Teng and Tang, 2013). Similar results were obtained in our experiment. At the end of the reproductive season, two transcripts of the *rtn4a* gene were upregulated in the gonads of the algae-fed group. This upregulated pathway seemed to be helping fish cope with a stress oxidation status inflicted by prolonged exposure to a putative unknown algae component. Thus, we hypothesize that the algae used for feed formulation may have a specific compound that induces apoptotic and autophagic processes and inhibits DNA replication and spermatogenesis-associated mechanisms (cell proliferation and differentiation) but, at the same time, algae diet could upregulate some defense mechanisms through its antioxidant properties. Moreover, the fact that the algae feed is supplemented with two different species, and we could not test each alga-effects separately, leads us to suspect that this combination could be unfavorable for the overall fitness of the gonadal and sperm traits, as previously discussed regarding sperm quality biochemical analysis. Moreover, the downregulation of *ncoa1* gene was found to be an indicator of toxicity effects in mice testis exposed to bisphenol-A (BPA) (Tainaka et al., 2012), while in our experiment, two transcripts of *ncoa1* were exclusively expressed in algae fed fish, reinforcing our hypothesis and corroborating the apparently contradictory results in terms of antioxidant protection and apoptosis events, also observed by Teng and Tang (2013). Noteworthy, many important genes involved in caspases activation and apoptosis induction (*b4galt1l*, *card14*, *asph*, *tmem63c*) were downregulated. The proteins CARD11 and CARD14 are activators of the pro-apoptotic BCL10 protein (Bertin et al., 2001), but in our study *card14* was downregulated.

It is worth mentioning, that the incorporation of algae in fish feeds can be done using algae biomass or extracts. In the present study, dried algae were used, so there was no control over the algae-active compounds included in the diets. In the case of using algae extracts, the type of extraction process can affect the type of bioactive compounds obtained (Afonso et al., 2021). This could be a solution for the selection of the desirable algae properties and a safety procedure for the inclusion of algae in fish diets and obtention of standardized results.

5. Conclusion

This experiment explored the usage of an algae blend as a functional ingredient for broodstock feeds, namely the inclusion of *Gracilaria gracilis* and *Phaeodactylum tricornutum* dried algae. Although it did not decrease sperm variability during the breeding season, the sperm parameters and biochemical analyses showed that the algae diet diminished spermatozoa oxidation processes and prevented DNA fragmentation at some points of the reproductive period. Nonetheless, the gonadal transcriptomic analysis performed at the end of the breeding season, revealed interactions among different reproductive, DNA synthesis and lipid metabolism pathways, suggesting that algae-fed fish were probably undergoing a compensatory mechanism inflicted by an unknown algae component. More research is needed to comprehend which bioactive compounds were behind the observed effects and how to select the desirable ones for safe incorporation of algae in fish feeds.

CRediT authorship contribution statement

F. Félix: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **C. Raposo de Magalhães:** Writing – original draft, Validation, Software, Resources, Methodology, Formal analysis, Data curation. **C. Marrero-Alemán:** Investigation. **D. Duarte:** Investigation. **P. Parente:** Investigation. **E. Fatsini:** Validation, Methodology, Investigation. **C.C.V. Oliveira:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision. **E. Cabrita:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors 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

We have submitted the RNA-seq data to ArrayExpress (Accession E-MTAB-14218) although not available yet online

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Appendix A. Supplementary data

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