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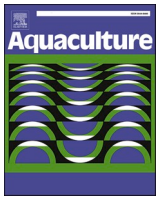

The use of egg quality parameters to evaluate the effect of a diet supplemented with algae and antioxidants in turbot (*Scophthalmus maximus*)

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ABSTRACT

Enhancing egg quality can be achieved by improving breeders' diet through the antioxidant content, as oxidative stress could adversely affect egg quality. Micro- and macroalgae species are natural sources of antioxidants and other essential nutrients that can be incorporated in fish feeds. In this study the quality of *Scophthalmus maximus* eggs were compared between breeders fed a commercial (non-supplemented) diet and those fed a diet enriched with 5 % *Arthrospira platensis* and 1 % of the iodine-rich *Laminaria digitata*, further fortified with antioxidants (astaxanthin, vit. C and E) (supplemented diet). Several egg parameters were evaluated. Hierarchical clustering of all the egg batches grouped them into two main categories: higher ($\geq$ 50 % buoyancy) and lower-quality eggs ($\leq$ 30 % buoyancy). The expression of transcripts related to egg quality (*ctsz*, *ccna2*), oxidative response (*nrf2*, *cat*), and apoptosis (*bax*, *casp3a*) was also measured in batches categorized by quality, as well as in higher-quality batches from females fed the commercial versus supplemented diet. Eggs of higher quality (greater buoyancy), regardless of females' diet, had significantly higher total antioxidant status (TAS) levels ($P < 0.05$), suggesting TAS as an egg quality marker. The expression of *ccna2* was upregulated, while *ctsz* showed almost no expression in higher-quality eggs but was down-regulated in lower-quality eggs, highlighting their potential as markers of egg quality in turbot. Higher-quality eggs from females fed the supplemented diet exhibited higher TAS, lower superoxide dismutase activity, and an upregulation of *nrf2* compared to higher-quality eggs from non-supplemented females. This suggests a more efficient cellular mechanism to restore oxidative homeostasis. Supplementing the diet increased the likelihood of achieving $\geq$80 % buoyant eggs and overall cumulative egg production, contributing to more effective and sustainable turbot farming.

1. Introduction

Turbot (*Scophthalmus maximus*) is an economically significant flatfish species farmed in specific regions, including China, the Netherlands, and Southern Europe, particularly Spain and Portugal. Over the past 30 years, China's annual production has stabilized at around 60,000 tons, representing about 80 % of global aquaculture turbot supply. In 2022, Europe produced approximately 12,600 tons of turbot, with Spain contributing 8700 tons and Portugal 3800 tons, accounting for almost all the European total (FAO, 1950–2022). The natural reproductive season of this batch-spawning fish (McEvoy, 1984) is from April to August, but in captivity, this period can be extended throughout the year by environmental manipulation through temperature and photoperiod (Girin and Devauchelle, 1978). However, turbot do not exhibit spontaneous spawning behaviour in captivity and rely on hand-stripping of breeders followed by artificial fertilization.

Numerous studies on turbot have explored various aspects of reproduction, including the effects of environmental factors such as salinity and temperature (Nissling et al., 2006) and photoperiod (Forés et al., 1990; Imsland et al., 2003; Polat et al., 2021), hormone

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administration (Mugnier et al., 2000; Alvariño et al., 2001), artificial fertilization methods (Chereguini et al., 1995), and sperm cryopreservation (Chereguini et al., 2003; Chen et al., 2004). More recent studies have focused on sex control (Taboada et al., 2018) and the functions of HPG axis-related genes during the reproductive cycle (Hu et al., 2018; Gao et al., 2019; Huang et al., 2019; Jia et al., 2016, 2019). Despite extensive research, much conducted two decades ago, variable larval survival rates, partly due to the gamete quality, continue to challenge production. Producing an elevated number of high-quality eggs is essential for the aquaculture industry (Migaud et al., 2013). Enhanced egg quality can lead to better fertilization rates, reducing the need for a large number of breeders, which in turn can optimize production and lower maintenance costs.

Egg quality is affected by several factors, including nutrition, photoperiod, temperature, salinity, husbandry practices, and stress (Bobe and Labbe, 2010; Bobe, 2015; Mechaly et al., 2024). Nutrition plays a crucial role in optimizing reproductive success, as it can improve egg quality, in turn enhancing the survival and development of the offspring. Formulating feeds that improve reproductive efficiency is crucial, particularly during periods of high energy demand such as gametogenesis. During these critical periods, fish gametes are particularly vulnerable to oxidative stress due to their high lipid content and limited antioxidant defenses (Félix et al., 2021; Shastak and Pelletier, 2023; Mechaly et al., 2024). Oxidative stress can lead to lipid peroxidation, protein alterations, and DNA damage, impairing the structural and functional integrity of the gametes. Such damage can diminish fertilization rates and lead to offspring developmental problems (Samarin et al., 2019a; Bhat et al., 2023). Antioxidants play a crucial role in neutralizing reactive oxygen species (ROS), thus protecting gametes from oxidative damage and maintaining their functionality and viability. By manipulating antioxidant intake through dietary strategies, it is possible to regulate ROS levels and reduce the harmful effects of oxidative stress during gametogenesis, ultimately improving reproductive performance (Félix et al., 2021; Saffari et al., 2022; Shastak and Pelletier, 2023). Particularly in turbot, previous studies have demonstrated that vitamins E and C in diets supplementation enhanced hatching rates (Lavens et al., 1999). Additionally, certain compounds, such as microminerals (Saffari et al., 2022) and the carotenoid pigment astaxanthin (Shastak and Pelletier, 2023), when included in fish broodstock diets, can help protect against oxidative damage and directly improve egg quality. Some of these compounds can be synthesized and added to the feeds, but they are also naturally found in various micro- and macroalgae species. Among microalgae, *Arthrospira platensis*, a blue-green algae, has gained significant attention in recent years as an immune stimulant in aquaculture feeds (Rana et al., 2024) and, to a lesser extent, for its potential to enhance reproductive performance in fish (Khanzadeh et al., 2016; Rajasekar et al., 2019; Lu and Takeuchi, 2004). *A. platensis* contains many pigments, such as β-carotene, phycocyanin, allophycocyanin, phycoerythrin, and zeaxanthin, which are capable of quenching free radicals to protect biomolecules from oxidative damage. In addition to pigments, it contains phenols, vitamins, polyphenols, and polysaccharides that contribute to enhancing antioxidant enzyme activity in fish. Moreover, *A. platensis* is a source of proteins (up to 55–70 % of dry weight) and fatty acids (Rana et al., 2024) which are crucial for the proper development of gonads, gametes and larvae (Izquierdo et al., 2001; Lubzens et al., 2010). It is also the most common cultured microalgae being produced at a commercial scale. Among macroalga, the brown algae *Laminaria digitata* is notable for its high iodine content, polyphenols, and carbohydrate richness (Schiener et al., 2015). Although limited research has explored the impact of *L. digitata* in aquaculture feeds, recent studies have highlighted its positive effects on immune and antioxidant parameters, particularly in juvenile fish (Purcell-Meyerink et al., 2021; Marmelo et al., 2024).

This study aims to identify factors determining the quality of ovulated eggs (unfertilized eggs) in turbot, with a particular focus on parameters related to oxidative stress and protection. In addition, it assesses the impact of incorporating sustainable marine-origin compounds such as micro- and macroalgae and increasing the antioxidant supplementation in the diet, on the antioxidant metabolism and quality of turbot eggs, and overall production efficiency.

2. Materials and methods

2.1. Broodfish and experimental design

Captive-reared broodstocks from the semi-intensive aquaculture farm FLATLANTIC - Actividades Piscícolas S.A. (Praia de Mira, Portugal) were maintained in 15 m^3 fiber-glass tanks at a constant water temperature of 14.0 ± 0.5 °C and under a simulated natural photoperiod. A total of 57 females (weight range 4.0–10.1 kg) fed a commercial diet, and 32 females (weight range 5.4–11.1 kg) fed an algae antioxidant-supplemented diet, were checked for ovulation at different dates during the spawning period. Eggs were stripped from females according to external visual examination of gonadal maturation and gentle palpation. Females were classified into five stages based on the criteria of García-López et al. (2006) for other flatfish species, such as the Senegalese sole (*Solea senegalensis*): 0) no visible or palpable gonadal development; 1) ovary detectable only by abdominal palpation; 2) slight ovarian swelling, visible in the abdominal region; 3) intermediate level of ovarian swelling; and 4) maximum ovarian development, with readiness for egg release upon gentle abdominal pressure. Since the study was conducted under production conditions, routine activities continued in parallel. Egg collection followed standard procedures and was conducted uniformly across tanks with broodstock fed the commercial non-supplemented feed and tanks with broodstock fed the supplemented diet.

The commercial feed (Sparos Lda., Portugal) was a semi-most feed that contained 47.6 % crude protein and 14.3 % crude fat. The algae antioxidant-supplemented diet (Sparos Lda., Portugal) was isoproteic and isoenergetic and consisted of the commercial feed with 5 % *Arthrospira platensis* and 1 % of the iodine-rich *Laminaria digitata* incorporated at the expenses of fishmeal, fish protein hydrolysate, squid meal, wheat gluten and technical ingredients (starches, etc.) (**Suppl. Table S1**). Additionally, the diet was further fortified with antioxidants, with a double dose of astaxanthin (76.8 mg kg^{-1} feed), vitamin C (1553.4 mg kg^{-1}) and vitamin E (1524.7 mg kg^{-1}) (**Suppl. Table S2**). Feeding trials began four months before the spawning season, overlapping with the gametogenesis period, and continued during spawning, aligned with the corresponding photoperiod daylength. This diet is referred hereafter as the supplemented diet. All females were fed ad libitum.

2.2. Egg collection, fecundity and hatching

Egg batches were hand-stripped from ovulated females (total $n = 34$ batches), including those fed the commercial diet ($n = 17$) and those fed the supplemented diet (n = 17). For each female, eggs were collected into plastic bowls. Egg buoyancy, an initial indicator of egg quality in species with pelagic eggs (Migaud et al., 2013; Mechaly et al., 2024) and positively correlated with fertilization and hatching rates in turbot (Jia et al., 2014), was measured in 35 ppm seawater in a graduated cylinder and calculated using the following formula: Buoyancy (%) = 100 x vol. of buoyant eggs/vol. of total eggs in the cylinder. The diameter of 20 eggs per female was also recorded. Additionally, aliquots of eggs and ovarian fluid were stored separately at −80 °C for subsequent analyses of ovarian fluid pH and osmolality and egg antioxidant activity, lipid peroxidation, and for mRNA abundance of specific transcripts.

During routine production activities, the total number of stripped eggs collected was calculated by multiplying the weight of the egg mass from each female by the number of eggs present in 1 g. Egg counts were expressed as the total number of stripped eggs, and the following parameters were calculated: absolute stripping fecundity (ASF), defined as

the total number of stripped eggs per female, and relative stripping fecundity (RSF), calculated by dividing the total number of stripped eggs by the weight of the fish, as described by Adámek et al. (2004). Egg batches were artificially fertilized and incubated following standard production procedures (Chereguini et al., 1995). Hatching success was recorded as the percentage of embryos hatched from the total number of eggs incubated.

2.3. Total antioxidant status and antioxidant enzymatic activity of eggs

Egg extracts were prepared based on the method used by Martínez-Páramo et al. (2012) for sperm samples, with some modifications. Briefly, egg samples were rinsed with 0.01 M phosphate-buffered saline (PBS) and homogenized in 0.01 M PBS containing 0.1 % (*v*/v) Triton X-100. The homogenate was centrifuged at 10,000*g* for 10 min at 4 °C. All egg samples were used for the determination of total antioxidant status (TAS), superoxide dismutase activity (SOD), glutathione peroxidase activity (GPx), and glutathione reductase activity (GR), using the commercial assay kits and following the protocols of the manufacturer (Randox Laboratories Ltd. (Crumlin, United Kingdom). SOD activity was measured using the xanthine oxidase method, GPx activity was evaluated by the oxidation of NADPH in the presence of cumene hydroperoxide, and GR activity was determined by the oxidation of NADPH. Absorbance was read using a microplate reader (Synergy 4, BioTek, Vermont, United States) at 505 nm for SOD, and 340 nm for GPx and GR. The results were normalized against the protein content of each sample for intersample comparison. Protein content was quantified using the BioRad DC Protein Assay Kit (Bio-Rad Laboratories, United States), according to the manufacturer's instructions and absorbance was read at 750 nm. Enzyme activities were expressed as units of enzyme per gram of protein (U/g protein) for each egg sample.

Total Antioxidant Status (TAS) was evaluated in egg homogenates using the TAS Randox kit (Randox Laboratories, Ltd., UK), according to the manufacturer's instructions. This assay is based on a colorimetric method where ABTS, a stable radical cation, is generated from a reaction involving metmyoglobin and hydrogen peroxide (H_2O_2), which then interacts with the chromogen ABTS. Absorbance was measured at 600 nm and TAS was reported as mmol/g of protein.

2.4. Lipid peroxidation analysis

Samples were prepared following the method of Buege and Aust (1978) with modifications. Eggs were rinsed and homogenized by a manual homogenizer in cold Ringer's solution (1:2) until no eggs were visible in the solution (approximately 20 s). The homogenate was then centrifuged at 10,000*g* at 4 °C for 10 min to remove debris, and the supernatant was collected.

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) levels using the Bioxytech MDA-586 Kit (Oxis Research, Portland, OR, USA). The concentration of MDA was determined using a colorimetric assay as described by Martínez-Páramo et al. (2012) for sperm samples. Briefly, diluted homogenates in Ringer (3:10) were incubated with 200 μM sodium ascorbate and 40 μM ferrous sulfate for 30 min at 37 °C in the dark. Homogenates were then treated according to the manufacturer's protocol, incubated for 1 h at 45 °C in the dark, and centrifuged at 10,000*g* for 10 min at 4 °C. Supernatants (200 μL of each homogenate) were transferred to a 96-well transparent plate (Nunc) and absorbance was read at 586 nm (Synergy 4, Biotek Instruments Inc.). MDA concentrations were calculated from a standard curve (0 to 14 μM) and expressed as μM of MDA per egg. The number of eggs per 100 μL subsample was counted in triplicate.

2.5. mRNA abundance

2.5.1. Extraction of total RNA and cDNA synthesis

Egg samples, grouped into two distinct clusters based on hierarchical clustering methodology (see Section 2.7.), and displaying differing quality according to buoyancy, were selected. These samples were chosen regardless of whether they were obtained from females fed the commercial diet or from those fed the supplemented diet, to evaluate specific mRNA abundance profiles as potential markers of egg quality in turbot. Nine samples with 72 ± 9 % buoyancy, referred to as "higher-quality", and ten with 11 ± 12 % buoyancy, referred to as "lower-quality", were used for this analysis. To evaluate the effects of dietary supplementation, egg samples from the same hierarchical cluster and, thus, of similar quality based on buoyancy were selected from females fed the commercial diet (n = 7; 63 ± 9 % buoyancy) and those supplemented (n = 10; 66 ± 13 % buoyancy).

RNA was extracted from each egg sample using TRI Reagent RNA Isolation (Merck, Lisbon, Portugal), according to the manufacturer's instructions. RNA concentrations and quality absorbance ratios were measured with a Nanodrop spectrophotometer (NanoDrop 1000, Thermo Scientific, Porto Salvo, Portugal). Complementary DNA (cDNA) was synthesized using the Thermo Scientific Maxima First Strand cDNA Synthesis Kit with dsDNase (Thermo Scientific, Porto Salvo, Portugal), following the manufacturer's protocol. The synthesis reactions were performed in a Bio-Rad Thermocycler (Bio-Rad, Portugal) under the following conditions: 2 min at 37 °C, 10 min at 25 °C, 15 min at 50 °C, and 5 min at 85 °C.

2.5.2. Target mRNAs

Transcripts identified as putative indicators of egg quality (*ctsz, ccna2*) (Reading et al., 2018), along with those associated with oxidative response (*nrf2, cat*) (Shastak and Pelletier, 2023; Tao et al., 2023; Dettleff et al., 2021), and apoptosis (*bax, casp3a*) (Qiang et al., 2022) were selected. After identifying these transcripts in turbot within the GenBank database, primer sequences were designed using Primer3 software targeting the 3'UTR region (Rozen and Skaletsky, 1999). All primers were ordered from STAB vida (Caparica, Portugal) and are listed in Table 1. Primer functionality, efficiency, and optimal concentration were validated prior to real-time PCR. To determine the appropriate dilution of cDNA samples for PCR and to assess reaction efficiency, standard dilution curves were generated for each primer pair.

2.5.3. Real-time PCR

The real-time PCR was conducted using a Bio-Rad CFX96™ Thermocycler (Bio-Rad, Portugal) in 96-well plates, with each reaction ran in duplicate and including a systematic negative control (NTC; no template control) containing water instead of cDNA. Reactions were performed in a total volume of 20 μL, consisting of 10 μL of SsoFast EvaGreen Supermix (Bio-Rad, Portugal), 2 μL of primers (0.5 mM), 5 μL of cDNA for target genes, and RNase/DNase-free water to make up the remaining volume. Standard amplification conditions were as follows: an initial denaturation step at 95 °C for 30 s; 40 cycles of denaturation at 95 °C for 5 s; annealing at 57 °C for 5 s; and a melt curve with a 0.5 °C increase (from 65 °C to 95 °C) for 2–5 s. Results were normalized using as housekeeping gene ubiquitin (*ubq*) (Robledo et al., 2014) and were obtained by calculating the geometric average (Vandesompele et al., 2002). Relative mRNA expression for each gene was determined using the method (1 + ET)^(ΔCt)/(1 + ER)^(ΔCt) (Pfaffl, 2001). When evaluating mRNA expression from eggs with higher vs. lower quality, since the origin of samples was independent on female diet, a pooled sample from the population was used as the reference to which the expression levels of the two groups were normalized. When evaluating mRNA expression from eggs of similar quality based on buoyancy from females fed the commercial diet vs. females fed the supplemented diet, the non-supplemented group was set as the reference acting as a control.

2.6. Statistical analysis

All data were analysed using R v4.4.1. As an exploratory data analysis, Principal Component Analysis (PCA) was performed on the

Table 1
Transcripts of mRNA targets, biological gene functions, GenBank accession numbers, and primer sequences used for Real-Time PCR primers.

Transcript	Abbreviation	Gene Function	Accession #	Forward Sequence	Reverse Sequence
Nuclear factor erythroid 2-related factor 2	*nrf2*	Central overseer of the cellular antioxidant response	MT023796	TTCAGCAGGTTGAGAGACGAG	AGTGGTTTTCTTCGCGCTTG
Catalase	*cat*	Cellular antioxidant defense	MG253621	CGTTTGGCTACTTTGAGGTGAC	TTGCCGACATGCTCAAACAC
BCL2-associated X	*bax*	Apoptosis regulator	XM_035615645	AGAAATTCAGCCCTCGGATCC	TGCAACACAAATCCCAGCAC
Cysteinyl aspartate specific proteinase 3 cysteinyl aspartate specific proteinase 3	*casp3a*	Apoptosis regulator	XM_035637276	TGTGCGTTCTGTTGAGTCAC	TGCAAGCCTGGATGAAGAAG
Cathepsin z	*ctsz*	Protein degradation	MF780876	TGAATGCAAACGGACTCAGC	AAATCCTGCATCGGGTTGTG
Cyclin-a2	*ccna2*	Cell cycle regulator	XM_035650323	ATGCTGCTGAAATCCACACG	CTCATGCTGTTGGTGATGTCTG
E3 Ubiquitin-protein ligase	*ubq*	Housekeeping	A0A8D3D560	AAAATTCCCCAATCAATCTCCT	CTTCACAAAGATCTGCATCTTGA

following variables: buoyancy and diameter of eggs, ovarian fluid pH and osmolality, SOD, GPx, GR activities, TAS, and MDA levels, to understand how these variables contribute to variation within samples. Correlations among variables were further examined using Pearson's correlation coefficients to identify linear relationships.

Hierarchical data clustering was applied to observe the classification of all egg batches. A two-way ANOVA was used to identify significant differences between the two main clusters obtained and to assess the effects of the supplemented diet on the different measured variables and egg production parameters. A Student *t*-test was used to compare mRNA abundance within grouping factors for each gene. Shapiro-Wilk and Levene tests were used to assess the normality of data distribution and homogeneity of variance, respectively. Data that did not follow normality or homogeneity of variances was log-transformed. Outliers were removed using the Interquartile Range (IQR) method. Results are presented as mean ± standard deviation (SD) unless stated otherwise. Significant differences were determined at a significance level of $P < 0.05$. GraphPad Prism 5 was used to create bar plot figures.

2.7. Ethics statement

Egg batches were obtained from the farm FLATLANTIC - Actividades Piscícolas S.A. (Praia de Mira, Portugal), where fish husbandry and handling were conducted in accordance with best practice standards and relevant EU legislation for farmed fish.

3. Results

3.1. Egg samples overview and variables interaction

A total of 34 egg batches, corresponding to 34 ovulations during the spawning period were analysed. Seventeen batches were stripped from females fed the commercial diet, while other 17 batches were obtained from females fed the supplemented diet. The Principal Component Analysis (PCA) revealed that the first three principal components (PC1, PC2, and PC3) collectively explained 58 % of the variance of the data. Egg batches were distributed along these three principal components (Fig. 1), but the pairwise scatter plots of PC1 vs. PC3 and PC2 vs. PC3 provided a clearer view of the separation according to their origin, whether from supplemented females or not. Based on the magnitudes of

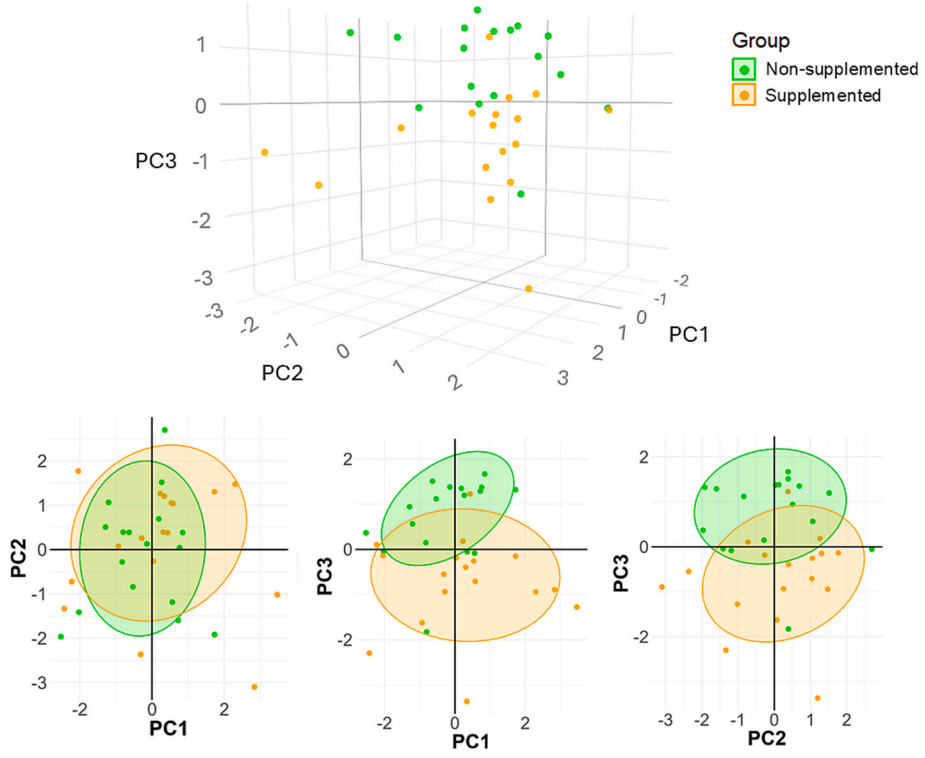

Fig. 1. The 3D PCA plot illustrates the distribution of the "Non-supplemented" and "Supplemented" groups along these three principal components. The visualization of the data on pairwise scatters plots of PC1 vs. PC3 and PC2 vs. PC3 provides a more detailed view of the group separation.

the loadings of the variables included in the principal component analysis (egg buoyancy and diameter, ovarian fluid osmolarity and pH, SOD, GPx and GR activities in eggs, MDA and total antioxidant status of eggs), the variables that contributed most to the separation in the pairwise comparison of PC1 vs. PC3 were egg diameter, GPx and GR activities, ovarian fluid osmolarity, and MDA in eggs. These variables had the highest absolute loadings on either or both PC1 and PC3, making them the primary drivers of variance and separation in this PCA plot (Suppl. Fig. 1). The variables that contributed most to the separation in the pairwise comparison of PC2 vs. PC3 were egg buoyancy, ovarian fluid osmolarity, SOD activity and TAS. These variables presented the highest absolute loadings on either or both PC2 and PC3, making them the primary drivers of variance and separation in this PCA plot. In the overall, PCA indicated that egg batches showed a tendency to group, but there was no clear grouping according to their origin whether from supplemented females or not. In contrast, hierarchical clustering analysis revealed two major clusters (Fig. 2), with subcategories, where each of the two clusters contained an even distribution of egg batches from both origins.

Correlations were performed among the evaluated variables (egg buoyancy and diameter, ovarian fluid osmolarity and pH, SOD, GPx and GR activities in eggs, MDA and TAS) to identify potential relationships linking ovulated eggs' quality with oxidative protection and damage parameters. Most of the observed correlations were relatively weak, suggesting that the variables are not strongly linearly related. Notable correlations included a moderate positive association between egg buoyancy and TAS, and between GR and GPx activities (Fig. 3). Specifically, a positive correlation (0.540; $P < 0.05$) indicated a tendency for higher buoyancy values to be associated with higher TAS values. Similarly, a positive correlation (0.542; $P < 0.05$) suggested that higher GR activity was associated with higher GPx values.

3.2. Comparative analysis of egg parameters across clusters and dietary effects

Comparison of the two primary clusters identified through hierarchical clustering revealed a significant difference in buoyancy ($P < 0.001$). Cluster 1 had a mean buoyancy of 13.6 ± 4.3 %, with values ranging from 0 % and 33 %, while Cluster 2 had a mean buoyancy of 66.5 ± 10.5 %, with values ranging from 50 % to 90 %. This difference in buoyancy likely distinguishes egg quality; with buoyancy below 33 % suggesting lower-quality egg batches and buoyancy above 50 % indicating higher-quality batches. Further subcategories within the two main clusters might include "lowest egg quality" with less than 10 % buoyancy and "highest egg quality" with more than 80 % buoyancy. TAS levels were also significantly different between the two major clusters, being higher ($P < 0.001$) in the cluster with high buoyancy levels.

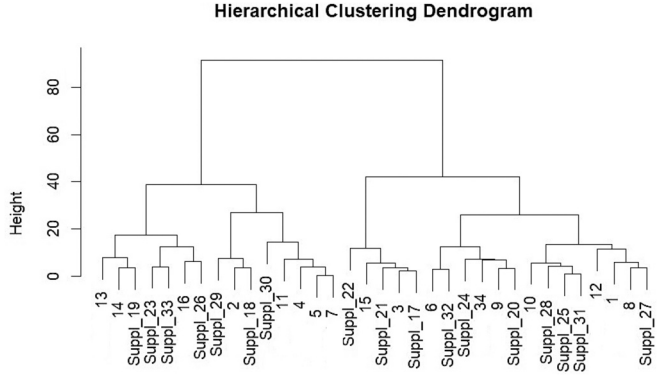

Fig. 2. Hierarchical clustering analysis of egg batches obtained from females fed the commercial diet (non-supplemented) and females fed the algae antioxidant-supplemented diet.

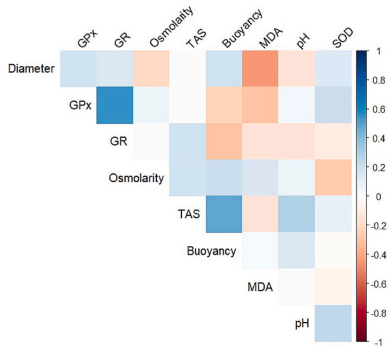

Fig. 3. Variables correlation analysis.

However, no significant differences were observed in egg diameter, ovarian fluid pH and osmolality, SOD, GPx and GR activity, and MDA levels between egg batches depending on the sub-setting of data into the two main clusters (Table 2).

Comparison of batches from non-supplemented and supplemented females, showed a significantly lower SOD activity, and higher TAS levels ($P < 0.01$) in eggs from females fed the supplemented diet. No significant differences were observed in egg diameter, ovarian fluid pH and osmolality, GPx activity and MDA levels between batches depending on their origin (Table 2).

While both major clusters contained an even distribution of egg batches from both origins, a slightly higher percentage of egg batches (60 %) from supplemented females fell into the "highest egg quality" category (>80 % buoyancy), whereas a slightly lower percentage (37.5 %) fell into the "lowest egg quality" category (<10 % buoyancy) (Suppl. Fig. 2).

3.3. Relative mRNA abundance of selected transcripts

When observing mRNA abundance in eggs with different quality regardless female diet (Fig. 4), the expression of *nrf2* and *cat* was upregulated in egg samples with higher quality, while *bax* and *casp3a* were downregulated across all samples, being more pronounced in lower quality samples. Significant differences ($P < 0.05$) were observed

Table 2
Values of each variable (mean ± SD) in egg batches classified in Cluster 1 "lower-quality eggs" in comparison with Cluster 2 "higher-quality eggs", and from females fed the commercial diet (non-supplemented) and females fed the algae antioxidant-supplemented diet. Statistical differences are indicated with lowercase letters for the factor "origin" and "cluster" (Two-way ANOVA, $P < 0.05$).

Variable	Cluster 1 "Lower Quality"	Cluster 2 "Higher Quality"	Commercial diet	Supplemented
Egg Buoyancy (%)	**13 ± 13^a**	**66 ± 11^b**	40 ± 31	46 ± 28
Ovarian fluid pH	7.49 ± 0.40	7.65 ± 0.32	7.55 ± 0.29	7.61 ± 0.42
Ovarian fluid Osmolarity (mOsm/L)	320 ± 30	315 ± 26	310 ± 20	326 ± 22
Egg diameter (cm)	1.09 ± 0.05	1.11 ± 0.05	1.12 ± 0.04	1.09 ± 0.06
SOD activity (U/g protein)	0.40 ± 0.18	0.40 ± 0.14	**0.51 ± 0.15^a**	**0.29 ± 0.13^b**
GR activity (U/g protein)	6.72 ± 2.17	5.84 ± 1.82	5.80 ± 2.20	6.66 ± 1.74
GPx activity (U/g protein)	20.61 ± 7.64	19.50 ± 4.86	19.78 ± 6.42	20.19 ± 6.08
TAS (mmol/g protein)	**0.13 ± 0.05^a**	**0.20 ± 0.02^b**	**0.13 ± 0.05^a**	**0.18 ± 0.03^b**
MDA μM/egg	0.16 ± 0.08	0.18 ± 0.06	0.18 ± 0.07	0.16 ± 0.07

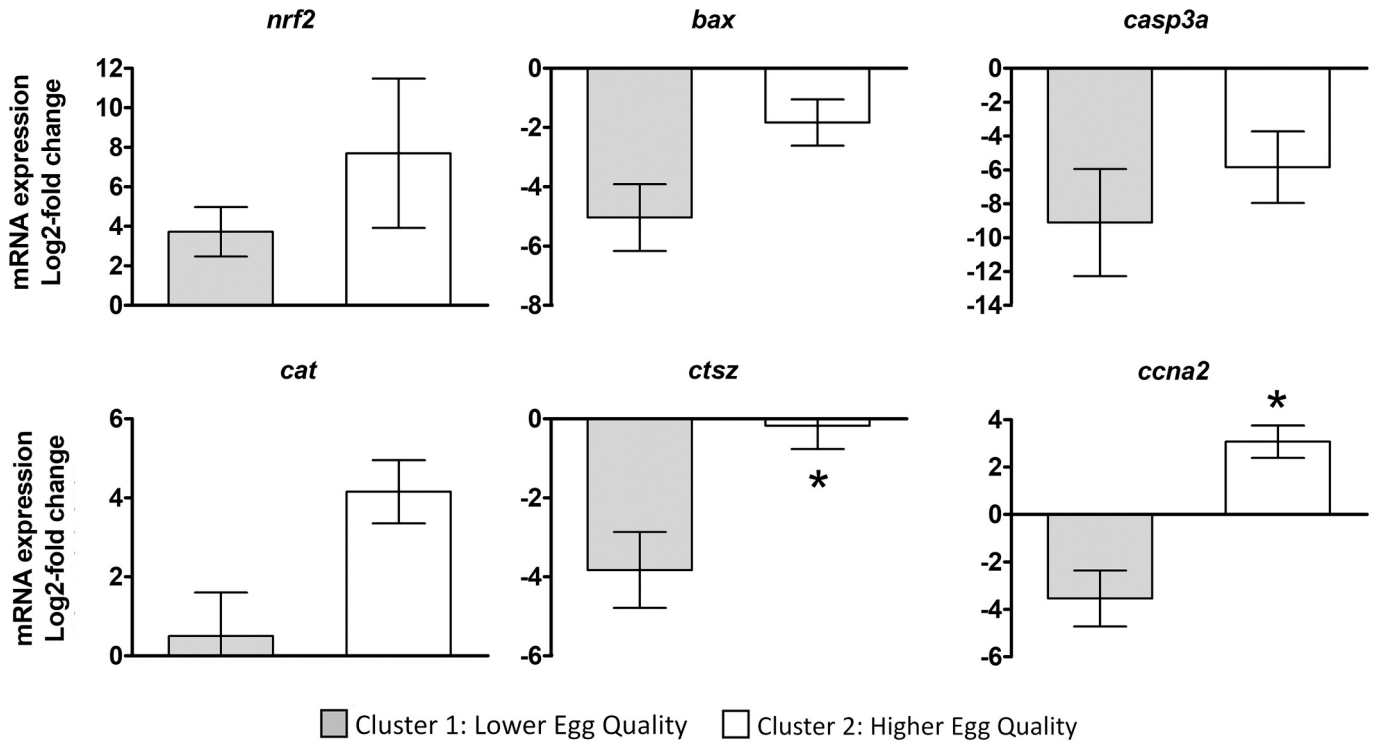

Fig. 4. mRNA levels of 6 selected transcripts quantified in eggs displaying extremes in quality as classified using the hierarchical clustering methodology. Cluster 1 refers to lower-quality eggs and Cluster 2 to higher-quality eggs. Results are presented as mean ± standard error of mean (SEM). Asterisk indicates significant differences (Student *t*-test, $P < 0.05$).

in *ctsz* expression, which was downregulated in all samples and showed a 4-fold downregulation in lower-quality eggs compared to higher-quality ones, where expression was nearly absent. The expression of *ccna2* was also significantly different between groups; this gene was downregulated in lower-quality eggs but upregulated in higher-quality eggs. When comparing expression levels of higher-quality eggs from females fed the commercial diet to those fed the supplemented diet (Fig. 5), *nrf2* was significantly upregulated in eggs of supplemented females, whereas its expression was almost absent in eggs of non-supplemented ones.

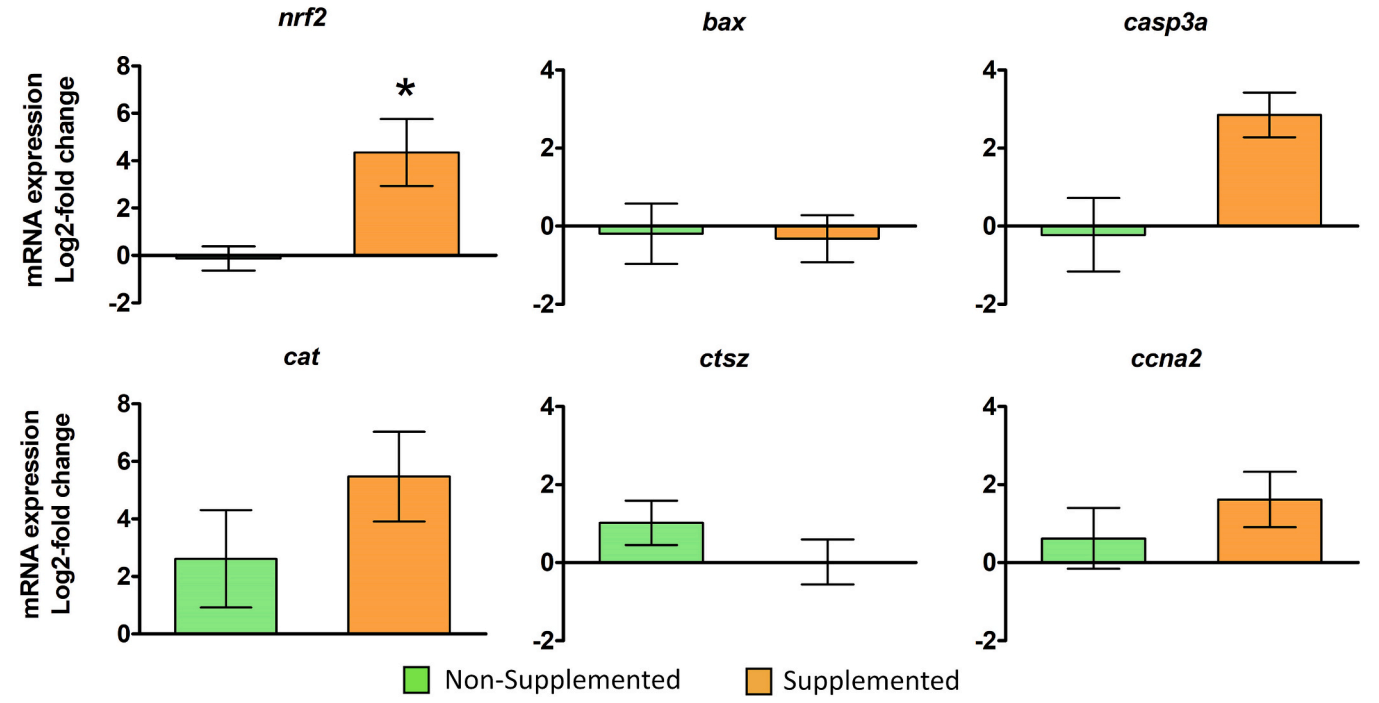

Fig. 5. mRNA levels of 6 selected transcripts quantified in higher-quality egg batches from females fed the commercial diet (non-supplemented) and from those fed the algae antioxidant-supplemented diet. Results are presented as mean ± SEM. Asterisk indicates significant differences (Student-t-test, $P < 0.05$).

3.4. Egg collection, fecundity and hatching

During routine activities, stripping was performed in 9 females fed the non-supplemented diet and 20 females from the supplemented group. The non-supplemented group produced numerically fewer batches of eggs (25 total strippings) compared to the supplemented group (93 total strippings). However, no significant differences were observed in the mean number of strippings per feeding diet, due to high variability within tanks (13 ± 11 in the non-supplemented; 47 ± 28 in the supplemented group). The mean absolute stripping fecundity (ASF: 590,741 ± 303,963 eggs per female) and relative stripping fecundity (RSF: 93,991 ± 78,683 eggs per kg) were not statistically different within groups. Despite this, the cumulative egg production from the supplemented group (10,355,000 eggs) doubled that of the non-supplemented (5,595,000 eggs). No significant differences were observed in hatching rates (58 ± 26 %) between diets.

4. Discussion

Producing high-quality eggs is vital for the success of the aquaculture industry (Migaud et al., 2013), nutrition being a key factor in optimizing reproductive success. Oxidative stress can interfere with pathways linked to fish reproduction, thus gaining insights into these interactions allows the development of biomarkers and antioxidant-focused strategies to enhance reproductive success. Given the significant role of breeders' nutrition on reproductive performance, optimizing diets or incorporating alternative nutrient sources can play a key role in improving broodstock performance. This study identified variables that can be used to predict egg quality in turbot and investigated the effects of a diet that incorporated algae, *A. platensis* and L. *digitata*, along with additional antioxidant supplementation (doubled doses of vitamins C and E, and astaxanthin) on egg quality and production.

A high variability in egg quality was observed, likely explaining why egg batches from females on the same diet did not cluster together in the principal component analysis. Hierarchical clustering further revealed an even distribution of eggs into two main clusters. Egg buoyancy, often associated with good quality and successful development in species with pelagic eggs (Bobe, 2015), was used as a quality indicator and differed significantly between the two main clusters. This led to a separation into lower-quality eggs (≤ 30 % buoyancy) and higher quality eggs (≥ 50 %). Turbot eggs have a high water content (91 %), with about 90 % of their buoyancy in sea water attributed to this aqueous content (Craik and Harvey, 1987). Consequently, a separation among floating and non-floating eggs was evident. Additionally, Jia et al. (2014) found a positive correlation between egg buoyancy and fertilization and hatching rates in turbot. Low buoyancy can be related with problems such as amino acid deficiency, indicating an egg with poor quality, uncapable to sustain fertilization and embryonic development (Dettleff et al., 2022). One probable explanation for the variability in egg quality, regardless female diet, could be the timing of egg stripping. In species that do not typically spawn spontaneously in captivity, oocytes retained in the ovarian cavity may become damaged due to overripening. Stripping the eggs at the optimal ripeness stage, when fertilization success is highest, is crucial for in vitro fertilization and breeding programs (Ramos-Júdez et al., 2019). Ovulation is a gradual process, beginning with egg ripening, followed by a window of optimal ripeness, and ending with overripening when egg viability decreases due to ageing effects. The duration of egg ripeness and maximum fertilization success varies between species and temperature (Samarin et al., 2015). Since turbot requires manual egg stripping after ovulation, obtaining eggs at their optimal quality window relies on the external examination of individuals. As established forty years ago, daily examination of each female in broodstock is crucial for improving fry output in production operations, since overripening begins approximately 10 h after ovulation. Freshly ovulated eggs can achieve fertilization rates greater than 90 % and hatching rates up to 97 %, but eggs retained in the ovary for one day before stripping results in 0 % hatching (McEvoy, 1984). Only females with fully developed ovaries (stage 4) and ready to release eggs upon gentle abdominal pressure were selected for egg collection, at each specific sampling points. However, this method did not prevent the possibility of missing the peak window of egg quality. While overriped eggs cannot be used for reproduction, it is essential to remove them, as they may harden and obstruct future spawning (Robledo et al., 2014).

In addition to high buoyancy, eggs classified with higher quality also exhibited significantly higher TAS, with a positive correlation observed between these two parameters. This suggests that TAS could be a potential marker for egg quality in turbot. While TAS has been used as a marker for sperm quality (Félix et al., 2021), to our knowledge, it has not been widely employed for fish eggs (Schaefer et al., 2016). Saffari et al. (2022) reported positive correlations between TAS levels in eggs, fertilization success, and normal embryogenesis in yellowfin sea bream (*Acanthopagrus arabicus*). In contrast, TAS was not predictive of egg quality in pikeperch (*Sander lucioperca*) (Schaefer et al., 2016). High TAS levels are expected to be beneficial to counteract oxidative stress (Saffari et al., 2022). However, it remains unclear whether TAS effectively protects against oxidative stress under hatchery conditions or if it mainly impacts other aspects during embryo development (Schaefer et al., 2016).

Oocyte ageing has been associated with increased ROS, leading to oxidative stress in some fish species (Samarin et al., 2019a). However, in goldfish, oxidative stress is unlikely to act as a trigger or promoter of oocyte ageing (Samarin et al., 2019b). In the present study, MDA levels, an indicator of lipid peroxidation and oxidative stress, showed no significant differences between eggs of varying quality across the two clusters. Similarly, in tench (*Tinca tinca*), age-related changes did not result in oxidative lipid damage, as reflected by stable MDA levels. However, tench oocytes exhibited a significant age-related increase in protein oxidation (Samarin et al., 2018). Future studies on turbot could include protein oxidation measurements to assess whether differences in egg quality correlate with oxidative stress and ageing.

Gene expression in higher and lower-quality eggs revealed significant differences in the transcripts of *ccna2* (Cyclin-A2) and *ctsz* (cathepsin Z), suggesting their potential use as markers for egg quality in turbot. Cyclins are a large group of proteins involved in cell division and are crucial for regulating mitosis and meiosis. Cyclin-A2 is identified as a maternal gene, most abundant in unfertilized eggs and during cleavage (Reading et al., 2018). In mouse embryos, *ccna2* mRNA is maternally supplied in the oocyte and remains present after fertilization, playing a crucial role in early embryonic development (Winston et al., 2000). Consistent with the findings of the present study, which observed downregulation of *ccna2* in lower-quality eggs, cyclins were also found to be downregulated in the ovaries of striped bass (*Morone saxatilis*) that produced poor-quality eggs (Chapman et al., 2014). Relative expression of *ccna2* was higher in high quality embryos in gilthead sea bream, indicated by higher fertilization rates and normal embryo development (Georgiou et al., 2022). Cathepsin Z is a papain-like cysteine protease and is thought to function primarily in intracellular protein degradation and turnover (Kao and Huang, 2008). This study found that *ctsz* mRNA levels were downregulated in lower-quality eggs, while minimal expression was observed in higher-quality eggs. Accordingly, in rainbow trout (*Oncorhynchus mykiss*), *ctsz* mRNA was more abundant in eggs with lower embryonic survival, reduced hatching success, and increased embryonic malformations (Aegerter et al., 2005).

Incorporating algae (both micro and macro) as sustainable marine sources into aquaculture feeds has shown promising potential, particularly in enhancing fish health (Nagappan et al., 2021; Naiel et al., 2021; Sheikhzadeh et al., 2024) and the relatively unexplored area of reproductive performance. Algae provide not only antioxidants but also other nutrients (Wan et al., 2019) that might be relevant during gametogenesis. For example, diets supplemented with *A. platensis* influenced the reproductive traits of yellowtail cichlid (*Pseudotropheus acei*) after three months of feeding (Güroy et al., 2012) and three-spot gourami

(*Trichopodus trichopterus*) after four months (Khanzadeh et al., 2016). Similarly, maternal supplementation with antioxidants such as nano-selenium (nano-Se) in Arabian yellowfin seabream (*Acanthopagrus arabicus*) over three months, improved egg antioxidant capacity and quality (Saffari et al., 2022). In Atlantic cod (*Gadus morhua*), astaxanthin supplementation for two months positively affected fertilization rates (Sawanboonchun et al., 2008). Therefore, dietary supplementation was expected to produce a positive impact in turbot females, when administered for four months, coinciding with gametogenesis and spawning season. In this study, the supplementation did not result in changes in egg diameter, ovarian fluid pH or osmolarity, compared to females fed the commercial diet. In turbot, egg diameter (Jia et al., 2014) and ovarian fluid pH (Fauvel et al., 1993) correlate with fertilization and hatching success. The lack of alteration in these morphological and biochemical parameters suggests that the supplemented diet is unlikely to compromise future fertilization processes. Consistent with these findings, *A. platensis* as a sole food source for zebrafish also did not affect egg size (Geffroy and Simon, 2013).

Regarding enzymatic activity, significantly higher SOD activity was noted in the non-supplemented group. SOD plays a crucial role in defending cells against ROS by catalyzing the conversion of superoxide into hydrogen peroxide and oxygen. When present in excess, ROS can damage cell membranes, inactivate enzymes, and alter RNA and DNA (Félix et al., 2021). The higher SOD activity in eggs from non-supplemented females might reflect a need to control ROS levels, possibly due to greater oxidative stress in these eggs, suggesting therefore an imbalance between free radicals and antioxidants. Saffari et al. (2022) found that fish fed lower nano-Se supplementation in plant-based diets had higher SOD activity in their eggs compared to those fed higher nano-Se supplementation or fishmeal-based diets. Although SOD is an important antioxidant enzyme in fish eggs, its activity does not always directly correlate with oxidative stress levels (Samarin et al., 2015). Lipid peroxidation, assessed via MDA levels, showed no statistical difference between the two diets. This contrasts with studies where lipid peroxidation was reduced in eggs from broodfish fed nano-Se-supplemented diets (Saffari et al., 2022). This lack of difference in turbot could be because oxidative stress in eggs might not be fully characterized by lipid peroxidation alone but also by protein oxidation (Samarin et al., 2018), a parameter that was not measured. Maternal supplementation of algae and antioxidant fortification in the diet had no significant effect on the activity of other antioxidant enzymes, such as GPx and GR, but remains to be determined if the benefits of these dietary antioxidants will be more pronounced after fertilization and during embryo development. Fontagné et al. (2008) suggests that antioxidant enzyme activity may become more prominent later in life.

On the other hand, TAS was significantly higher in eggs from supplemented females. TAS provides protection from oxidative stress, and previous studies have demonstrated a relation of nutrition and TAS levels (Cao et al., 1998; Ebeid et al., 2011; Kusano and Ferrari, 2008). Saffari et al. (2022) found elevated TAS levels in eggs from Arabian yellowfin seabream after nano-Se supplementation. In that study, TAS correlated with selenium concentration in the diet, as well as with fertilization success and normal embryogenesis. Considering TAS as a marker of egg quality in turbot, maternal supplementation with algae and high doses of antioxidants would enhance the protection of eggs against oxidative stress. Accordingly, in terms of transcript levels, *nrf2* was significantly upregulated in supplemented females' eggs compared to non-supplemented. Nuclear factor erythroid-2 related factor 2 is a critical transcription factor that regulates the expression of numerous antioxidants and cytoprotective genes, functioning as the master regulator of cellular defense against oxidative stress (Shastak and Pelletier, 2023). Lee et al. (2003) found that neural cells from mice lacking *nrf2* were more susceptible to oxidative stress compared to cells from normal mice. The upregulation of *nrf2* in the kidneys and liver of fish that received astaxanthin supplementation was observed to counteract oxidative stress induced by diazinon exposure (Shabanzadeh et al., 2023). Therefore, the upregulation observed in eggs from supplemented turbot suggests an efficient cellular mechanism to restore oxidative homeostasis. It is important to note that while *nrf2* upregulation generally indicates improved antioxidant defenses, prolonged or excessive oxidative stress can disrupt this system (Shastak and Pelletier, 2023).

While the primary reason affecting egg quality was likely the timing of egg collection, a higher percentage (60 %) of egg batches from females on the supplemented diet fell into the highest quality category ($\geq$ 80 % buoyancy), compared to a slightly lower percentage (37.5 %) in the lowest quality category ($\leq$ 10 % buoyancy). This suggests that algae antioxidant supplementation may improve the likelihood of achieving high-quality, buoyant eggs in turbot. In terms of production, dietary supplementation resulted in a higher obtention of egg batches, as more females were stripped, indicating a greater number of ovulating females. Similarly, astaxanthin supplementation in Atlantic cod positively impacted cumulative egg production (Sawanboonchun et al., 2008). If the supplementation of algae and antioxidants enhances the chances of obtaining high-quality eggs in turbot, the profitability of the eggs would increase. When combined with higher egg production, this could enhance overall larval output, as hatchability was not negatively affected by the supplemented diet. However, the results regarding the increased number of ovulated females leading to higher egg production should be interpreted cautiously due to high variability within tanks. Additional factors may influence the synchronization of turbot seasonal reproduction and maturation rhythms (Zhao et al., 2022). Furthermore, more detailed information on the oxidative damage status of eggs is required, and current parameters should be assessed in relation to fertilization capacity, embryo development, and the incidence of malformations. It is worth noting that hatching records may not effectively distinguish egg quality between broodstocks and that the current diet supplementation may instead be linked to the survival duration of unfed larvae.

5. Conclusion

Overall, supplementing the diets of turbot females with antioxidants and nutrients from sources such as micro- and macroalgae enhanced the total antioxidant status of eggs, thereby increasing their protection against oxidative stress. The compounds used in this study in breeder's diet supplementation are affordable, readily available, and environmentally safe, making them a nutritionally valuable source of natural antioxidants and nutrients. To comprehensively evaluate the impact of antioxidant supplementation on egg quality and its potential benefits for larval development, further analyses are needed. These should include measurements of total ROS levels, protein oxidation, indicators of mitochondrial dysfunction, and ATP content in the eggs. Additionally, future studies should explore optimal dosages and assess the long-term effects of supplementation on parameters such as immune response and the overall health of breeders. Such research will provide valuable insights to support sustainable aquaculture practices.

CRediT authorship contribution statement

S. Ramos-Júdez: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **E. Fatsini:** Writing – review & editing, Investigation, Data curation, Conceptualization. **C. Marrero-Alemán:** Methodology, Investigation. **C. García-Pichel:** Methodology, Investigation. **P. Parente:** Methodology, Investigation. **D. Medina:** Methodology, Investigation. **C. Castro:** Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **E. Cabrita:** Funding acquisition, Writing – review & editing, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **C. C. V. Oliveira:** Writing - review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Supervision.

Declaration of competing interest

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

Elsa Cabrita reports financial support and administrative support were provided by University of Algarve Centre of Marine Sciences. Diogo Medina and Carolina Castro are associated with the company FLATLANTIC, which developed the experiment within its facilities. Other than this, the authors declare no conflicts of interest. 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.

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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.2025.742306.

Data availability

Data will be made available on request.

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