Pedro Parente

Optimising sperm quality in flatfish through algae blend fish feed supplementation

UAlg
UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2024

Pedro Parente

Optimising sperm quality in flatfish through algae blend fish feed supplementation

Mestrado em aquacultura e pescas

(Especialidade em Aquacultura)

Trabalho efetuado sob a orientação de:

Professora Doutora Elsa Cabrita

Doutora Catarina Oliveira

UAlg
UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2024

Optimising sperm quality in flatfish through algae blend fish feed supplementation

Declaração de autoria

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Acknowledgements

Firstly I would like to give a big thank you to professor Elsa Cabrita for introducing me in the reproduction world. Elsa has provided me with the opportunity to develop my master thesis in the Aquagroup where I was certainly well received. Thank you Elsa for the opportunity, all the advisement and all the knowledge you provided me. Thank you for being the best teacher I could have had, your passion for reproduction was contagious and without you nothing of this would have been possible.

To Catarina Oliveira for having the biggest patience in the world, while also being extremely kind and calm. Thank you for listening to me speak non-stop when we went sampling to Aveiro, some countries might have considered it torture. Thank you for teaching me and giving me all the support I required to finish this thesis.

To Elvira Fatsini for all the advice given along this year. Thank you for motivating me when I needed it the most, while being rough enough to keep me on my toes. Your guidance during the practical work gave me the basis needed to accomplish my thesis. I ~~won’t~~ will not make the same mistakes again.

To the Aquagroup team, which always made my day a little bit happier while also having to put up with me in my off days. A big special thank you to Carlos Marrero and Candela Pichel, my partners in crime, I will never forget the moments we spent together. Candela you will for sure have a special place in heaven waiting for you, for putting up with me and Carlos.

To my friends, which are too many to mention here, for all the good times and specially for being my pillars during the hard times. Without many of you I would for sure had given up at some point, so I will be forever grateful.

To my dear parents, for all these years of support even when things were going south. Thank you for putting up with me and being patient enough for me to be able to find my preferred area.

The authors gratefully acknowledge Carina Mónico and CCMAR Imaging Platform for support & assistance in this work and Flatlantic - Actividades Piscícolas S.A. (Praia de Mira, Portugal) for providing access to their facilities and biological material.

This study received Portuguese national funds from “Programa Crescimento Azul (#4)” - PT-INNOVATION-0080 (EEA grants project-BREEDFLAT), FCT - Foundation for Science and Technology through project UIDB/04326/2020, UIDP/04326/2020 and LA/P/0101/2020, and from the operational programmes CRESC Algarve 2020 and COMPETE 2020 through project EMBRC.PT ALG-01-0145-FEDER-022121. It had also the contribution of national funds through fellowships (CCMAR/BM/12/2023 and CCMAR/BM/03/2024) conceded to Pedro Parente.

Abstract

Flatfish production is a growing sector in the Atlantic region, with turbot (*Scophthalmus maximus*) and Senegalese Sole (*Solea senegalensis*) being two important species with high market value in southern European countries. However, reproductive dysfunctions like the inability of captive bred individuals spawning spontaneously, poor sperm quality and quantity, still affect the obtention of progeny. Broodstock nutrition impacts reproductive performance, determining gonadal maturation, fecundity and larval survival. Sustainable supplements like micro and macroalgae, rich in essential compounds like vitamins and carotenoids are used in aquafeeds as they are known to enhance sperm quality and antioxidant defences. This study aimed to determine the effects of an algae blend supplementation on turbot and sole breeders' diet, particularly on sperm quality. Two broodstocks of each species were fed either a control or an algae-supplemented diet for 3 months. For turbot, the algae diet was supplemented with 1% *Laminaria digitata* and 5% *Arthrospira platensis,* whereas sole's diet was supplemented with 3% of *Gracilaria birdiae* and 3% of *Phaeodactylum tricornutum*. Sperm quality was evaluated through the analysis of motility, DNA fragmentation and lipid peroxidation (MDA). Total antioxidant status and the circulating levels of sex steroids were assessed in blood plasma samples. The algae diet improved sperm quality in turbot by decreasing DNA fragmentation and enhancing curvilinear and straight-line velocity. However, total motility and 11-ketotestosterone levels decreased, with an increase in malondialdehyde levels, indicating a possible shortening of the spawning cycle. In *S. senegalensis* the supplemented diet positively affected oxidative stress markers and showed normal oscillations of sperm kinetic parameters and DNA fragmentation. Nonetheless, testosterone levels decreased in the algae group, suggesting a shortening of the steroid cycle. Further studies should perform a deeper analysis by increasing the number of samplings, analyse antioxidant enzymes and algae's specific compounds effects on each species.

Keywords: Senegalese Sole; Turbot; Sperm quality; algae; antioxidant, breeders nutrition

Resumo português

A produção de peixes planos é um sector em crescimento na região Atlântica, com o pregado (*Scophtalmus maximus*) e o linguado Senegalês (*Solea senegalensis*) a terem um elevado valor de mercado nos países do sul da Europa. No entanto, ambos apresentam disfunções reprodutivas em cativeiro, como a incapacidade de realizar posturas espontâneas e a fraca qualidade e quantidade de sémen, o que afeta a obtenção de juvenis. A nutrição dos reprodutores tem um impacto direto no seu desempenho reprodutivo, influenciando a maturação das gónadas, a taxa de fecundidade e a sobrevivência das larvas. Compostos essenciais, como as vitaminas e os carotenoides, são utilizados como suplementos na alimentação dos peixes para melhorar a qualidade do sémen e reforçar as defesas antioxidantes. Suplementos como a vitamina E e C, além de terem propriedades antioxidantes, ajudam na proteção do sémen durante a espermatogéneses e fertilização, sendo que a sua deficiência na dieta origina uma redução da motilidade espermática. Similarmente, carotenoides como a fucoxantina, demonstraram ter uma função inibidora de produção de espécies reativas de oxigénio e em consequência, uma redução do stress oxidativo. As micro e macroalgas são alternativas sustentáveis para incorporação em rações de reprodutores, sendo ricas em vitaminas e carotenoides, pelo que uma mistura de algas pode resultar numa combinação de benefícios. Este estudo teve como objetivo determinar os efeitos da suplementação de uma mistura de algas na dieta de reprodutores de pregado e linguado, em particular na melhoria da qualidade do sémen. Para este estudo, dois lotes de reprodutores de pregado e de linguado foram alimentados com uma dieta controlo ou com uma dieta suplementada com algas durante 3 meses. No caso do pregado, a dieta suplementada com algas foi constituída por 1% de *Laminaria digitata* e 5% de *Arthrospira platensis*, enquanto no caso do linguado, a dieta foi suplementada com 3% de *Gracilaria gracilis* e 3% de *Phaeodactylum tricornutum*. Em ambas as espécies foram recolhidas amostras de sémen e de plasma sanguíneo durante a época reprodutiva. A qualidade do sémen foi avaliada através da análise dos parâmetros de motilidade, da avaliação da percentagem de ADN fragmentado na cauda dos espermatozóides e da análise dos níveis de malondealdeído (MDA). Adicionalmente, a capacidade antioxidante (TAS) e os níveis de esteroides sexuais foram avaliados em amostras de plasma sanguíneo. Com esta análise de qualidade pretendeu-se identificar de que forma as dietas suplementadas afetam as caraterísticas do sémen, a capacidade antioxidante total e a produção de esteroides sexuais, determinando consequentemente se produzem um impacto positivo no desempenho reprodutivo destas espécies de peixes planos. No caso do pregado, a motilidade total dos espermatozoides, apresentou valores mais baixos no grupo alimentado com a dieta de algas na segunda amostragem, quando comparado com o controlo. Adicionalmente, a velocidade curvilínea e em linha reta dos espermatozoides do grupo das algas foi positivamente afetada na primeira amostragem, demonstrando uma descida na amostragem seguinte. Os parâmetros cinéticos do sémen apresentam habitualmente um padrão de melhoria de qualidade durante o início da época reprodutiva, seguindo-se de um decréscimo de qualidade para o final da época. Portanto, é possível que na segunda amostragem o grupo alimentado com a dieta suplementada já apresentava um padrão de decréscimo de qualidade característico do final da época de reprodução. Quanto ao linguado, os parâmetros de motilidade do sémen apresentaram uma oscilação, característica da espécie ao longo da época de reprodução, com um aumento de qualidade ao início seguido de um decréscimo da mesma. Apesar disto, a mobilidade progressiva e a velocidade curvilínea parecem ter sido afetadas negativamente pela dieta suplementada. Este resultado sugere uma possível falta de ATP intracelular no sémen e/ou um encurtamento da época reprodutiva. Em ambas as espécies as dietas suplementadas com algas tinham como objetivo melhorar a atividade antioxidante, através das vitaminas e carotenoides, combatendo o stress oxidativo. Todavia, este efeito não é observado quando olhamos para os resultados do pregado onde o grupo das algas apresenta uma maior concentração de malondealdeido no sémen e uma ausência de diferenças significativas quanto à capacidade antioxidante no plasma sanguíneo. Os resultados observados levam à hipótese que a suplementação de 1% de *Laminaria digitata* pode não ser suficiente para produzir um efeito positivo, dado que alguns compostos específicos como a laminaria aparentam ter um intervalo específico de ação que varia de espécie para espécie. Outra suposição com base na bibliografia existente é que as duas algas utilizadas para suplementar a dieta do pregado apresentam um efeito não sinergético quando combinadas. No caso do linguado, a dieta suplementada induziu uma resposta positiva, promovendo uma melhor resposta antioxidante quando comparado com o grupo controlo. Os resultados da última amostragem apresentam níveis de malondealdeido significativamente mais baixos que o controlo, enquanto que a capacidade antioxidante teve valores superiores. No que diz respeito à integridade do ADN do pregado, observou-se um efeito benéfico da dieta rica em algas, através da descida da percentagem de fragmentação. Este resultado deve-se muito provavelmente a uma melhoria da integridade da membrana plasmática do espermatozoide, relacionada com a presença de vitamina E na composição das algas suplementadas. No linguado, pelo contrário, os níveis de fragmentação do ADN apresentaram uma oscilação natural com uma subida gradual até ao fim da época de reprodução. Isto deve-se não só ao facto de o sémen sofrer um processo de envelhecimento natural que provoca o aumento da descondensação da cromatina, mas também ao aumento da temperatura que afeta diretamente este parâmetro em reprodutores. Quanto aos esteroides sexuais no pregado, o grupo alimentado com a dieta suplementada em algas não apresentou variações quanto aos níveis de testosterona, mas observou-se uma redução de 11-ketotestosterona da primeira para a segunda amostragem. Estes resultados diferem das oscilações apresentadas na bibliografia para a mesma espécie, o que nos leva a crer que a dieta pode ter de facto reduzido o ciclo reprodutivo. No que diz respeito à variação de hormonas sexuais no linguado, a 11-ketotestosterona não exibe efeitos da dieta, mas a testosterona no grupo das algas apresenta diferenças significativas com o controlo. Os linguados alimentados com a dieta suplementada com algas apresentaram valores de testosterona significativamente superiores ao controlo a meio do ensaio, com valores inferiores no final. Tendo em conta alguns parâmetros obtidos nesta tese, a suplementação com uma mistura de algas demonstra-se como uma abordagem promissora para utilizar em rações de reprodutores. Estudos futuros devem ter em conta a percentagem de inclusão de cada alga, considerando os efeitos específicos que cada composto tem nas espécies alvos. Adicionalmente, para verificar as hipóteses aqui apresentadas, experiências subsequentes necessitariam de análises adicionais, como por exemplo determinar a atividade de enzimas antioxidantes e os níveis de ATP intracelular, de forma a entender e otimizar o efeito da mistura de algas nos ciclos reprodutivos de peixes planos.

Palavras-chave: linguado Senegalês, pregado qualidade seminal; algas; antioxidante; nutrição de reprodutores

Table of Contents

Abbreviations

RAS – recirculating aquaculture systems

F1 males – first-generation males of broodstock bred and reared in captivity

ROS – reactive oxygen species

LPO – lipid peroxidation

CASA – computer assisted sperm analysis

TM – percentage of total motile cells

PM – progressively motile spermatozoa

VCL – curvilinear velocity

VSL – straight-line velocity

VAP – average path velocity

LIN – linearity

TAS – total antioxidant status

MDA – malondialdehyde

SCGE – single cell gel electrophoresis

% tDNA – percentage of tail DNA

OTM – Olive Tail Moment

ELISA – solid-phase immunoenzyme assay

Ctrl – control diets

T – Testosterone

11-KT – 11-ketotestosterone

PBS – Phosphate buffer solution

H_2O_2 – Hydrogen peroxide

SOD – superoxide dismutase

CAT – catalase

GPx – glutathione peroxidase

GSR – glutathione reductase

1. Introduction

In the Atlantic region, flatfish production has been following a steady growth, with production being dominated mainly by south European countries, such as Spain and Portugal (Daures et al., 2018). Two of the species with the biggest market value are turbot (*Scophthalmus maximus*) and Senegalese Sole (*Solea senegalensis*), although both still present different reproductive dysfunctions, affecting the availability of progeny (Sánchez et al., 2019). In captivity, first-generation breeders of the two species are unable to spawn spontaneously (Chereguini et al., 1999; Guzmán et al., 2008), leading to the necessity of hand-stripping the gametes. Moreover, males produce sperm in lower volumes and lower quality (Beirão et al., 2009; Svåsand et al., 2007). Izquierdo et al., (2001) explained that the main factor determining fish maturation, fecundity and larval survival is nutrition, as the gonadal development and larval survival are directly affected by the absence of essential compounds in the diet. Vitamins and carotenoids are compounds used as supplements or functional additives in aquafeeds, due to their role in maintaining high-quality spawning (Hernandez de-Dios et al., 2022). These additives take the role of protecting sperm during spermatogenesis and fertilization, reducing oxidative stress damage in sperm cells, by boosting the fish's antioxidant system (Izquierdo et al., 2001; Kang et al., 2013). Algae (micro and macro) are rich in these compounds, comprising antibacterial and antioxidant capabilities, whilst being a sustainable alternative to plant meal in aquafeeds (Wan et al., 2019). These characteristics make them good candidates for feed supplementation targeting sperm quality (Abu-Ghannam & Rajauria, 2013; Wan et al., 2019). Studies on the benefits of algae supplementation have focused mostly on the potential of a single algae, while the use of an algae blend may result in a combination of benefits, and therefore high nutritional blends (Amaro et al., 2019; Ferreira et al., 2021).

1.1. Turbot and sole aquaculture

Flatfish aquaculture is a commercially valuable sector which grew in importance in Europe and Asia, thanks to the exhaustion of the fisheries targeting these species (Cerdà & Manchado, 2013). Turbot (*Scophthalmus maximus*) and Senegalese sole (*Solea senegalensis*), are examples of species presenting a remarkable market acceptance while displaying innate characteristics which translate into an easy adaptation to production in intensive systems

(Barreto et al., 2021; Fajardo et al., 2023; Fernández-González et al., 2021; Morais et al., 2016; Wang et al., 2016).

Turbot aquaculture started in 1970 in Scotland, but only in the 90's technological developments in juvenile's production allowed the expansion of the number of farms and subsequent production (Svåsand et al., 2007). This fish displays a fast growth rate and great capability of adaptation to intensive production conditions, being farmed in Europe and Asia, of which Spain, Portugal and China are the biggest producers (Fajardo et al., 2023; Fernández-González et al., 2021; Wang et al., 2016). European turbot farming is monopolized by Galicia and the north of Portugal, with 75% and 21% of the total production, respectively (Vázquez et al., 2020). China leads turbot production worldwide in open systems but has been shifting to recirculating aquaculture systems to reduce water consumption and environmental impact (Guo et al., 2024). In the wild, turbot spawning is dependent on environmental factors to obtain maximum reproductive success and survival of the offspring (Polat et al., 2021). The spawning period varies according to the geographical area, taking place from April to June in the Black Sea (Aydin et al., 2020), March to May in the Adriatic Sea (Caputo et al., 2001) and May to August in the North Sea (Jones, 1974). The manipulation of photoperiod is used mainly with the objective of promoting oocyte growth (Hansen et al., 1992), maturation (Imsland et al., 1997) and controlling spawning time (Akhoundian et al., 2020), where Imsland et al. (2013) discovered that 16 hours of light promotes oocyte growth and maturation. This manipulation might be used to simulate the reproductive and hatchery performance obtained with natural photoperiod year-round (Polat et al., 2021).

Differently, Senegalese sole farming started through the need to diversify the marine aquaculture industry (Howell, 1997). The existing tradition of extensive rearing in Portugal and Spain (Dinis et al., 1999), combined with the saturation of seabream and seabass in the markets and the high market value of sole, lead to an increased interest in this species by researchers and producers (Bjørndal et al., 2016). Its capability of adjustment to intensive production, good growth rates and high larval survivability led to a quick development in production, having from 2007 to 2021 grown from 55t to 1480t, respectively (APROMAR, 2022). Sole production was traditionally done through polyculture in salt marshes, changing in the last decades to intensive systems of fibreglass, concrete tanks or shallow raceways with a recent implementation of recirculating aquaculture systems (RAS) (Morais et al., 2016). Broodstocks maintained in the latter-mentioned systems are mainly of wild origin, caught in the southern Atlantic coast of Spain and Portugal, being able to spawn in the same year of capture after undergoing quarantine and acclimation (Dinis et al., 1999; Imsland et al., 2003). Both photoperiod and temperature influence the spawning timing in this species, but the recommendation is to apply natural photoperiod (Dinis et al., 1999), as a disruption of gonadal maturation was observed in individuals under constant light (Garcia-Lopez et al., 2006). Conversely, temperature manipulation has been used to extend the spawning season and achieve spawning for 8 consecutive months (Martín et al., 2014). Nevertheless, both turbot and sole present several reproductive limitations, which affect their overall production (Fatsini et al., 2020; Svåsand et al., 2007).

1.2. Reproductive Dysfunctions

To establish a sustainable aquaculture industry, fish's reproductive processes must be controlled. One of the major limitations in this sector has been the difficulties in obtaining a steady supply of gametes, as most captive fish species display some degree of reproductive dysfunction, due to stress or inappropriate environmental conditions (Mañanós et al., 2009; Migaud et al., 2013). The lack of seed quality limits reproductive success, influenced by various external factors, for instance, temperature and nutrition, as the factors which affect reproduction are highly species-specific, harnessing an increased interest in the study of fish reproduction and gamete quality research (Alavi, 2008; Köprücü et al., 2015). High-quality gametes should produce high egg survival and hatching, as well as quality larvae, leading offspring to reach adulthood in proper commercialization conditions (Bobe & Labbé, 2010; Valdebenito et al., 2015). When it comes to males, not only the quality but also the quantity of sperm may translate into its capacity to successfully reproduce and fertilize in both a natural and artificial scenario (Kowalski & Cejko, 2019).

In the case of turbot, spontaneous spawning is not possible in first generation males in captivity, having the necessity to hand-strip the gametes (Sánchez et al., 2019). Moreover, males produce sperm in low quantities and of low quality, when compared with other teleosts, while females show no difficulty in producing 5 to 10 million eggs (Svåsand et al., 2007). Fry production also shows problems as low larval survival is still found in production (Svåsand et al., 2007).

In captivity, the reproduction of Senegalese sole is mostly achieved using wild-captured individuals, but this method has proven to be unsustainable in the long term (Fatsini et al., 2020; Morais et al., 2016). The alternative of using broodstock bred and reared in captivity (F1 males), is seen as the main reproductive limitation for this species, as they produce viable gametes but lack the capability to produce courtship behaviour (Fatsini et al., 2020). Additionally, the collection of gametes from cultured males frequently results in low volumes, poor quality, and high variability in sperm samples (Cabrita et al., 2006). The small size of the F1 males' testis, when compared with wild specimens, and the low participation in spawning behaviour, might be possible explanations for the limited amount of milt produced (Carazo et al., 2017; Fatsini et al., 2020; González López et al., 2019). As temporary solution, artificial fertilization protocols have been developed and employed with the application of hormones (Guzmán et al., 2009; Rasines et al., 2013). Besides, cryopreservation techniques have been used to take full advantage of the low quantity of sperm (Riesco et al., 2017). Researchers have been focusing on inducing natural spawning (Fatsini et al., 2020), increasing sperm volume (Ramos-Júdez et al., 2021) and most importantly improving aquafeeds, in an effort to augment reproductive status (Fernández et al., 2019), and consequently gametogenesis quality (Beirão et al., 2015).

1.3. Broodstock Nutrition

Broodstock nutrition influences the result of maturation, fecundity and even larval survival as the absence of essential compounds in the diet limits gonadal development and fertility (Izquierdo et al., 2001). Not only the quantity but also the quality of the ingredients present in fish feeds reflect changes in fish growth and reproduction, leading to the use of macronutrients from different sources (Hernandez de-Dios et al., 2022). But as the aquaculture industry strives to deliver diets with optimal nutritional requirements, the need for the addition of functional additives grows, with the addition of proteins, vitamins, lipids and inorganic compounds (Fernández-Palacios et al., 1997; Kjørsvik et al., 1990; Smith et al., 1979). Functional additives are substances that benefit certain biological functions, and an example of this is the crucial role of vitamins and carotenoids in maintaining high-quality spawning (Hernandez de-Dios et al., 2022).

Vitamins are a crucial element for optimal suitable growth, health and reproduction, as vitamin E (alpha-tocopherol) and vitamin C (ascorbic acid) have been shown to have antioxidant properties, protecting sperm during spermatogenesis, fertilization and reducing lipid peroxidation. Besides, their absence has been seen to be related with a reduction in sperm motility (Ahmadi et al., 2016; Izquierdo et al., 2001). Lipid peroxidation (LPO) or oxidative stress happens in consequence of an imbalance between the natural antioxidant defences and the production of reactive oxygen species (ROS) (Valko et al., 2016). ROS, although necessary to maintain some biological functions, are a result of aerobic metabolism, leading the feed industry to supplement diets with antioxidant-rich compounds, helping the antioxidant system to maintain the oxy-redox balance (Valko et al., 2016; Xu et al., 2017). Carotenoids, such as fucoxanthin, are a prime example of compounds frequently found in marine algae that have been proven to inhibit the production of intracellular ROS, as part of their antioxidant activity (Kang et al., 2013).

Marine algae present themselves as good candidates for feed supplementation targeting sperm quality, comprising the compounds previously referred, with antibacterial and antioxidant capabilities (Abu-Ghannam & Rajauria, 2013). Microalgae, as primary producers in marine ecosystems, give critical support to aquatic animals through their components, such as fatty acids, sterols, phenolic compounds, enzymes and pigments (Muller-Feuga, 2000; Pasquet et al., 2011). One promising microalgae already used in aquafeeds is *Phaeodactylum tricornutum*, a diatom, characterized by a high content in fucoxanthin (Cerezuela et al., 2012; Kim et al., 2012). This diatom has already been used as a dietary supplement in seabream (*Sparus aurata*), resulting in the stimulation of the immune system (Cerezuela et al., 2012) and improvement of external pigmentation (Ribeiro et al., 2017). Conversely, Spirulina (*Arthrospira platensis*), is a cyanobacterium with commercial relevance already used in aquafeeds, with a rich antioxidant and anti-inflammatory profile, resulting in benefits in terms of growth, feed utilization, physiological condition and resistance to stress and disease (Abdelkhalek et al., 2015; Hosseini et al., 2013). El-Habashi and co-workers (2019) observed that the inclusion of Spirulina in Nile Tilapia (*Oreochromis niloticus*) feeds improved not only the fish's immune response, but also increased enzymatic antioxidants.

Macroalgae or seaweeds, commonly used in human and animal food, biofuel and fertilizers production, caught the interest of the fish nutrition sector, as their characteristics allow high yields of biomass and biologically active phytochemicals (Francavilla et al., 2013; Ghadiryanfar et al., 2016; Tiwari & Troy, 2015). Among them is *Gracilaria gracilis*, a red macroalgae from the *Gracilaria* genus, known for their high yields and cultivated for the production of commercially valuable compounds such as agar-agar, sulphated agaran and other bioactive molecules (Capillo et al., 2018; Imjongjairak et al., 2016; Kassila et al., 2019; Maciel et al., 2008; Marinho-Soriano, 2017). Agaran has already been described as an antioxidant agent, while also showing anti-inflammatory properties (Oliveira et al., 2020; Vanderlei et al., 2011). *Laminaria digitata*, a brown seaweed, is known for its rich bioconstituents, namely vitamins, carotenoids and laminarin (Abdel-Tawwab et al., 2024). *L. digitata* polysaccharides' biological properties go from anti-oxidant, anti-apoptotic, anti- tumour, and anti-coagulant, to anti-inflammatory activities (Holdt & Kraan, 2011; Kadam et al., 2015; Kim et al., 2006; Kuda et al., 2005; Miao et al., 1995; Ngo et al., 2011). Therefore, laminarin sources seem to be good food ingredients for aquafeeds. Despite the potential demonstrated by the use of micro or macroalgae in aquafeeds, most of the studies only focus on the benefits of supplementation with a single algae, although the mix of two or more algae might help reach highly nutritional blends, reinforcing antibacterial and antioxidant properties (Amaro et al., 2019; Ferreira et al., 2021).

1.4. Techniques for gamete quality analysis

In aquaculture, to optimize the production of both commercial species or new species with commercial interest, the control and identification of the specific biomarkers for sperm quality, proved to be an essential analysis (Cabrita et al., 2014). The evaluation of spermatozoa motility has been proven to be the most common tool for the assessment of sperm quality, as motility is directly related to fertilization success (Gallego & Asturiano, 2018). Computer assisted sperm analysis (CASA) systems determine sperm kinetic parameters through objective, sensitive and accurate techniques (Gallego & Asturiano, 2018). These systems have been used since the 1970s for mammalian sperm and adapted for fish in the 2000s, employing an imaging tracking software coupled with a visualization equipment, for instance, a small video camera (Cabrita et al., 2009). The percentage of motile (TM) and progressively motile spermatozoa (PM), can relay a quality overview of a sperm sample, where TM identifies any spermatozoa showing movement and PM only those who swim progressively (Gallego & Asturiano, 2018). Other researchers refer to velocity parameters as a more preferable biomarker in sperm quality, analysing curvilinear velocity (VCL), sperm's velocity within its real trajectory, straight-line velocity (VSL), the distance between the beginning and end of the movement in a straight-line divided by time, and average path velocity (VAP), the velocity of the spermatozoa during a derived smoothed path (Gallego & Asturiano, 2018). Although VCL and VSL might be identic if the sperm's trajectory is a straight line, due to the different environments in which fertilisation takes place, spermatozoa can follow erratic paths making both parameters useful (Kime & Tveiten, 2002; Rurangwa et al., 2004). Additionally, CASA systems provides information from each individual spermatozoa, allowing the identification of sperm subpopulations, characterized by distinct motility patterns (Beirão et al., 2009).

Fish sperm cells are also prone to suffer damage related to oxidative stress, as they have a high content of polyunsaturated fatty acids, targeting the membrane, proteins and DNA (Cabrita et al., 2014). The defence against the oxidative damage in fish sperm is done through the seminal plasma, as the spermatozoa present low content of cytoplasm (Shiva et al., 2011). This has led authors to analyse the antioxidants and oxidants defensive enzymes present in both sperm and seminal plasma (Cabrita et al., 2014). One of these analyses is the total antioxidant status (TAS) of sperm samples, which identifies the overall antioxidant status through the presence of metabolites and antioxidant enzymes (Cabrita et al., 2014). Commercial kits are used to determine the overall antioxidant status, characterizing these levels in both seminal and blood plasma. In Atlantic cod (*Gadus morhua* L.) a possible relation between the antioxidant capacity and motility parameters was suggested in thawed sperm samples (Butts et al., 2011). Another biomarker to evaluate oxidative stress is lipid peroxidation, which is quantified by the measurement of the amount of malondialdehyde (MDA), one of the final products of lipid oxidation. This technique can be performed by determining thiobarbituric acid-reactive substances (TBARS) or by using commercial kits to determine directly MDA, which in contrast with older procedures, was developed minimizing interferences of non-target compounds (Cabrita et al., 2014).

As previously mentioned, oxidative damage also targets DNA, fragmenting its strands and resulting in chromatin damage. The comet assay or single cell gel electrophoresis (SCGE), is the technique most frequently employed to identify the resulting fragments of packaging failures (Cabrita et al., 2014). This assay bases itself on the migration pattern of DNA fragments, depending on their size, and is developed in individual cells (Cabrita et al., 2014). This migration often forms a comet-like tail, which will be analysed on a cell-by-cell basis using specific software, giving information on parameters such as percentage of tail DNA (% tDNA) and Olive Tail Moment (OTM) (Beirão et al., 2008).

Broodstock nutrition interacts directly with the endocrine system of fish, as the latter recognizes external and internal conditions in order to complete the reproductive development and spawn, or delay until optimal conditions are achieved (Volkoff & London, 2018). To understand this interaction it is important to study the cycle of sex steroid hormones present in blood plasma (Lee & Yang, 2002). These hormones have a role in the development and maturation of gonads in teleost, with testosterone (T) and 11-ketotestosterone (11-KT) being accountable for male's secondary characteristics (Bhat et al., 2022; Ueda et al., 1984). Androgens are known to stimulate the proliferation of germ cells while regulating reproductive and agonistic behaviours, producing feedback effects in the brain and pituitary (Borg, 1994; Miura et al., 1991; Schulz & Goos, 1999). A solid-phase immunoenzyme assay (ELISA), has been used to quantify sex steroid levels in blood plasma in several fish species (Oliveira et al., 2020).

1.5. Objectives

The aim of this thesis was to improve sperm quality of both flatfish species, turbot and Senegalese sole, through the incorporation of macro and microalgae in breeder's diets. More specifically:

- For turbot the diet will be supplemented with *Laminaria digitata* and *Arthrospira platensis.*
- For Senegalese sole the diet will be supplemented with *Gracilaria gracilis* and *Phaeodactylum tricornutum*.

2. Material and Methods

2.1. Ethics statement

The experimental procedures were conducted in accordance with Portuguese legislation for use of laboratory animals (PORT 1005/92) by the Portuguese direction for veterinary and food services (Direção Geral de Alimentação e Veterinária, DGAV) while following ARRIVE guidelines. DGAV authorized the experimental procedures (license number 003289) and it is certified that the animals involved in the present study were maintained and handled according to the best practices.

2.2. Broodstock Management

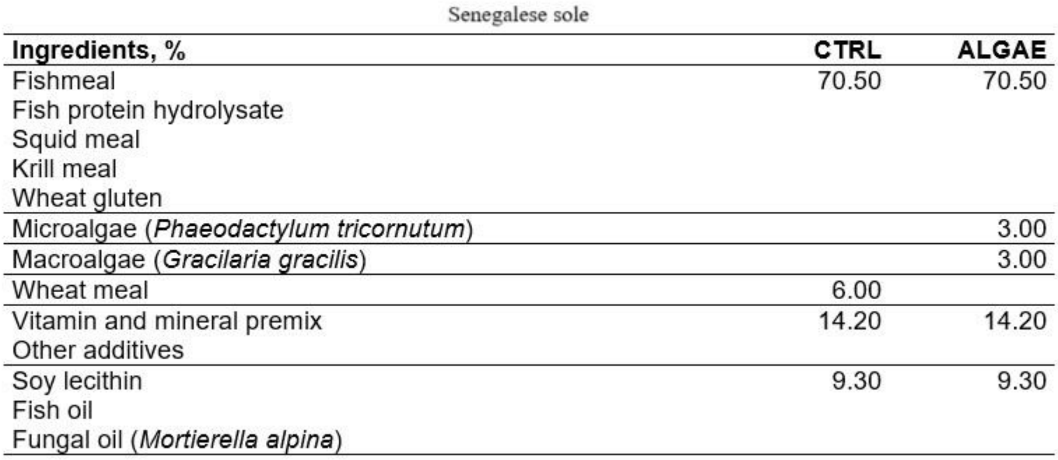
For this experiment, different broodstocks of turbot and Senegalese sole were used. A broodstock of 180 turbot males and females with the mean body weight (BW) of 6336 ± 1644g, were sampled *in loco* at the semi-intensive aquaculture farm Flatlantic - Actividades Piscícolas S.A. (Praia de Mira, Portugal). These fish were maintained indoors in fibre-glass tanks of 15 m3, under a controlled photoperiod simulating natural conditions and a constant temperature of 14.3 ± 0.2 ºC.

The Senegalese sole broodstock used in this study was an established stock of F1 breeders (1014 ± 221 g BW), divided in 4 fiber-glass tanks of 5.89 m3 (Ø2.5 m, 1.2 height), each one with 18 animals, at Ramalhete experimental station of the University of Algarve and CCMAR (37.00658, −7.96731 - Faro, Portugal). This research facility maintained fish on a semi-closed system with a 2:1 sex ratio (male:female), necessary for reproduction in this species. During the experiment, photoperiod naturally oscillated between 12 L:12D to 14 L:10D, and water temperature ranged from 14.6 to 20.4 ºC.

2.3. Experimental Diets

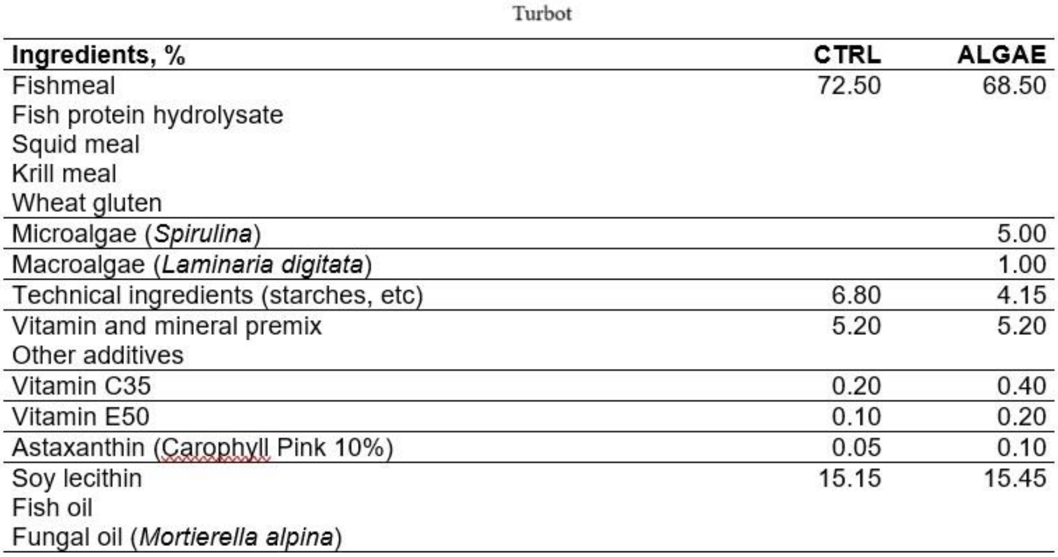
The semi-moist experimental diets were produced by Sparos Lda (Olhão, Portugal). The commercial feeds made by SPAROS were used as control diets (Ctrl), containing the essential protein, fat and gross energy believed to be essential for the species. For turbot, the formulated algae diet was supplemented with 1% *Laminaria digitata* and 5% *Arthrospira platensis*, while for Senegalese sole the diet was supplemented with 3% of *Gracilaria gracilis* and 3% *Phaeodactylum tricornutum.* The formulation and substitutions made in order to supplement with the same percentage of macro and microalgae can be seen in Table 2.1. Both diets are isoenergetic and isoproteic.

Table 2.1: Diet formulation (% inclusion levels) of the experimental diets (Control and Algae) for Senegalese sole (*Solea senegalensis*) and Turbot (*Scophtalmus maximus*).



Senegalese sole

Ingredients, %	CTRL	ALGAE
Fishmeal	70.50	70.50
Fish protein hydrolysate		
Squid meal		
Krill meal		
Wheat gluten		
Microalgae (*Phaeodactylum tricornutum*)		3.00
Macroalgae (*Gracilaria gracilis*)		3.00
Wheat meal	6.00	
Vitamin and mineral premix	14.20	14.20
Other additives		
Soy lecithin	9.30	9.30
Fish oil		
Fungal oil (*Mortierella alpina*)		

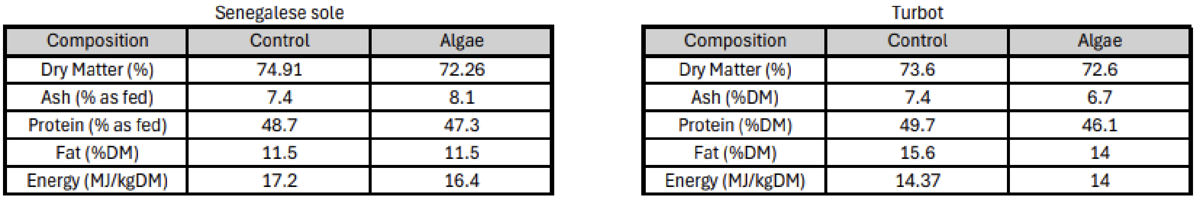


Turbot

Ingredients, %	CTRL	ALGAE
Fishmeal	72.50	68.50
Fish protein hydrolysate		
Squid meal		
Krill meal		
Wheat gluten		
Microalgae (*Spirulina*)		5.00
Macroalgae (*Laminaria digitata*)		1.00
Technical ingredients (starches, etc)	6.80	4.15
Vitamin and mineral premix	5.20	5.20
Other additives		
Vitamin C35	0.20	0.40
Vitamin E50	0.10	0.20
Astaxanthin (Carophyll Pink 10%)	0.05	0.10
Soy lecithin	15.15	15.45
Fish oil		
Fungal oil (*Mortierella alpina*)		

All powder ingredients were mixed accordingly 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, which were humidified with 25% water and made into 22 or 6 mm (for turbot and sole, respectively) 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 research site, where they were stored frozen at -20ºC until use. Samples of diets were taken for analytical characterization (Table 2.2).

Table 2.2: Proximate composition (% of dry weight) of the experimental diets (Control and Algae) for Senegalese sole (*Solea senegalensis*) and Turbot (*Scophtalmus maximus*).



Senegalese sole

Composition	Control	Algae
Dry Matter (%)	74.91	72.26
Ash (% as fed)	7.4	8.1
Protein (% as fed)	48.7	47.3
Fat (%DM)	11.5	11.5
Energy (MJ/kgDM)	17.2	16.4

Turbot

Composition	Control	Algae
Dry Matter (%)	73.6	72.6
Ash (%DM)	7.4	6.7
Protein (%DM)	49.7	46.1
Fat (%DM)	15.6	14
Energy (MJ/kgDM)	14.37	14

2.4. Experimental Design

With the objective of analysing the changes in sperm parameters and blood plasma from fish fed with different feeds, each species broodstock was divided into four tanks. Of these four tanks, two were fed with the control diet while the other two were fed the algae diet, starting on the 9th and 23rd of February for turbot and sole, respectively. In the case of turbot, each tank was sampled twice, once during the photoperiod cycle of 15 hours of light (mid-April) and another after the change to the 15:30h photoperiod cycle (mid-May), mimicking the natural photoperiod for the species. Senegalese sole samplings occurred approximately every 15 days during the breeding season from April to June.

2.5. Sperm collection

While turbot did not require any type of anaesthetic, Senegalese sole individuals were anesthetized with 300 ppm phenoxyethanol for the collection of biological samples. The procedure for sampling retrieval was similar for both species, with small adjustments. Phosphate buffer solution (1x PBS) has been used to clean the genital pore, aiming to avoid any type of contamination by anaesthesia, urine or mucus. Through abdominal massage, sperm samples were collected with a sterile syringe and stored in an eppendorf placed in a styrofoam box on ice, at approximately 10 ºC, for posterior analysis. Blood was then collected with heparinised syringes directly from the caudal vein and centrifuged at 3000 g for 15 min at 4 ºC for plasma separation. Collected plasma was separated in two aliquots for further TAS determination and sex steroids analysis and stored at -80 ºC.

2.6. Sperm motility analysis using CASA system

Spermatozoa motility parameters were evaluated using a CASA system (ISAS software, Proiser, Valencia, Spain) paired with a phase-contrast microscope (Nikon E-200; Nikon, Tokyo, Japan) equipped with an ISAS camera (25 fps).

To assess spermatozoa motility, sperm samples were activated using 1 µL of sperm sample and 10 µL of artificial seawater. Immediately after activation and during 60 s, various parameters were recorded, including total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), and linearity (LIN, %). A total of 7-17 samples for turbot and 15-21 samples for S. sole were analysed for each group / sampling point.

2.7. Lipid peroxidation

Lipid peroxidation assessment was performed, following the optimized protocols by our group (Riesco et al., 2017), by quantification of MDA through a colorimetric assay (Oxis BIOXYTECH MDA-586) and following the manufacturer's protocol. Sperm samples from sole and turbot were diluted following an incubation for 30 min at 37ºC, with a 200 µM sodium ascorbate solution containing 40 µM FeSO4, in the dark. During this incubation a standard curve was prepared by diluting MDA standard solution (20 µM) in MiliQ water (Table 2.3).

Table 2.3: Standard curve points used for the MDA assay.

Standard curve points	R7	R6	R5	R4	R3	R2	R1
MDA (µM)	14	10	8	4	2	1	0

Afterwards, the reagents provided in the kit were added to 100 µL of cell suspension and a second incubation of 1 hour at 45 ºC in the dark was performed. Samples were then put through a centrifugation (10 000 g for 10 min at 4 ºC), with a subsequent transfer of 200 µL of the supernatant into a 96-well flat-bottom transparent plate (Nunc). Absorbances were then read in a microplate reader (Synergy 4, Biotek Instruments. Inc.) at 586 nm and the standard curved was used to calculate MDA concentrations presented as nM of MDA per million spermatozoa. A total of 7-16 samples for turbot and 12-21 samples for S. sole were analysed for each group / sampling point.

2.8. Total Antioxidant Status

The total antioxidant status of blood plasma in sole and turbot was evaluated using the commercial TAS kit (Randox Laboratories Limited, United Kingdom). In the case of sole, the analysis was performed following the manufacturer's instructions and the protocol established by our group (Martínez-Páramo et al., 2013), while for turbot the protocol had to be optimized by lowering the amount of sample used. For this procedure, two absorbance reads were made at 600 nm and 37 ºC. The first read was done following the reaction of 2 or 0.5 µL of the sample, for sole and turbot respectively, with 100 µL of chromogen (R2), readied by the kit. Afterwards, 20 µL of substrate (R3) was added and followed by an incubation of 3 min to complete the second read. During this process, a control sample (TAS control, REF 2331, Randox Laboratories Ltd., United Kingdom) was included to validate the quality of the results obtained. Following the calculations, the results were reported in units of mmol/L. A total of 6-12 samples for turbot and 12 samples for Senegalese sole were analysed for each group / sampling point.

2.9. Evaluation of DNA fragmentation

Spermatozoa DNA fragmentation was analyzed through the technique of comet assay (single-cell gel electrophoresis), with modifications by Cabrita et al. (2005). Buffers, solutions and agarose gel slides used for the procedure of cell lysis, DNA denaturation and electrophoresis were prepared the day before the sampling. Sperm samples were diluted in Ringer solution and 20 µL of diluted sperm was added to 5% low melting point agarose (Invitrogen). Sixty microliters from this solution were introduced in the previously made agarose slides and kept at 4 ºC for 20 min to solidify. Afterwards the slides were placed in a coplin jar containing a lysis buffer (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, 1% Triton X-100, 1% lauryl sarcosine) for 1 hour at 4 ºC. An electrophoresis tray (BioRad, Spain) was then filled with electrophoresis solution (0.3 M NaOH, 1 mM Na_2-EDTA, pH 13), in which the volume was adjusted to achieve 300 mA. The slides were placed in the tray for 20 minutes, allowing the unwinding of the DNA, after which were subjected to an electrophoresis for 10 min 25 V and 300 mA at 4 ºC. Two more coplin jars were prepared with neutralizing solution (0.4 M Tris, pH

7.5), in which, the slides were washed for 5 min at 4 ºC after electrophoresis, to eliminate any possible detergent or alkali followed by the fixation in pure ethanol for 3 min. Slides were subsequently air-dried in the hotte and then refrigerated at 4 ºC until additional analysis. To observe the DNA fragmentation a solution of 10% Propidium iodide in PBS was made, from which 10 µL were added to each slide. For turbot sperm samples, the analysis was performed in a fluorescence microscope (Nikon Eclipse E200) at a magnification of 40x with an excitation filter of 450 nm, capturing an average of 100 images through a digital camera (VisiCam 16 Plus, VWR). The analysis of Senegalese sole samples was performed in an upright fluorescence microscope (Zeiss Axioscope 5) with 40 X A-plan 0.65 objective and red channel filter cube (Excitation Bandpass 560/40, Beam splitter 585, Emission Bandpass 630/75). Fifty images on average were captured per sample, with a digital camera Axiocam 202 mono through the acquisition software Zen PRO 3.1. Two slides were prepared for each sample. The final step to calculate tail DNA percentage was performed using the KOMET software, version 6.0 (Andor Technology, Belfast, Ireland), as described by Cabrita et al. (2011). A total of 8-15 samples for turbot and 3-20 samples for S. sole were analysed for each group / sampling point.

2.10. Sex Steroids Determination

Circulating levels of testosterone and 11-ketotestosterone were assessed in male blood plasma samples, for each species and through enzyme-linked immunosorbent assay (ELISA). The procedure followed the manufacturer's instructions, having the assay been performed with the respective commercial ELISA kits from Cayman Chemicals (Ann Arbor, Michigan, United States), applied previously by Oliveira et al., (2020). For each hormone, a total of 6-12 samples for turbot and 10-12 samples for S. sole were analysed for each group / sampling point.

2.11. Statistical Analysis

The statistical analysis was performed with SPSS Statistics 29.0 software (IBM Co., Hong Kong). All results were presented with means ± standard deviation (mean ± SD). Shapiro-Wilks test was used to analyse data normality, while the Levene test was used to analyse the homogeneity of variance. The level of significance considered was 0.05 ($p < 0.05$). A Kruskal Wallis test was used to evaluate the effects of the diet on the sperm motility, for both species, lipid peroxidation and DNA fragmentation in Senegalese sole. A Mann-Whitney U test was used to analyse lipid peroxidation, DNA fragmentation and sexual steroid levels in turbot. A two-way ANOVA followed by a Tukey HSD test was used to analyse TAS for both species and sexual steroid levels in sole.

3. Results

3.1. Sperm Motility Parameters

In the case of *S. maximus* individuals, which were fed a diet supplemented with macro and micro algae, total and progressive sperm motility exhibited a comparable pattern displaying significantly (Two-way ANOVA, Tukey HSD, $P<0.001$) lower motility percentage than the fish under the control diet at the second sampling (45.17 ± 18.91 *vs* 75.94 ± 13.49 %; for TM and 28 ± 15.23 *vs* 48.05 ± 7.86 %, for PM) (Figure 3.1 A and B). On both VLC and VSL, fish fed the algae diet showed significantly faster spermatozoa (Two-way ANOVA, Tukey HSD, $P<0.05$) in the 1st sampling (146.57 ± 23.46 µm/s and 89.71 ± 22.42 µm/s, respectively) in comparison with the turbot fed the control diet (117.67 ± 27.05 µm/s and 71.07 ± 13.49 µm/s, respectively) (Figure 3.1 C and D). In the second sampling, the opposite was observed with spermatozoa being significantly slower for the algae group in comparison with the control for both VCL and VSL (75.17 ± 14.90 *vs* 122.35 ± 27.23 µm/s and 60.05 ± 13.37 *vs* 80.47 ± 20.41 µm/s; respectively). Linearity results showed that fish subjected to the algae diet displayed significant higher (Mann-Whitney U Test, $P<0.05$) linearity at the second sampling, when compared to the control (79.83 ± 5.71 *vs* 65.76 ± 11.55, respectively) (Figure 3.1 E). Finally, looking at differences of the same treatment between sampling points, a pattern of significant decrease (Two-way ANOVA, Tukey HSD, $P<0.05$) was seen from 1st to 2nd sampling on fish fed with then algae feed for in all parameters, except linearity where the opposite was seen with a significant increase (Mann-Whitney U Test, $P<0.05$) in values over time.

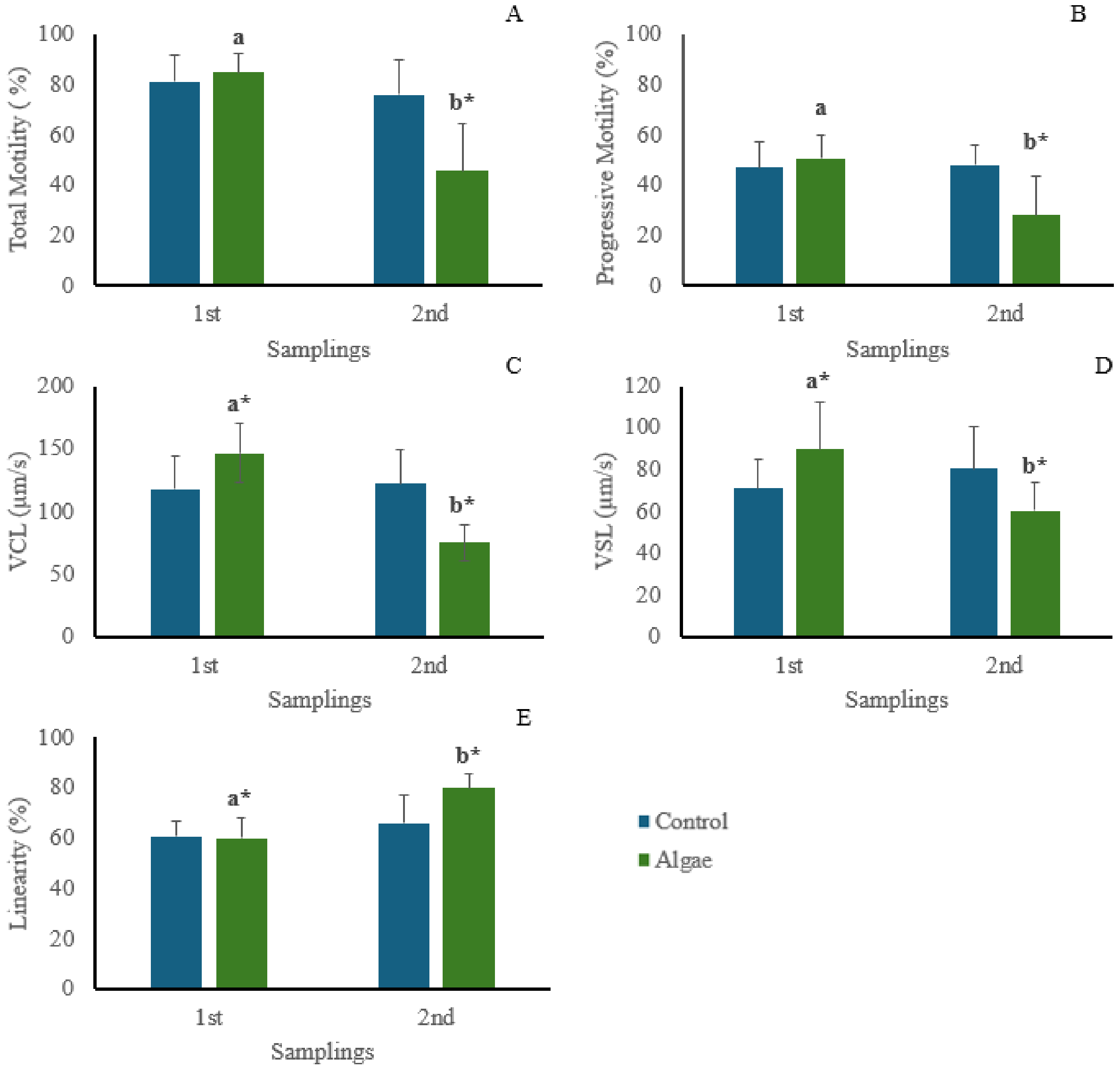


Figure 3.1: Motility parameters determined in *S. maximus* sperm from individuals fed on a control (n=15-17) or an algae supplemented (n=7-8) diet. The blue columns correspond to the control group and the green columns to the algae group. The following parameters were evaluated: A) Total motility (%); B) Progressive motility (%); C) Curvilinear velocity (µm/s); Straight-line velocity (µm/s) and E) Linearity (%). Analyses were performed at the 1st (April) and 2nd (May) samplings. Data is shown as mean ± SD, with the asterisk indicating significant differences between treatments in a specific sampling point, while letters show differences between samplings of the same treatment (Kruskal Wallis, $P<0.001$).

When considering the analysis done to Senegalese sole fed an algae enriched diet, no significant differences between treatments in both sperm TM and LIN (Mann-Whitney U Test, $P>0.05$) were found (Figure 3.2 A and E). On PM the individuals feed an algae supplemented diet showed significant lower (Mann-Whitney U Test, $P<0.05$) results in the 4th sampling (39.27 ± 14.75 *vs* 51.6 ± 7.85 % for algae and control, respectively) (Figure 3.2 B). Likewise, VCL presented significant lower results (Mann-Whitney U Test, $P<0.05$) in fish fed on algae diet on the 3rd sampling (166.94 ± 14.16 *vs* 181 ± 14.47 µm/s for algae and control, respectively) (Figure 3.2 C). Regarding VSL, spermatozoa of fish fed the supplemented diet had significantly higher velocity (Mann-Whitney U Test, $P<0.05$) than control fish, at the 1st sampling point (20.8 ± 4.75 *vs* 17.22 ± 4.61 µm/s for algae and control, respectively) (Figure 3.2 D). When assessing differences between samplings within the same treatment, the same pattern could be seen for almost all parameters (except total motility): a significant increase (Kruskal Wallis Test, $P<0.05$) of values was seen from the 2nd to the 3rd samplings, followed by a decrease at the 5th sampling (PM and VCL) or a stabilization for both 4th and 5th samplings (VSL and Linearity).

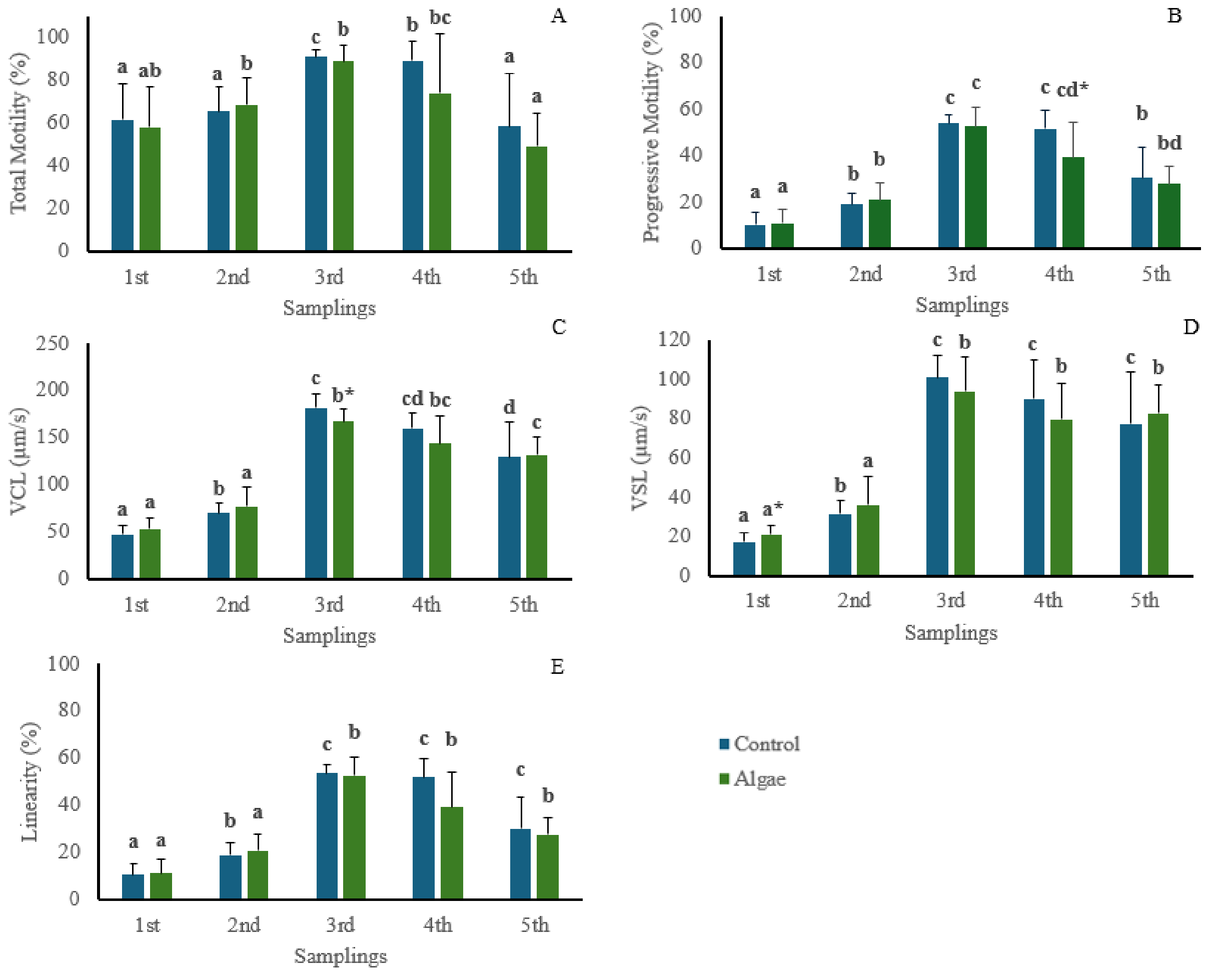


Figure 3.2: Motility parameters determined in *S. senegalensis* sperm from individuals fed on a control (n=15-18) or an algae supplemented (n=15-21) diet. A) Total motility (%); B) Progressive motility (%); C) Curvilinear velocity (µm/s); Straight-line velocity (µm/s) and E) Linearity (%). Analyses were performed at the 1st (19/04/23), 2nd (12/05/23), 3rd (22/05/23), 4th (13/06/23) and 5th (27/06/23) samplings. Data is shown as mean ± SD, with the asterisk indicating significant differences between treatments in a specific sampling point, while letters show differences between samplings of the same treatment (Kruskal Wallis, $P<0.001$).

3.2. Lipid Peroxidation

In turbot, the concentration of MDA present in sperm was significant higher (Mann-Whitney U Test, $P<0.05$) in the algae supplemented group at the first sampling (24.01 ± 4.36 nM of MDA per million spermatozoa), when compared with the control (19.85 ± 7.76 nM of MDA per million spermatozoa) (Figure 3.3). Differently, *S. senegalensis* [MDA] showed significant differences in the 5th and last sampling, with the algae group presenting lower concentration when compared with the control (57.38 ± 38.96 *vs* 127.64 ± 104.21 µM of MDA per million spermatozoa; Mann-Whitney U Test, $P<0.05$) (Figure 3.4).

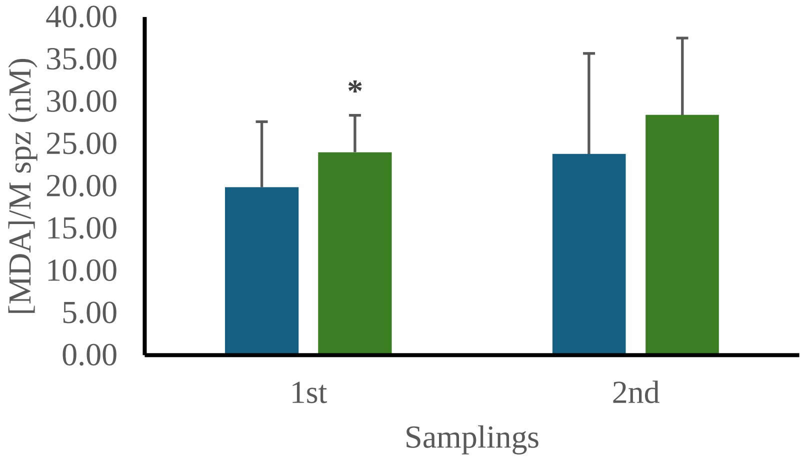


Figure 3.3: MDA concentration per million spermatozoa (nM) determined in *S. maximus* spermatozoa fed on a control (n=15-16) or an algae (n=7-8) supplemented diet. Analyses were performed at the 1st (April) and 2nd (May) samplings. Data is shown as mean ± SD, with the asterisk indicating significant differences between treatments in a specific sampling point (Mann-Whitney U Test, $P< 0.02$).

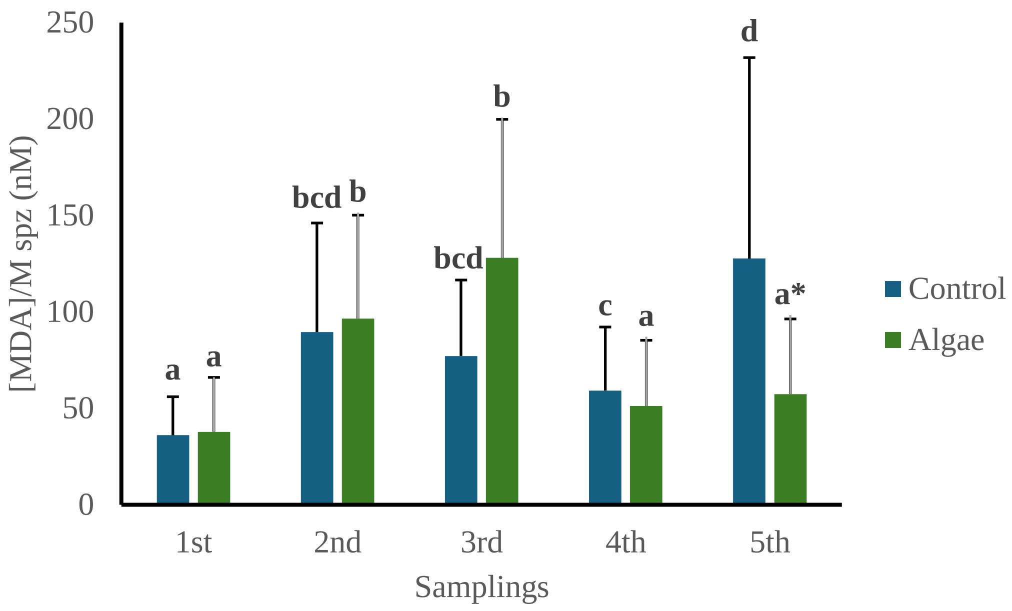


Figure 3.4: MDA concentration per million spermatozoa (nM) determined in *S. senegalensis* sperm from individuals fed on a control (n=16-21) or an algae (n=12-17) supplemented diet. Analysis were performed at the 1^{st} (19/04/23), 2^{nd} (12/05/23), 3^{rd} (22/05/23), 4^{th} (13/06/23) and 5^{th} (27/06/23) samplings. Data is shown in mean ± SD, with the asterisk indicating significant differences between treatments in a specific sampling point (Mann-Whitney U Test, P< 0.05) while letters show differences between samplings of the same treatment (Kruskal Wallis, P<0.001).

3.3. Total Antioxidant Status

Concerning turbot individuals, no significant differences were found between treatments nor samplings in the blood plasma antioxidant status (Two-way ANOVA, P>0.05) (Figure 3.5). As for Senegalese soles fed algae supplemented feed, at the third sampling, a significant higher level of antioxidants (Two-way ANOVA, Tukey HSD, P<0.05) was observed when compared with the control group (1.53 ± 0.48 vs 1.19 ± 0.33 mmol/l, respectively), although no differences were found between samplings of the same treatment (Two-way ANOVA, P>0.05) (Figure 3.6).

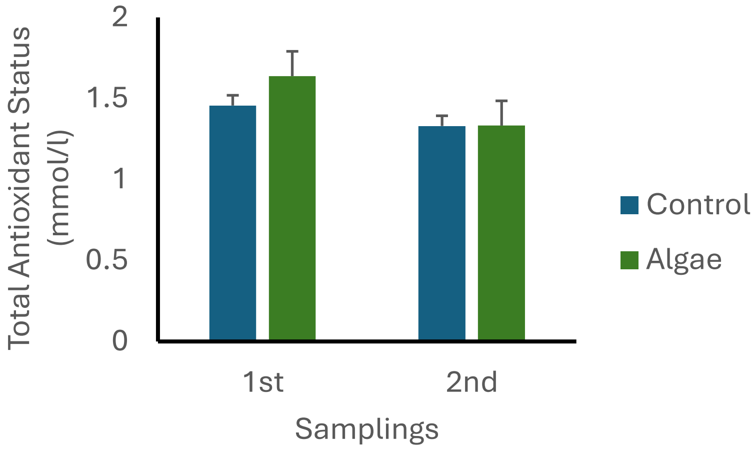


Figure 3.5: Total Antioxidant Status determined in *S. maximus* blood plasma from individuals fed on a control (n=12) or an alga (n=6) supplemented diet. Analyses were performed at the 1st (April) and 2nd (May) samplings. Data is shown as mean ± SD, with no significant differences being found (Two-way ANOVA, P>0.05)

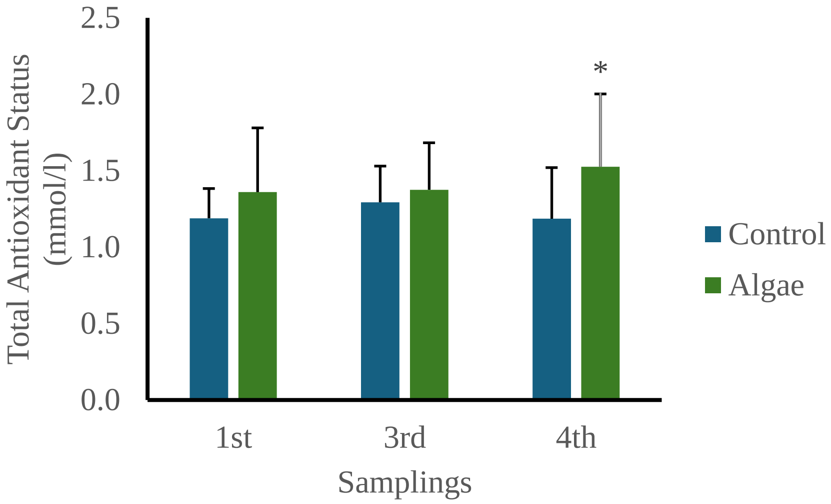


Figure 3.6: Total Antioxidant Status determined in *S. senegalensis* blood plasma from individuals fed on a control (n=12) or an alga (n=12) supplemented diet. Analyses were performed at the 1st (19/04/23), 3rd (22/05/23) and 4th (13/06/23) samplings. Data is shown as mean ± SD, with the asterisk indicating significant differences between treatments at a specific sampling point (Two-way ANOVA, Tukey HSD, P<0.001).

3.4. DNA Fragmentation

The percentage of DNA fragmentation in the comet's tail was significant lower (Mann-Whitney U Test, P<0.001) for the turbot fed on the algae supplemented feed in comparison with the control group, at both 1st (15.86 ± 10.01 *vs* 25.18 ± 15.20 %, respectively) and 2nd samplings (7.17 ± 8.31 *vs* 15.24 ± 13.24 %, respectively). Furthermore, both turbot fed on control and algae diets presented a significant decrease in % of fragmented DNA from the first to the second sampling (P< 0.001 and P< 0.001, respectively) (Figure 3.7).

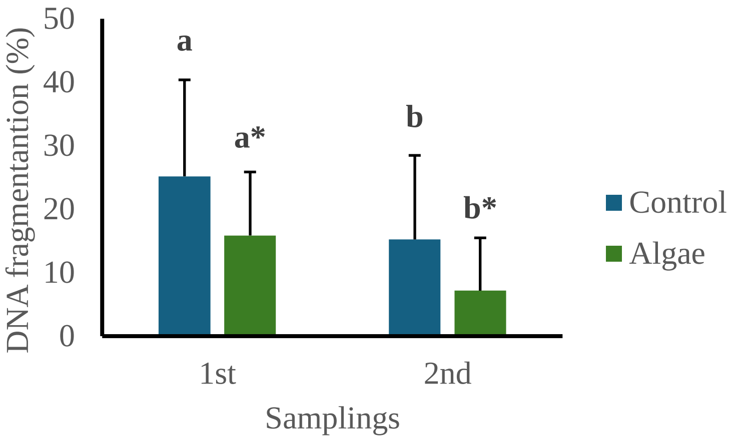


Figure 3.7: Percentage of DNA fragmentation determined in *S. maximus* spermatozoa from individuals fed on a control (n=12-15) or an alga (n=8) supplemented diet. Results were obtained for the 1st (April) and 2nd (May) samplings. Data is shown as mean ± SD with the asterisk indicating significant differences between treatments in a specific sampling point, while letters show differences between samplings of the same treatment (Mann-Whitney U Test, p<0.01).

In the case of Senegalese sole breeders, significant differences between groups were only found at the second and third samplings (Mann-Whitney U Test, P< 0.001), where sole under algae supplemented feeding (41.69 ± 17.62 % and 27.22 ± 15.86 %, for the 2nd and 3rd samplings respectively) presented a higher amount of fragmentation at the second sampling and lower at the third, relative to the control group (39.88 ± 17.31 % and 32.28 ± 16.84 %, for the 2nd and 3rd samplings respectively) (Figure 3.8). Additionally, significant differences (Kruskal Wallis, P<0.001) could be seen between some samplings of the same treatment.

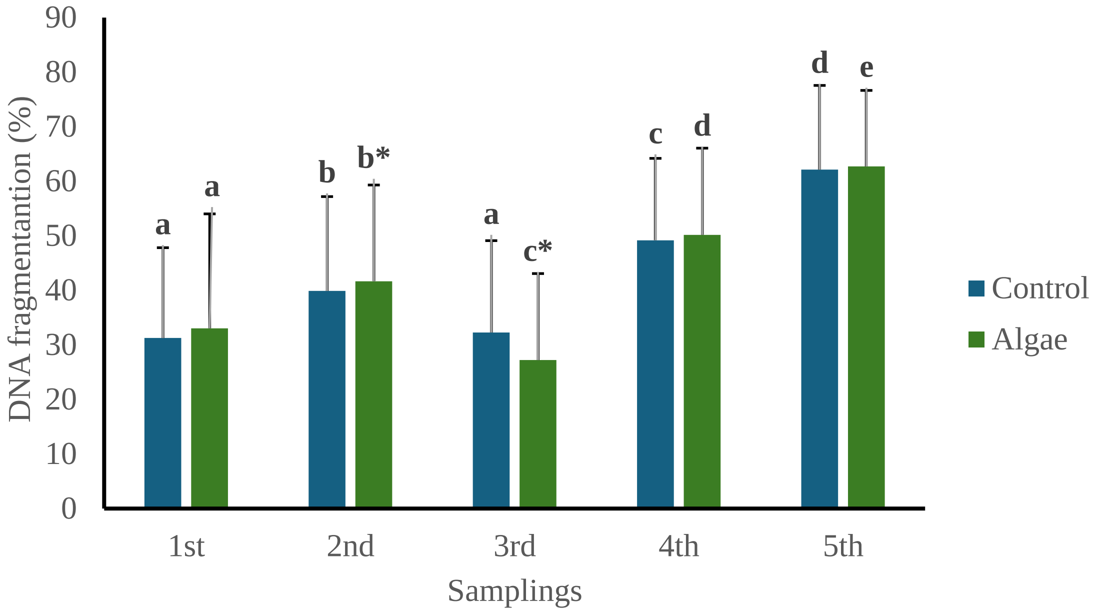


Figure 3.8: Percentage of DNA fragmentation determined in *S. senegalensis* spermatozoa fed on a control (n=3-20) or an algae (n=7-17) supplemented diet. Results were obtained for the 1st (19/04/23), 2nd (12/05/23), 3rd (22/05/23), 4th (13/06/23) and 5th (27/06/23) samplings. Data is shown as mean ± SD, with the asterisk indicating significant differences between treatments in a specific sampling point (Mann-Whitney U Test, $P= 0.004$ and $P< 0.001$ for the 2nd and 3rd samplings respectively) while letters show differences between samplings of the same treatment (Kruskal Wallis, $P<0.001$).

3.5. Sex Steroids Determination

Testosterone plasma concentration in *S. maximus* bloodstream showed no influence under dietary treatment in both sampling points (Two-way ANOVA; $P>0.05$) (Figure 3.9). In the case of sole, at the third sampling point, the T concentration was significant higher (Two-way ANOVA, Tukey HSD, $P<0.001$) in fish fed with the algae supplemented diet in comparison with control (1.4 ± 0.46 *vs* 0.9 ± 0.23 ng/ml, respectively), showing the inverse pattern at the 5th sampling point (1.3 ± 0.55 *vs* 1.8 ± 0.48 ng/ml) (Figure 3.10). When comparing samplings of the same treatment, a pattern of significant increase (Two-way ANOVA; $P<0.001$) in T concentration could be seen from the 1st to the 2nd sampling for the algae fed fish, while control presented a similar increase but only from the second to the third sampling.

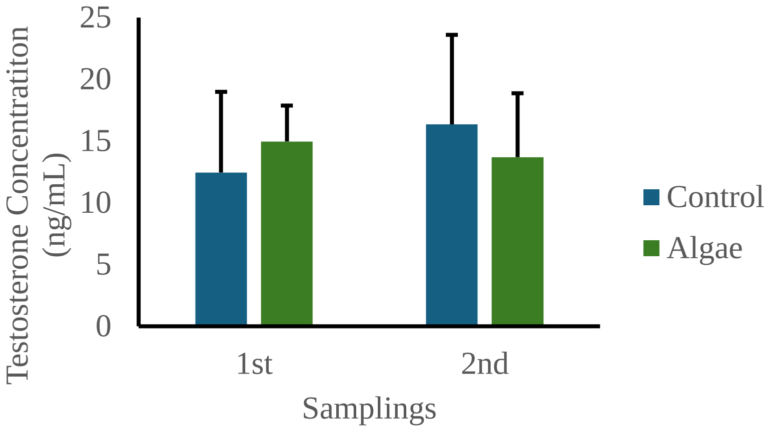


Figure 3.9: Plasma levels of Testosterone determined in *S. maximus* blood plasma from individuals fed on a control (n=12) or algae (n=6) supplemented diet. Results were obtained for the 1st (April) and 2nd (May) samplings. Data is shown as mean ± SD, with no significant differences being found (Mann-Whitney U Test, P>0.05).

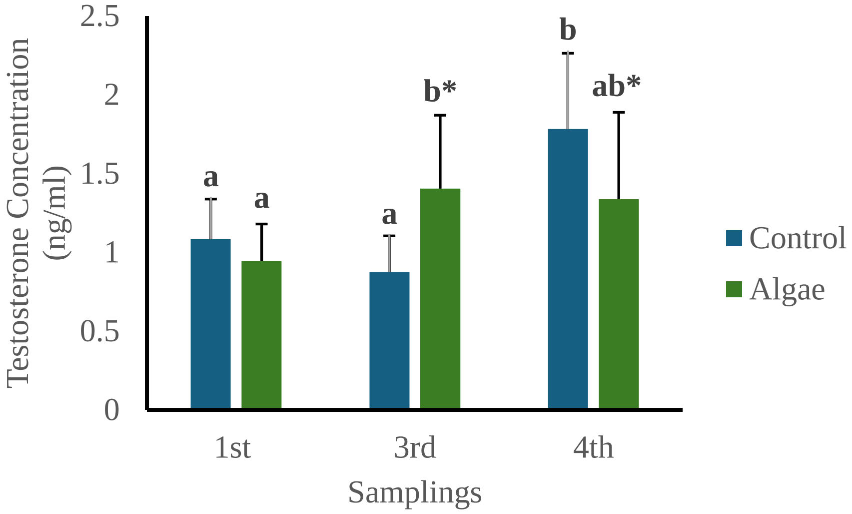


Figure 3.10: Plasma levels of Testosterone determined in *S. senegalensis* blood plasma from individuals fed on a control (n=11-12) or algae (n=12) supplemented diet. Results were obtained for the 1st (19/04/23), 3rd (22/05/23) and 4th (13/06/23) samplings. Data is shown as mean ± SD, with the asterisk indicating significant differences between treatments at a specific sampling point while letters show differences between samplings within the same treatment (Two-way ANOVA, Tukey HSD, P<0.001).

The circulating levels of 11-KT in the blood plasma in turbot presented significant higher concentration from those fed the algae diet (6.01 ± 4.24 ng/ml; Mann-Whitney U Test, P<0.05) in the 1st sampling when comparing with control fed specimens (Figure 3.11). Moreover, a significant decrease (Mann-Whitney U Test, P<0.05) in the concentration of 11-KT could be seen from 1st to 2nd samplings in the algae fed group, with a significant (Mann-Whitney U Test, $P<0.05$) inverse pattern being seen in fishes fed the control diet. Senegalese sole showed no significant differences (Two-way ANOVA, $P>0.05$) between treatments throughout the three samplings (Figure 3.12). However, a significant decrease in the blood plasma levels of 11-KT could be seen along the samplings for the control group and between the first and both second and third samplings of the algae fed group (Two-way ANOVA, Tukey HSD, $P<0.001$*)*.

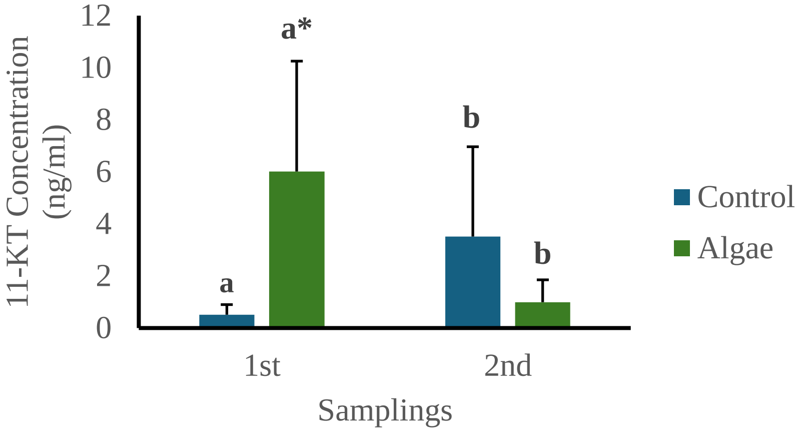


Figure 3.11: Plasma levels of 11-KT determined in *S. maximus* blood plasma from individuals fed on a control (n=12) or algae (n=6) supplemented diet. Results were obtained for the 1st (April) and 2nd (May) samplings. Data is shown as mean ± S.D. with the asterisk indicating significant differences between treatments at a specific sampling point while letters show differences between samplings within the same treatment (Mann-Whitney U Test, $P>0.05$).

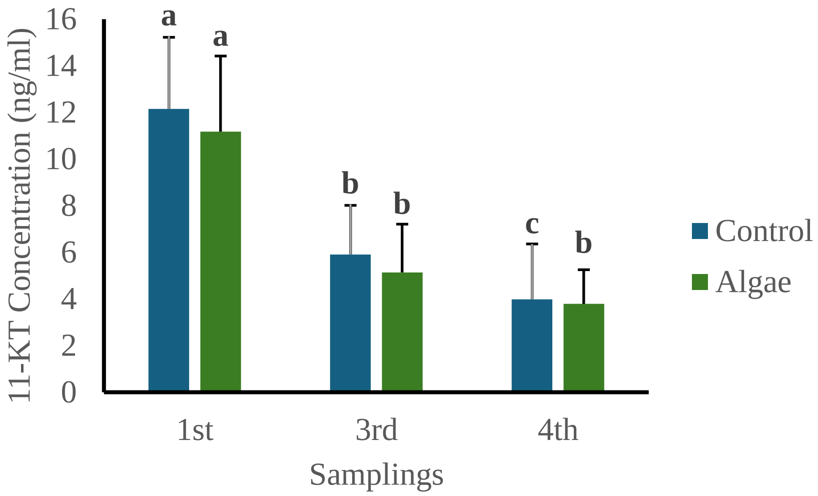


Figure 3.12: Plasma levels of 11-KT determined in *S. senegalensis* blood plasma from individuals fed on a control (n=10-12) or algae (n=11-12) supplemented diet. Results were obtained for the 1st (19/04/23), 3rd (22/05/23) and 4th (13/06/23) samplings. Data is shown as mean ± S.D. with letters showing significant differences between samplings within the same treatment (Two-way ANOVA, Tukey HSD, $P<0.001$).

4. Discussion

In aquaculture, gonadal maturation, gamete quality and larvae survival are affected by the presence or absence of essential compounds, such as vitamins and carotenoids (Hernandez de-Dios et al., 2022; Izquierdo et al., 2001). Not only do these compounds offer protection to the sperm during spermatogenesis and fertilization, but also boosts the animal's antioxidant system (Izquierdo et al., 2001; Kang et al., 2013). Algae present themselves as a good supplementation option when looking into sperm quality as they are rich in the compounds addressed before (Abu-Ghannam & Rajauria, 2013; Wan et al., 2019). Although most of the literature relies on the application of one single algae in the diets, the present study looked into the potential effects of algae blends. With this objective, several techniques were used to assess the effects on sperm quality, oxidative stress and hormonal levels. In turbot, the effect of the algae supplementation elicited a positive effect by lowering DNA fragmentation and improving spermatozoa velocity. Additionally, no benefits could be seen in other sperm kinetic parameters, oxidative stress markers and hormone levels, with the differences found possibly revealing variances in stages in the reproductive cycle. Differently, sole sperm kinetic parameters and hormonal levels did not suffer any effects from the algae blend, but potential benefits could be seen in terms of lipid peroxidation.

Concerning sperm kinetic parameters, turbot fed on the algae diet lowered these values from the first to the second sampling point, while also presenting lower motility than control at the second sampling. This decrease might be explained by a possible shortening in the spawning season related to the supplementation with *L. digitata* and spirulina. Sperm parameters are known to have oscillations during the spawning season on species with an annual reproductive cycle. In turbot it has already been proven that sperm motility presents an increase at the beginning of the reproductive season, with a decrease in kinetic values as the season progresses (Suquet et al., 1998). Similar oscillation patterns have previously been reported for species like Senegalese sole (Beirão et al., 2011) and brown trout (Salmo trutta) (Hajirezaee et al., 2010). Thus, it is possible that at the second sperm sampling, the algae fed group were already in a pattern of quality descent, related to the end of the spawning season, putatively detailing a shortening of the reproductive cycle. To further support this hypothesis both the VCL and VSL at the first sampling were higher on algae supplemented turbot, representing the possible quality peak. Moreover, the time-frame in the turbot experiment might require additional samplings as when dealing with gonadal maturation and broodstock nutrition results might only be visible in a longer time spawn (Izquierdo et al., 2001).

Under the influence of the algae blend, *S. senegalensis* sperm kinetic parameters exhibited a typical pattern of increase until reaching a peak, followed by a decrease. The same pattern was seen in the control group. This was in accordance with other studies, as several parameters of semen quality, such as motility, are known to increase during the spawning period achieving its highest point between March and May, followed by a decrease (Beirão et al., 2011). This decrease during the spawning season was also observed in other teleost such as seabass (*Dicentrarchus labrax*) (Rainis et al., 2003) and in two flatfish species, turbot (Suquet et al., 1998) and halibut (*Hippoglossus hippoglossus*) (Babiak et al., 2006). When comparing the effects of both diets, fish fed with the algae blend were negatively influenced in terms of PM and VCL at the 4th and 3rd sampling, respectively. This indicates that by the end of May the algae fed fish were already experiencing a decrease pattern in sperm quality possibly indicating that the algae blend elicited a shortening of the spawning season. Both these parameters directly influence fertilization, as slow and nonlinear sperm lead to the unlikelihood of achieving fertilization after 1 minute of ejaculation (Beirão et al., 2009; Casselman et al., 2006; Rudolfsen et al., 2005). Similarly to our results, Hassaan, (2022) found no differences in sperm motility between Nile tilapia fed a commercial control diet and those fed a diet supplemented with *Cyclotella* spp. However, Félix et al., (2024) experimented the inclusion of two different macroalgae, *Sargassum vulgare* and *Plocamium cartilagineum* and observed a significant increase in TM and sperm viability with 5 % inclusion of *P. cartilagineum*, by the end of the experiment, corresponding to the end of the spawning season. Typically, higher motility can be related to sperm cells having a richer intracellular ATP content, but our diet might not be optimal to deliver the essential compounds to partake these changes (Kowalski & Cejko, 2019; Suquet et al., 2012). In future studies it would be interesting to test this hypothesis by measuring the ATP content of the sperm cell throughout the spawning cycle, when comparing dietary treatments.

The imbalance between oxidant and antioxidant status is known to result in oxidative stress which, among several consequences, produces cell degradation through the action of radical oxygen species or its by-products such as MDA (Passos et al., 2021). The antioxidant response of the organisms is therefore an essential parameter to analyze, leading us to evaluate two important indicators, lipid peroxidation (through MDA levels) and TAS. Lipid peroxidation represents the chain reaction which causes cellular damage via the oxidative degradation of lipids (Gutteridge & Halliwell, 1990) and TAS displays the overall ability of the organism to neutralize ROS and fight against oxidative stress (Silvestrini et al., 2023). Both these indicators are directly connected, since a consequential increase in TAS rises cellular protection by antioxidant enzymes and may lead to a decrease in lipid peroxidation levels, as they fight against oxidative stress (Marmelo et al., 2024). In both species, the diet supplementation with algae had the objective of reducing ROS effects in spermatozoa, as the vitamins and carotenoids present in the selected algae are biological agents, known to contribute to the antioxidant activity against reactive oxygen species (Martin-Hidalgo et al., 2019; Sandoval-Vargas et al., 2021; Valentão et al., 2010). However, this effect is not noticeable when looking at *S. maximus* sperm lipid peroxidation, which showed significant higher concentration of MDA in sperm from turbot fed on spirulina and *L. digitata* in the first sampling, while no significant differences were observed on TAS. When considering both algae supplemented in turbot diet, spirulina is in fact the most studied one on its effects on oxidative stress, although with few reports of its effects on broodstock.

Mahmoud et al., (2018) recorded lower MDA levels in Nile tilapia serum, subjected to a challenge trial, when fed a diet supplemented with 1 % spirulina compared with a control diet or 2 % inclusion. Besides, Teimouri et al., (2019) tested increasing percentages of spirulina inclusion (0, 2.5, 5, 7.5 and 10 %) on rainbow trout feeds and observed an increase in TAS with the increase in spirulina, coupled with a decrease in LPO in the liver in the two highest inclusions. Additionally, Carneiro et al., (2022) preformed a full replacement of fish meal with spirulina on pacu (*Piaractus mesopotamicus*) and observed an increase in the hepatic activity of antioxidant enzymes such as CAT and SOD, while also denoting a rise in TAS. All these results suggested that spirulina, through its compounds such as Β-carotenes and phycocyanin should have an active role in the activation of the antioxidant system in order to prevent oxidative damage (Teimouri et al., 2019; Wu et al., 2016). Although the previous works seems to demonstrate that spirulina potentiates the hepatic antioxidant system, in the present study the analysis were performed on semen or blood plasma as we chose to use non-lethal methods on our broodstock. Therefore, future studies on broodstock might want to broaden the range of tissues and/or body fluids analyzed to reach better conclusions.

The turbot supplemented diet also contained *Laminaria digitata*. Several studies report positive and negative effects of the inclusion of *L. digitata* in the diet, being mostly dose dependent. Yin et al., (2014) experimented three inclusion rates for laminarin in the diet (0.5%, 1% and

1.5%) and observed that only the two lower inclusions offered a positive modulation of the immune response in orange-spotted grouper (*Epinephelus coioides*). Similarly, Abdel-Mawla et al., (2023) did not observe significant differences in MDA levels of thin lip grey mullet (*Liza ramada*) serum between control and its lowest and highest inclusion (200 and 800 mg/kg, respectively), while Wu et al., (2023) completed a 28 day feeding trial in juvenile largemouth bass (*Micropterus salmoides*) and observed that when supplementing with a medium (10g/kg) or high (15g/kg) level of laminarin, fish displayed a decreased antioxidant capacity. This led us to hypothesize that the beneficial inclusion of laminarin in the diets might have a species-specific range and, in our case, the supplemented 1 % might not be enough to produce a positive effect in this species. Other hypothesis might be a non-synergistic effect of both algae when combined. Batista et al., (2020) studied the effect of *G. gracilis* and *Nannochloropsis oceanica* isolated and as a blend, when supplemented in seabass diet. The results showed a significant lower activity of GPx (glutathione peroxidase), an antioxidant enzyme, when algaes were used in a blend comparing with the supplementation of a single algae. These results suggests that our algae blend might not be an optimal combination to elicit an antioxidant effect.

In sole, the inclusion of *Gracilaria gracilis* and *Phaeodactylum tricornutum* in the feed promoted an effective antioxidant response, when compared with the control, but just at the last sampling. At this point, MDA levels were significantly lower than control, while TAS was significantly higher. This goes in accordance with other authors such as, Hoseinifar et al., (2018), which verified in zebrafish that the inclusion of 1 % *Gracilaria gracilis* resulted in a significant rise in the expression of antioxidant enzyme genes, providing a positive effect on its antioxidant defense. In another study, Peixoto et al., (2016) also found an improvement of seabass redox status with 2.5 % and 7.5 % inclusion of *Gracilaria* spp in the diet. On the other hand, no other study has focused on the effect of supplementation of *P. tricornutum* on lipid peroxidation or TAS, having most of the authors focused on the immunostimulation against a challenge, on fish meal replacement or growth and feed utilization (González et al., 2012; Reis et al., 2021; Sørensen et al., 2016). The lack of more specific effects on the improvement of sperm quality in both species could be associated with the type of inclusion of our algae in the diet, which were included as a whole. However, recent studies made by Barreto et al., (2021) who tested different percentages of inclusion of both whole microalgae and broken cells, observed no differences in oxidative stress. Nevertheless, these authors made further analysis comparing the same oxidative stress results between smaller larvae (with less than 11.5 mg of dry weight) *vs* heavier larvae (with more than 11.5 mg of dry weight), and found out that lipid peroxidation was significantly higher on smaller larvae. Our results showing an improvement of antioxidant status by the end of the experiment, are in line with Barreto et al., (2021) hypothesis, demonstrating that *P. tricornutum* may be more adequate for older life stages, as it has already shown improvements for juveniles of other species (Cerezuela et al., 2012; Sørensen et al., 2016).

As Cabrita et al., (2014) showed, spermatozoa from wild-reared sole breeders are highly susceptible to ROS actions, producing a negative influence in the quality of sperm. However, our results might not give the full image on lipid peroxidation as the MDA assay kit indicates that in the possible presence of hydrogen peroxide (H_2O_2), MDA concentration can be reduced until 13% (Gérard-Monnier et al., 1998). The presence of hydrogen peroxide, a ROS, also signals lipid oxidation activity as it is part of the lipid peroxidation chain reaction, originated from the decomposition of 0^{2-} by the superoxide dismutase (SOD) and being further broke down into H_2O and O_2 by catalase (CAT) (Halliwell & Gutteridge, 2015). For further studies it will be important to consider a more thorough assessment of the antioxidant enzymes such as SOD, glutathione peroxidase (GPx) and redutase (GSR) and CAT, giving a deeper analysis on other ROS generation pathways.

As previously indicated, the production of ROS by sperm cells is considered a normal physiological process, but in the case it is decompensated can lead to cell damage, loss of motility, infertility and even DNA damage (Lahnsteiner et al., 2010). Likewise, DNA integrity and stability is considered a priority, as the transmission of genetic information to the embryo is the main goal of spermatozoa (Cabrita et al., 2019). In our experiment, both the control and algae diets used for turbot resulted in a reduction of sperm DNA fragmentation from the first to the second samplings. Moreover, the fish fed on algae diet displayed significantly lower fragmentation on both occasions when compared with control. This may be due to an improvement of the plasma membrane integrity of the sperm cell through the supplementation of vitamin E, which is known to be present in both algae (Costa et al., 2022; Göksan, 2006).

Jeuthe et al., (2022) observed a moderate correlation between DNA fragmentation and membrane integrity. These findings together with the results of Xu et al., (2015) showing improvements of plasma membrane integrity in turbot fed a diet supplemented with dietary vitamin E, corroborate our hypothesis on why we observed higher DNA integrity in turbot fed the algae blend diet. On the other hand, Senegalese sole presented a gradual increase in DNA fragmentation, except for the third sampling (end of May), where both control and algae groups showed a significant decrease. This data goes in accordance with Beirão et al., (2011) results, where he observed a lower DNA fragmentation for the same period (May) when compared with the end of spawning season (June). As sperm undergoes an aging process it is normal to find an increase in chromatin decondensation, where the extracted sperm cells might be overripe or even apoptotic (Suquet et al., 1998). Another important factor to take into account when analyzing DNA integrity, is the effect of breeders rearing temperature as it has already been proven to affect this parameter in mammalians sperm (Banks et al., 2005). When analysing our results for DNA fragmentation the former is confirmed as fragmentation increases with the end of the spawning season, which is also the beginning of summer, where mean temperatures tend to increase.

Gonadal steroid hormones have key functions in the regulation of the reproductive cycle. Two of the most important androgens are testosterone, which takes the role of promoting spermatogonia proliferation, and 11-KT, by mainly regulating gamete final maturation. In turbot, authors hypothesized that T might be the dominant hormone when compared to 11-KT, since this hormone had lower levels than T and no significant changes throughout the spawning cycle, comparing with other species (Feng et al., 2018). Additionally, the same authors correlated 11-KT levels with sperm motility and found that turbot males which presented sperm with lower activity had higher levels of 11-KT hormone (Feng et al., 2018). These results differ from the obtained in our study, where sperm motility from turbot fed on algae diet followed the same pattern as the 11-KT hormone, lowering from the first to the second sampling, while T concentration showed no variations. When looking at results from other turbot species, Reyes et al., (2012) observed in California horny head turbot males (*Pleuronichthys verticalis*) a seasonal peak in 11-KT in the spring, the spawning season, with a significant decrease towards summer, while in *Scophthalmus rhombus* Hachero-Cruzado et al., (2013) had similar results to ours, with significant decrease in 11-KT levels starting at the end of April. This leads us to the hypothesis mentioned before, that in fact the algae diet shortened the reproductive cycle. The peak in hormonal levels observed, in the turbot fed the algae diet, at the first sampling, with a sharp decline afterwards, indicates the ending of the spawning season at the second sampling, and agrees with the oscillation of motility parameters.

Cabrita et al., (2011) observed a seasonal variation of sex steroids in sole during the year displaying a peak for both 11-KT and T by March, followed by a decrease until the end of the spawning season (June). Our results for 11-KT agree with the information relayed before, where although no significant differences were found between treatments, we can clearly see a decrease in hormonal levels from the beginning of the experiment until its end. Regarding treatments, we observed significantly higher T levels in algae supplemented sole *vs* control at the middle of the experiment, and significantly lower levels than the control by the end of this period. The concentration of T was also higher when compared with another study performed in Senegalese sole by Félix et al., (2024), being more in line with the values reported by Oliveira et al., (2020) in males from the same species treated with kisspeptin hormone. This might mean that the supplementation through our algae blend did elicit an effect on the T levels, indicating again a possible shortening of the sex steroids cycle during the reproductive season, as testosterone peaked earlier than control.

Altogether this study allowed us to gain insights regarding the effects of algae blend supplementation in the gamete quality of flatfish species, showing the algae species used as a promising supplement to use in broodstock aquafeeds. Future research should have into account the percentage of inclusion of each alga, having in mind the compounds that will positively interact with gamete quality, in order to understand the species-specific effects of an algae blend. Moreover, additional analysis such as antioxidant enzymes and ATP levels on sperm, should be made to have a better understanding of the algae effects in the reproductive cycle.

5. Conclusion

The supplementation of Spirulina and *L. digitata* in *S. maximus* feed elicited the following responses:

- Positive impact in sperm quality in terms of sperm velocity and DNA fragmentation.
- Oxidative stress markers did not show any improvement.
- Sex steroids levels oscillation, specifically 11-KT, may indicate a shortening of the reproductive cycle.

In the future, for turbot, additional samplings should be done to obtain the full picture of the spawning season oscillations, while having into account the species specific effects of algae compounds.

The supplementation of *P. tricornutum* and *G. gracilis* in *S. senegalensis* feed elicited the following responses:

- No impact on sperm kinetic parameters was seen, except for VCL and PM, presenting a possible lack of ATP delivered by the diet.
- A positive effect on oxidative stress markers was seen, with the reduction of MDA and improvement of TAS.
- DNA fragmentation presented a normal oscillation previously seen by other authors during the spawning season.
- Testosterone levels in the blood plasma decrease sooner on the algae group than control, possibly indicating a shortening of the sex steroid cycle

For further studies a more thorough assessment of the antioxidant enzymes and ATP levels in sperm of S. sole should be made.

6. References

Abdelkhalek, N. K. M., Ghazy, E. W., & Abdel-Daim, M. M. (2015). Pharmacodynamic interaction of Spirulina platensis and deltamethrin in freshwater fish Nile tilapia, *Oreochromis niloticus*: Impact on lipid peroxidation and oxidative stress. *Environmental Science and Pollution Research*, *22*(4), 3023–3031. https://doi.org/10.1007/s11356-014-3578-0

Abdel-Mawla, M. S., Magouz, F. I., Khalafalla, M. M., Amer, A. A., Soliman, A. A., Zaineldin, A. I., Gewaily, M. S., & Dawood, M. A. O. (2023). Growth performance, intestinal morphology, blood biomarkers, and immune response of Thinlip Grey Mullet (*Liza ramada*) fed dietary laminarin supplement. *Journal of Applied Phycology*, *35*(4), 1801–1811. https://doi.org/10.1007/s10811-023-02973-6

Abdel-Tawwab, M., Harikrishnan, R., Devi, G., Bhat, E. A., & Paray, B. A. (2024). Stimulatory effects of seaweed *Laminaria digitata* polysaccharides additives on growth, immune-antioxidant potency and related genes induction in Rohu carp (*Labeo rohita*) during *Flavobacterium columnare* infection. *Aquaculture*, *579*, 740253. https://doi.org/10.1016/j.aquaculture.2023.740253

Abu-Ghannam, N., & Rajauria, G. (2013). 8—Antimicrobial activity of compounds isolated from algae. In H. Domínguez (Ed.), *Functional Ingredients from Algae for Foods and Nutraceuticals* (pp. 287–306). Woodhead Publishing. https://doi.org/10.1533/9780857098689.2.287

Ahmadi, S., Bashiri, R., Ghadiri-Anari, A., & Nadjarzadeh, A. (2016). Antioxidant supplements and semen parameters: An evidence based review. *International Journal of Reproductive Biomedicine*, *14*(12), 729–736.

Akhoundian, M., Salamat, N., Savari, A., Movahedinia, A., & Salari, M. A. (2020). Influence of photoperiod and temperature manipulation on gonadal development and spawning in Caspian roach (*Rutilus rutilus caspicus*): Implications for artificial propagation. *Aquaculture Research*, *51*(4), 1623–1642. https://doi.org/10.1111/are.14509

Amaro, H. M., Rato, A., Matias, D., Joaquim, S., Machado, J., Gonçalves, J. F. M., Vaz-Pires, P., Ozorio, R. O. A., Pereira, L. F., Azevedo, I. C., Sousa-Pinto, I., & Catarina Guedes, A. (2019). Alga diet formulation – An attempt to reduce oxidative stress during broodstock conditioning of Pacific oysters. *Aquaculture*, *500*, 540–549. https://doi.org/10.1016/j.aquaculture.2018.10.060

APROMAR. (2022). Aquaculture in Spain 2022. Asociasión Empresarial de Acuicultura de España. www.apromar.es

Aydin, İ., POLAT, H., Küçük, E., & Ozdemir, M. (2020). *Turbot and flounder aquaculture* (pp. 106–126).

Babiak, I., Ottesen, O., Rudolfsen, G., & Johnsen, S. (2006). Quantitative characteristics of Atlantic halibut, *Hippoglossus hippoglossus* L., semen throughout the reproductive season. *Theriogenology*, *65*(8), 1587–1604. https://doi.org/10.1016/j.theriogenology.2005.09.004

Banks, S., King, S. A., Irvine, D. S., & Saunders, P. T. K. (2005). *Impact of a mild scrotal heat stress on DNA integrity in murine spermatozoa*. https://doi.org/10.1530/rep.1.00531

Barreto, A., Pinto, W., Rodrigues, A., Rocha, R. J. M., Unamunzaga, C., Silva, T., Dias, J., & Conceição, L. E. C. (2021). *Phaeodactylum tricornutum* biomass in microdiets enhances Senegalese sole (*Solea senegalensis*) larval growth performance during weaning. *Journal of Applied Phycology*, *33*(4), 2233–2240. https://doi.org/10.1007/s10811-021-02444-w

Batista, S., Pereira, R., Oliveira, B., Baião, L. F., Jessen, F., Tulli, F., Messina, M., Silva, J. L., Abreu, H., & Valente, L. M. P. (2020). Exploring the potential of seaweed *Gracilaria gracilis* and microalga *Nannochloropsis oceanica*, single or blended, as natural dietary ingredients for European seabass *Dicentrarchus labrax*. *Journal of Applied Phycology*, *32*(3), 2041–2059. https://doi.org/10.1007/s10811-020-02118-z

Beirão, J., Cabrita, E., Soares, F., Herráez, M. P., & Dinis, M. T. (2008). Cellular damage in spermatozoa from wild-captured *Solea senegalensis* as detected by two different assays: Comet analysis and Annexin V–Fluorescein staining. *Journal of Applied Ichthyology*, *24*(4), 508–513. https://doi.org/10.1111/j.1439-0426.2008.01144.x

Beirão, J., Soares, F., Herráez, M. P., Dinis, M. T., & Cabrita, E. (2009). Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level. *Theriogenology*, *72*(9), 1251–1261. https://doi.org/10.1016/j.theriogenology.2009.07.021

Beirão, J., Soares, F., Herráez, M. P., Dinis, M. T., & Cabrita, E. (2011). Changes in *Solea senegalensis* sperm quality throughout the year. *Animal Reproduction Science*, *126*(1), 122–129. https://doi.org/10.1016/j.anireprosci.2011.04.009

Bhat, R. A., Saini, S., Saoca, C., Maricchiolo, G., & Fazio, F. (2022). Analysis of fatty acids and sex steroid hormones in rainbow trout testes (*Oncorhynchus mykiss*) during the reproductive process. *Aquaculture Research*, *53*(12), 4426–4436. https://doi.org/10.1111/are.15940

Bjørndal, T., Guillen, J., & Imsland, A. (2016). The potential of aquaculture sole production in Europe: Production costs and markets. *Aquaculture Economics & Management*, *20*(1), 109–129. https://doi.org/10.1080/13657305.2016.1124939

Borg, B. (1994). Androgens in teleost fishes. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, *109*(3), 219–245. https://doi.org/10.1016/0742-8413(94)00063-G

Butts, I. A. E., Babiak, I., Ciereszko, A., Litvak, M. K., Słowińska, M., Soler, C., & Trippel, E. A. (2011). Semen characteristics and their ability to predict sperm cryopreservation potential of Atlantic cod, *Gadus morhua L. Theriogenology*, *75*(7), 1290–1300. https://doi.org/10.1016/j.theriogenology.2010.11.044

Cabrita, E., Martínez-Páramo, S., Gavaia, P. J., Riesco, M. F., Valcarce, D. G., Sarasquete, C., Herráez, M. P., & Robles, V. (2014). Factors enhancing fish sperm quality and emerging tools for sperm analysis. *Aquaculture*, *432*, 389–401. https://doi.org/10.1016/j.aquaculture.2014.04.034

Cabrita, E., Riesco, M., & Mañanós, E. (2019). *Sperm Physiology and Artificial Fertilization* (pp. 142–168). https://doi.org/10.1201/9781315120393-8

Cabrita, E., Soares, F., Beirão, J., García-López, A., Martínez-Rodríguez, G., & Dinis, M. T. (2011). Endocrine and milt response of Senegalese sole, *Solea senegalensis*, males maintained in captivity. *Theriogenology*, *75*(1), 1–9. https://doi.org/10.1016/j.theriogenology.2010.07.003

Cabrita, E., V, R., & Herráez, M. (2009). *Methods in Reproductive Aquaculture: Marine and Freshwater Species, Cabrita, E., Robles V., Herraez, M.P. (Eds.)* (Vol. 1, pp. 93–148). Taylor and Francis.

Capillo, G., Savoca, S., Costa, R., Sanfilippo, M., Rizzo, C., Lo Giudice, A., Albergamo, A., Rando, R., Bartolomeo, G., Spanò, N., & Faggio, C. (2018). New Insights into the Culture Method and Antibacterial Potential of *Gracilaria gracilis*. *Marine Drugs*, *16*(12), Article 12. https://doi.org/10.3390/md16120492

Caputo, V., Candi, G., Colella, S., & Arneri, E. (2001). Reproductive biology of turbot (*Psetta maxima*) and brill (*Scophthalmus rhombus*) (Teleostei, Pleuronectiformes) in the Adriatic Sea. *Italian Journal of Zoology*, *68*(2), 107–113. https://doi.org/10.1080/11250000109356393

Carneiro, W. F., Castro, T. F. D., Reichel, T., de Castro Uzeda, P. L., Martínez-Palacios, C. A., & Murgas, L. D. S. (2022). Diets containing *Arthrospira platensis* increase growth, modulate lipid metabolism, and reduce oxidative stress in pacu (*Piaractus* *mesopotamicus*) exposed to ammonia. *Aquaculture*, *547*, 737402. https://doi.org/10.1016/j.aquaculture.2021.737402

Casselman, S. J., Schulte-Hostedde, A. I., & Montgomerie, R. (2006). Sperm quality influences male fertilization success in walleye (*Sander vitreus*). *Canadian Journal of Fisheries and Aquatic Sciences*, *63*(9), 2119–2125. https://doi.org/10.1139/f06-108

Cerdà, J., & Manchado, M. (2013). Advances in genomics for flatfish aquaculture. *Genes & Nutrition*, *8*(1), 5–17. https://doi.org/10.1007/s12263-012-0312-8

Cerezuela, R., Guardiola, F. A., González, P., Meseguer, J., & Esteban, M. Á. (2012). Effects of dietary *Bacillus subtilis*, *Tetraselmis chuii*, and *Phaeodactylum tricornutum*, singularly or in combination, on the immune response and disease resistance of sea bream (*Sparus aurata L.*). *Fish & Shellfish Immunology*, *33*(2), 342–349. https://doi.org/10.1016/j.fsi.2012.05.004

Cerezuela, R., Guardiola, F. A., Meseguer, J., & Esteban, M. Á. (2012). Enrichment of gilthead seabream (*Sparus aurata L.*) diet with microalgae: Effects on the immune system. *Fish Physiology and Biochemistry*, *38*(6), 1729–1739. https://doi.org/10.1007/s10695-012-9670-9

Chereguini, O., De La Banda, I. G., Rasines, I., & Fernandez, A. (1999). Artificial fertilization in turbot, *Scophthalmus maximus* (L.): Different methods and determination of the optimal sperm–egg ratio. *Aquaculture Research*, *30*(5), 319–324. https://doi.org/10.1046/j.1365-2109.1999.00326.x

Costa, M. M., Lopes, P. A., Assunção, J. M. P., Alfaia, C. M. R. P. M., Coelho, D. F. M., Mourato, M. P., Pinto, R. M. A., Lordelo, M. M., & Prates, J. A. M. (2022). Combined effects of dietary *Laminaria digitata* with alginate lyase on plasma metabolites and hepatic lipid, pigment and mineral composition of broilers. *BMC Veterinary Research*, *18*(1), 153. https://doi.org/10.1186/s12917-022-03250-3

Daures, F., Girard, S., Dieudonné, E., & Mardle, S. (2018). *Comparative analysis of production systems in fisheries and aquaculture*. https://archimer.ifremer.fr/doc/00486/59721/

Dinis, M. T., Ribeiro, L., Soares, F., & Sarasquete, C. (1999). A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. *Aquaculture*, *176*(1), 27–38. https://doi.org/10.1016/S0044-8486(99)00047-2

El-Habashi, N., Fadl, S. E., Farag, H. F., Gad, D. M., Elsadany, A. Y., & El Gohary, M. S. (2019). Effect of using Spirulina and Chlorella as feed additives for elevating immunity status of Nile tilapia experimentally infected with *Aeromonas hydrophila*. *Aquaculture Research*, *50*(10), 2769–2781. https://doi.org/10.1111/are.14229

Fajardo, C., Santos, P., Passos, R., Vaz, M., Azeredo, R., Machado, M., Fernández-Boo, S., Baptista, T., & Costas, B. (2023). Early Molecular Immune Responses of Turbot (*Scophthalmus maximus L.*) Following Infection with *Aeromonas salmonicida* subsp. Salmonicida. *International Journal of Molecular Sciences*, *24*(16), Article 16. https://doi.org/10.3390/ijms241612944

Fatsini, E., González, W., Ibarra-Zatarain, Z., Napuchi, J., & Duncan, N. J. (2020). The presence of wild Senegalese sole breeders improves courtship and reproductive success in cultured conspecifics. *Aquaculture*, *519*, 734922. https://doi.org/10.1016/j.aquaculture.2020.734922

Félix, F., Silva, N., Oliveira, C. C. V., Cabrita, E., & Gavaia, P. J. (2024). Effects of dietary supplementation with macroalgae on sperm quality and antioxidant system in Senegalese sole. *Aquaculture*, *590*, 741069. https://doi.org/10.1016/j.aquaculture.2024.741069

Feng, C., Xu, S., Liu, Y., Wang, Y., Wang, W., Yang, J., Zhao, C., Liu, Q., & Li, J. (2018). Progestin is important for testicular development of male turbot (*Scophthalmus maximus*) during the annual reproductive cycle through functionally distinct progestin receptors. *Fish Physiology and Biochemistry*, *44*(1), 35–48. https://doi.org/10.1007/s10695-017-0411-y

Fernández-González, R., Pérez-Pérez, M. I., & Garza-Gil, M. D. (2021). Main issues and key factors for development of turbot aquaculture in Spanish regions: A social-ecological perspective. *Aquaculture*, *544*, 737140. https://doi.org/10.1016/j.aquaculture.2021.737140

Fernández-Palacios, H., Izquierdo, M., Robaina, L., Valencia, A., Salhi, M., & Montero, D. (1997). The effect of dietary protein and lipid from squid and fish meals on egg quality of broodstock for gilthead seabream (*Sparus aurata*). *Aquaculture*, *148*(2), 233–246. https://doi.org/10.1016/S0044-8486(96)01312-9

Ferreira, M., Teixeira, C., Abreu, H., Silva, J., Costas, B., Kiron, V., & Valente, L. M. P. (2021). Nutritional value, antimicrobial and antioxidant activities of micro- and macroalgae, single or blended, unravel their potential use for aquafeeds. *Journal of Applied Phycology*, *33*(6), 3507–3518. https://doi.org/10.1007/s10811-021-02549-2

Francavilla, M., Franchi, M., Monteleone, M., & Caroppo, C. (2013). The Red Seaweed *Gracilaria gracilis* as a Multi Products Source. *Marine Drugs*, *11*(10), Article 10. https://doi.org/10.3390/md11103754

Gallego, V., & Asturiano, J. F. (2018). Sperm motility in fish: Technical applications and perspectives through CASA-Mot systems. *Reproduction, Fertility and Development*, *30*(6), 820–832. https://doi.org/10.1071/RD17460

Garcia-Lopez, A., Anguis, V., Couto, E., Canario, A. V. M., Canavate, J. P., Sarasquete, C., & Martinez-Rodriguez, G. (2006). Non-invasive assessment of reproductive status and cycle of sex steroid levels in a captive wild broodstock of Senegalese sole *Solea senegalensis* (Kaup). *AQUACULTURE*, *254*(1–4), 583–593. https://doi.org/10.1016/j.aquaculture.2005.10.007

Gérard-Monnier, D., Erdelmeier, I., Régnard, K., Moze-Henry, N., Yadan, J. C., & Chaudière, J. (1998). Reactions of 1-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Analytical applications to a colorimetric assay of lipid peroxidation. *Chemical Research in Toxicology*, *11*(10), 1176–1183. https://doi.org/10.1021/tx9701790

Ghadiryanfar, M., Rosentrater, K. A., Keyhani, A., & Omid, M. (2016). A review of macroalgae production, with potential applications in biofuels and bioenergy. *Renewable and Sustainable Energy Reviews*, *54*, 473–481. https://doi.org/10.1016/j.rser.2015.10.022

Göksan, T. (2006). The Effect of the Environmental Factors on the Vitamin C (Ascorbic Acid), E (Alpha-tocopherol), β-carotene Contents and the Fatty Acid Composition of Spirulina platensis. https://www.semanticscholar.org/paper/The-Effect-of-the-Environmental-Factors-on-the-C-E-G%C3%B6ksan/84630f91a64045d0022645ddc2fa4ea4d7bcd8cb

Guo, T., Wang, Y., Li, J., Guo, X., Xu, S., Han, H., Yu, J., Li, J., & Liu, Q. (2024). Accumulated CO2 affects growth, acid-base regulation and ion balance of turbot (*Scophthalmus maximus*) in a recirculating aquaculture system. *Aquaculture*, *578*, 740070. https://doi.org/10.1016/j.aquaculture.2023.740070

Gutteridge, J. M., & Halliwell, B. (1990). The measurement and mechanism of lipid peroxidation in biological systems. *Trends in Biochemical Sciences*, *15*(4), 129–135. https://doi.org/10.1016/0968-0004(90)90206-q

Guzmán, J. M., Norberg, B., Ramos, J., Mylonas, C. C., & Mañanós, E. L. (2008). Vitellogenin, steroid plasma levels and spawning performance of cultured female Senegalese sole (*Solea senegalensis*). *General and Comparative Endocrinology*, *156*(2), 285–297. https://doi.org/10.1016/j.ygcen.2008.02.002

Hachero-Cruzado, I., Forniés, A., Herrera, M., Mancera, J. M., & Martínez-Rodríguez, G. (2013). Sperm production and quality in brill *Scophthalmus rhombus L.*: Relation to circulating sex steroid levels. *Fish Physiology and Biochemistry*, *39*(2), 215–220. https://doi.org/10.1007/s10695-012-9692-3

Hajirezaee, S., Amiri, B. M., & Mirvaghefi, A. R. (2010). Changes in Sperm Production, Sperm Motility, and Composition of Seminal Fluid in Caspian Brown Trout, *Salmo trutta caspius*, Over the Course of a Spawning Season. *Journal of Applied Aquaculture*, *22*(2), 157–170. https://doi.org/10.1080/10454431003736482

Halliwell, B., & Gutteridge, J. M. C. (2015). *Free Radicals in Biology and Medicine*. Oxford University Press.

Hansen, T., Stefansson, S., & Taranger, G. L. (1992). Growth and sexual maturation in Atlantic salmon, *Salmon, salar L*., reared in sea cages at two different light regimes. *Aquaculture Research*, *23*(3), 275–280. https://doi.org/10.1111/j.1365-2109.1992.tb00770.x

Hassaan, M. S. (2022). Effects of Algal Diets Supplementation on Reproductive Performance Parameters of Nile Tilapia Broodstock. *Annals of Agricultural Science, Moshtohor*, *60*(3), 779–786. https://doi.org/10.21608/assjm.2022.257016

Hernandez de-Dios, M. A., Tovar-Ramírez, D., Maldonado García, D., Galaviz-Espinoza, M. A., Spanopoulos Zarco, M., & Maldonado-García, M. C. (2022). Functional Additives as a Boost to Reproductive Performance in Marine Fish: A Review. *Fishes*, *7*(5), Article 5. https://doi.org/10.3390/fishes7050262

Holdt, S. L., & Kraan, S. (2011). Bioactive compounds in seaweed: Functional food applications and legislation. *Journal of Applied Phycology*, *23*(3), 543–597. https://doi.org/10.1007/s10811-010-9632-5

Hoseinifar, S. H., Yousefi, S., Capillo, G., Paknejad, H., Khalili, M., Tabarraei, A., Van Doan, H., Spanò, N., & Faggio, C. (2018). Mucosal immune parameters, immune and antioxidant defence related genes expression and growth performance of zebrafish (*Danio rerio*) fed on *Gracilaria gracilis* powder. *Fish & Shellfish Immunology*, *83*, 232–237. https://doi.org/10.1016/j.fsi.2018.09.046

Hosseini, S. M., Khosravi-Darani, K., & Mozafari, M. R. (2013). Nutritional and medical applications of spirulina microalgae. *Mini Reviews in Medicinal Chemistry*, *13*(8), 1231–1237. https://doi.org/10.2174/1389557511313080009

Howell, B. R. (1997). A re-appraisal of the potential of the sole, *Solea solea* (L.), for commercial cultivation. *Aquaculture*, *155*(1), 355–365. https://doi.org/10.1016/S0044-8486(97)00103-8

Imjongjairak, S., Ratanakhanokchai, K., Laohakunjit, N., Tachaapaikoon, C., Pason, P., & Waeonukul, R. (2016). Biochemical characteristics and antioxidant activity of crude and purified sulfated polysaccharides from *Gracilaria fisheri*. *Bioscience, Biotechnology, and Biochemistry*, *80*(3), 524–532. https://doi.org/10.1080/09168451.2015.1101334

Imsland, A. K., Folkvord, A., Jónsdóttir, Ó. D. B., & Stefansson, S. O. (1997). Effects of exposure to extended photoperiods during the first winter on long-term growth and age at first maturity in turbot (*Scophthalmus maximus*). *Aquaculture*, *159*(1), 125–141. https://doi.org/10.1016/S0044-8486(97)00152-X

Imsland, A. K., Foss, A., Concei□□o, L. E. C., Dinis, M. T., Delbare, D., Schram, E., Kamstra, A., Rema, P., & White, P. (2003). A review of the culture potential of *Solea solea* and *S. senegalensis*. *Reviews in Fish Biology and Fisheries*, *13*(4), 379–408. https://doi.org/10.1007/s11160-004-1632-6

Imsland, A. K., Gunnarsson, S., Roth, B., Foss, A., Le Deuff, S., Norberg, B., Thorarensen, H., & Helming, T. (2013). Long-term effect of photoperiod manipulation on growth, maturation and flesh quality in turbot. *Aquaculture*, *416–417*, 152–160. https://doi.org/10.1016/j.aquaculture.2013.09.005

Izquierdo, M. S., Fernández-Palacios, H., & Tacon, A. G. J. (2001). Effect of broodstock nutrition on reproductive performance of fish. *Aquaculture*, *197*(1), 25–42. https://doi.org/10.1016/S0044-8486(01)00581-6

Jeuthe, H., Palaiokostas, C., & Johannisson, A. (2022). DNA fragmentation and membrane integrity in sperm of farmed Arctic charr (*Salvelinus alpinus*). *Aquaculture*, *547*. https://doi.org/10.1016/j.aquaculture.2021.737537

Jones, A. (1974). Sexual Maturity, Fecundity and Growth of the Turbot *Scophthalmus Maximus L*. *Journal of the Marine Biological Association of the United Kingdom*, *54*(1), 109–125. https://doi.org/10.1017/S0025315400022104

Kadam, S. U., Tiwari, B. K., & O'Donnell, C. P. (2015). Extraction, structure and biofunctional activities of laminarin from brown algae. *International Journal of Food Science & Technology*, *50*(1), 24–31. https://doi.org/10.1111/ijfs.12692

Kang, M.-J., Kim, S. M., Jeong, S.-M., Choi, H.-N., Jang, Y.-H., & Kim, J.-I. (2013). Antioxidant effect of *Phaeodactylum tricornutum* in mice fed high-fat diet. *Food Science and Biotechnology*, *22*(1), 107–113. https://doi.org/10.1007/s10068-013-0015-y

Kassila, J., Nhhala, H., Givernaud, T., Monsouri, M., Abrehouch, A., & Mosfioui, A. (2019). Opportunities for the development of seaweed farming as a supplementary income for small-scale fishermen in Nador lagoon: Experimental cultivations of *Gracilaria gracilis* (Stackhouse). *Mediterranean Fisheries and Aquaculture Research*, *2*(1), Article 1.

Kim, K.-H., Kim, Y.-W., Kim, H. B., Lee, B. J., & Lee, D. S. (2006). Anti-apoptotic Activity of Laminarin Polysaccharides and their Enzymatically Hydrolyzed Oligosaccharides from *Laminaria japonica*. *Biotechnology Letters*, *28*(6), 439–446. https://doi.org/10.1007/s10529-005-6177-9

Kim, S. M., Jung, Y.-J., Kwon, O.-N., Cha, K. H., Um, B.-H., Chung, D., & Pan, C.-H. (2012). A Potential Commercial Source of Fucoxanthin Extracted from the Microalga *Phaeodactylum tricornutum*. *Applied Biochemistry and Biotechnology*, *166*(7), 1843–1855. https://doi.org/10.1007/s12010-012-9602-2

Kime, D. E., & Tveiten, H. (2002). Unusual motility characteristics of sperm of the spotted wolffish. *Journal of Fish Biology*, *61*(6), 1549–1559. https://doi.org/10.1111/j.1095-8649.2002.tb02497.x

Kjørsvik, E., Mangor-Jensen, A., & Holmefjord, I. (1990). Egg Quality in Fishes. In J. H. S. Blaxter & A. J. Southward (Eds.), *Advances in Marine Biology* (Vol. 26, pp. 71–113). Academic Press. https://doi.org/10.1016/S0065-2881(08)60199-6

Kowalski, R. K., & Cejko, B. I. (2019). Sperm quality in fish: Determinants and affecting factors. *Theriogenology*, *135*, 94–108. https://doi.org/10.1016/j.theriogenology.2019.06.009

Kuda, T., Yano, T., Matsuda, N., & Nishizawa, M. (2005). Inhibitory effects of laminaran and low molecular alginate against the putrefactive compounds produced by intestinal microflora in vitro and in rats. *Food Chemistry*, *91*(4), 745–749. https://doi.org/10.1016/j.foodchem.2004.06.047

Lahnsteiner, F., Mansour, N., & Plaetzer, K. (2010). Antioxidant systems of brown trout (*Salmo trutta f. Fario*) semen. *Animal Reproduction Science*, *119*(3), 314–321. https://doi.org/10.1016/j.anireprosci.2010.01.010

Lee, W.-K., & Yang, S.-W. (2002). Relationship between ovarian development and serum levels of gonadal steroid hormones, and induction of oocyte maturation and ovulation in the cultured female Korean spotted sea bass *Lateolabrax maculatus* (Jeom-nong-eo). *Aquaculture*, *207*(1), 169–183. https://doi.org/10.1016/S0044-8486(01)00728-1

Maciel, J. S., Chaves, L. S., Souza, B. W. S., Teixeira, D. I. A., Freitas, A. L. P., Feitosa, J. P. A., & de Paula, R. C. M. (2008). Structural characterization of cold extracted fraction of soluble sulfated polysaccharide from red seaweed *Gracilaria birdiae*. *Carbohydrate Polymers*, *71*(4), 559–565. https://doi.org/10.1016/j.carbpol.2007.06.026

Mahmoud, M. M. A., El-Lamie, M. M. M., Kilany, O. E., & Dessouki, A. A. (2018). Spirulina (*Arthrospira platensis*) supplementation improves growth performance, feed utilization, immune response, and relieves oxidative stress in Nile tilapia (*Oreochromis niloticus*) challenged with Pseudomonas fluorescens. *Fish & Shellfish Immunology*, *72*, 291–300. https://doi.org/10.1016/j.fsi.2017.11.006

Marinho-Soriano, E. (2017). Historical context of commercial exploitation of seaweeds in Brazil. *Journal of Applied Phycology*, *29*(2), 665–671. https://doi.org/10.1007/s10811-016-0866-8

Marmelo, I., Dias, M., Grade, A., Pousão-Ferreira, P., Diniz, M. S., Marques, A., & Maulvault, A. L. (2024). Immunomodulatory and antioxidant effects of functional aquafeeds biofortified with whole *Laminaria digitata* in juvenile gilthead seabream (*Sparus aurata*). *Frontiers in Marine Science*, *11*. https://www.frontiersin.org/articles/10.3389/fmars.2024.1325244

Martín, I., Rasines, I., Gómez, M., Rodríguez, C., Martínez, P., & Chereguini, O. (2014). Evolution of egg production and parental contribution in Senegalese sole, *Solea senegalensis*, during four consecutive spawning seasons. *Aquaculture*, *424–425*, 45–52. https://doi.org/10.1016/j.aquaculture.2013.12.042

Martin-Hidalgo, D., Bragado, M. J., Batista, A. R., Oliveira, P. F., & Alves, M. G. (2019). Antioxidants and Male Fertility: From Molecular Studies to Clinical Evidence. *Antioxidants*, *8*(4), Article 4. https://doi.org/10.3390/antiox8040089

Miao, H.-Q., Ishai-Michaeli, R., Peretz, T., & Vlodavsky, I. (1995). Laminarin sulfate mimics the effects of heparin on smooth muscle cell proliferation and basic fibroblast growth factor-receptor binding and mitogenic activity. *Journal of Cellular Physiology*, *164*(3), 482–490. https://doi.org/10.1002/jcp.1041640306

Miura, T., Yamauchi, K., Takahashi, H., & Nagahama, Y. (1991). Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). *Proceedings of the National Academy of Sciences of the United States of America*, *88*(13), 5774–5778.

Morais, S., Aragão, C., Cabrita, E., Conceição, L. E. C., Constenla, M., Costas, B., Dias, J., Duncan, N., Engrola, S., Estevez, A., Gisbert, E., Mañanós, E., Valente, L. M. P., Yúfera, M., & Dinis, M. T. (2016). New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. *Reviews in Aquaculture*, *8*(3), 227–263. https://doi.org/10.1111/raq.12091

Muller-Feuga, A. (2000). The role of microalgae in aquaculture: Situation and trends. *Journal of Applied Phycology*, *12*(3), 527–534. https://doi.org/10.1023/A:1008106304417

Ngo, D.-H., Wijesekara, I., Vo, T.-S., Van Ta, Q., & Kim, S.-K. (2011). Marine food-derived functional ingredients as potential antioxidants in the food industry: An overview. *Food Research International*, *44*(2), 523–529. https://doi.org/10.1016/j.foodres.2010.12.030

Oliveira, C. C. V., Fatsini, E., Fernández, I., Anjos, C., Chauvigné, F., Cerdà, J., Mjelle, R., Fernandes, J. M. O., & Cabrita, E. (2020). Kisspeptin Influences the Reproductive Axis and Circulating Levels of microRNAs in Senegalese Sole. *International Journal of Molecular Sciences*, *21*(23), Article 23. https://doi.org/10.3390/ijms21239051

Pasquet, V., Chérouvrier, J.-R., Farhat, F., Thiéry, V., Piot, J.-M., Bérard, J.-B., Kaas, R., Serive, B., Patrice, T., Cadoret, J.-P., & Picot, L. (2011). Study on the microalgal pigments extraction process: Performance of microwave assisted extraction. *Process Biochemistry*, *46*(1), 59–67. https://doi.org/10.1016/j.procbio.2010.07.009

Passos, R., Correia, A. P., Pires, D., Pires, P., Ferreira, I., Simões, M., do Carmo, B., Santos, P., Pombo, A., Afonso, C., & Baptista, T. (2021). Potential use of macroalgae *Gracilaria gracilis* in diets for European seabass (*Dicentrarchus labrax*): Health benefits from a sustainable source. *Fish & Shellfish Immunology*, *119*, 105–113. https://doi.org/10.1016/j.fsi.2021.09.033

Peixoto, M. J., Salas-Leitón, E., Pereira, L. F., Queiroz, A., Magalhães, F., Pereira, R., Abreu, H., Reis, P. A., Gonçalves, J. F. M., & Ozório, R. O. de A. (2016). Role of dietary seaweed supplementation on growth performance, digestive capacity and immune and stress responsiveness in European seabass (*Dicentrarchus labrax*). *Aquaculture Reports*, *3*, 189–197. https://doi.org/10.1016/j.aqrep.2016.03.005

Polat, H., Öztürk, R. Ç., Terzi, Y., Aydin, İ., & Küçük, E. (2021). Effect of Photoperiod Manipulation on Spawning Time and Performance of Turbot (Scophthalmus maximus). *Aquaculture Studies*, *21*, 109–115. https://doi.org/10.4194/2618-6381-v21_3_03

Rainis, S., Mylonas, C. C., Kyriakou, Y., & Divanach, P. (2003). Enhancement of spermiation in European sea bass (*Dicentrarchus labrax*) at the end of the reproductive season using GnRHa implants. *Aquaculture*, *219*(1), 873–890. https://doi.org/10.1016/S0044-8486(03)00028-0

Reis, B., Ramos-Pinto, L., Martos-Sitcha, J. A., Machado, M., Azeredo, R., Fernández-Boo, S., Engrola, S., Unamunzaga, C., Calduch-Giner, J., Conceição, L. E. C., Silva, T., Dias, J., Costas, B., & Pérez-Sánchez, J. (2021). Health status in gilthead seabream (*Sparus aurata*) juveniles fed diets devoid of fishmeal and supplemented with *Phaeodactylum tricornutum*. *Journal of Applied Phycology*, *33*(2), 979–996. https://doi.org/10.1007/s10811-021-02377-4

Reyes, J. A., Vidal-Dorsch, D. E., Schlenk, D., Bay, S. M., Armstrong, J. L., Gully, J. R., Cash, C., Baker, M., Stebbins, T. D., Hardiman, G., & Kelley, K. M. (2012). Evaluation of reproductive endocrine status in hornyhead turbot sampled from Southern California's urbanized coastal environments. *Environmental Toxicology and Chemistry*, *31*(12), 2689–2700. https://doi.org/10.1002/etc.2008

Ribeiro, A. R., Gonçalves, A., Barbeiro, M., Bandarra, N., Nunes, M. L., Carvalho, M. L., Silva, J., Navalho, J., Dinis, M. T., Silva, T., & Dias, J. (2017). *Phaeodactylum tricornutum* in finishing diets for gilthead seabream: Effects on skin pigmentation, sensory properties and nutritional value. *Journal of Applied Phycology*, *29*(4), 1945–1956. https://doi.org/10.1007/s10811-017-1125-3

Riesco, M. F., Oliveira, C., Soares, F., Gavaia, P. J., Dinis, M. T., & Cabrita, E. (2017). *Solea senegalensis* sperm cryopreservation: New insights on sperm quality. *PLoS ONE*, *12*(10), e0186542. https://doi.org/10.1371/journal.pone.0186542

Rudolfsen, G., Figenschou, L., Folstad, I., Tveiten, H., & Figenschou, M. (2005). Rapid adjustments of sperm characteristics in relation to social status. *Proceedings of the Royal Society B: Biological Sciences*, *273*(1584), 325–332. https://doi.org/10.1098/rspb.2005.3305

Rurangwa, E., Kime, D. E., Ollevier, F., & Nash, J. P. (2004). The measurement of sperm motility and factors affecting sperm quality in cultured fish. *Aquaculture*, *234*(1), 1–28. https://doi.org/10.1016/j.aquaculture.2003.12.006

Sánchez, E. L. M., Duncan, N., Reiriz, C. S., López, A. G., Guzmán, J. M., & Cueto, J. A. M. (2019). Reproductive Physiology and Broodstock Management of Soles. In *The Biology of Sole*. CRC Press.

Sandoval-Vargas, L., Silva Jiménez, M., Risopatrón González, J., Villalobos, E. F., Cabrita, E., & Valdebenito Isler, I. (2021). Oxidative stress and use of antioxidants in fish semen cryopreservation. *Reviews in Aquaculture*, *13*(1), 365–387. https://doi.org/10.1111/raq.12479

Schulz, R. W., & Goos, H. J. T. (1999). Puberty in male fish: Concepts and recent developments with special reference to the African catfish (*Clarias gariepinus*). *Aquaculture*, *177*(1), 5–12. https://doi.org/10.1016/S0044-8486(99)00064-2

Shiva, M., Gautam, A. K., Verma, Y., Shivgotra, V., Doshi, H., & Kumar, S. (2011). Association between sperm quality, oxidative stress, and seminal antioxidant activity. *Clinical Biochemistry*, *44*(4), 319–324. https://doi.org/10.1016/j.clinbiochem.2010.11.009

Silvestrini, A., Meucci, E., Ricerca, B. M., & Mancini, A. (2023). Total Antioxidant Capacity: Biochemical Aspects and Clinical Significance. *International Journal of Molecular Sciences*, *24*(13), 10978. https://doi.org/10.3390/ijms241310978

Smith, C. E., Osborne, M. D., Piper, R. G., & Dwyer, W. P. (1979). Effect of Diet Composition on Performance of Rainbow Trout Brood Stock during a Three-year Period. *The Progressive Fish-Culturist*, *41*(4), 185–188. https://doi.org/10.1577/1548-8659(1979)41[185:EODCOP]2.0.CO;2

Sørensen, M., Berge, G. M., Reitan, K. I., & Ruyter, B. (2016). Microalga *Phaeodactylum tricornutum* in feed for Atlantic salmon (*Salmo salar*)—Effect on nutrient digestibility, growth and utilization of feed. *Aquaculture*, *460*, 116–123. https://doi.org/10.1016/j.aquaculture.2016.04.010

Suquet, M., Cosson, J., Donval, A., Labbé, C., Boulais, M., Haffray, P., Bernard, I., & Fauvel, C. (2012). Marathon vs sprint racers: An adaptation of sperm characteristics to the reproductive strategy of Pacific oyster, turbot and seabass. *Journal of Applied Ichthyology*, *28*(6), 956–960. https://doi.org/10.1111/jai.12061

Suquet, M., Dreanno, C., Dorange, G., Normant, Y., Quemener, L., Gaignon, J. L., & Billard, R. (1998). The ageing phenomenon of turbot spermatozoa: Effects on morphology, motility and concentration, intracellular ATP content, fertilization, and storage capacities. *Journal of Fish Biology*, *52*(1), 31–41. https://doi.org/10.1111/j.1095-8649.1998.tb01550.x

Svåsand, T., Crosetti, D., García-Vázquez, E., Verspoor, E., Bonhomme, F., Colombo, L., Lapégue, S., Marteinsdottir, G., Olesen, I., & Triantaphyllidis, C. (2007). Evaluation of genetic impact of aquaculture activities on native populations. In *2 s.* [Working paper]. ICES. https://imr.brage.unit.no/imr-xmlui/handle/11250/103238

Teimouri, M., Yeganeh, S., Mianji, G. R., Najafi, M., & Mahjoub, S. (2019). The effect of Spirulina platensis meal on antioxidant gene expression, total antioxidant capacity, and lipid peroxidation of rainbow trout (*Oncorhynchus mykiss*). *Fish Physiology and Biochemistry*, *45*(3), 977–986. https://doi.org/10.1007/s10695-019-0608-3

Tiwari, B. K., & Troy, D. J. (2015). Chapter 1—Seaweed sustainability – food and nonfood applications. In B. K. Tiwari & D. J. Troy (Eds.), *Seaweed Sustainability* (pp. 1–6). Academic Press. https://doi.org/10.1016/B978-0-12-418697-2.00001-5

Ueda, H., Hiroi, O., Hara, A., Yamauchi, K., & Nagahama, Y. (1984). Changes in serum concentrations of steroid hormones, thyroxine, and vitellogenin during spawning migration of the chum slamon, *Oncorhynchus keta*. *General and Comparative Endocrinology*, *53*(2), 203–211. https://doi.org/10.1016/0016-6480(84)90243-0

Valentão, P., Trindade, P., Gomes, D., Guedes de Pinho, P., Mouga, T., & Andrade, P. B. (2010). *Codium tomentosum* and *Plocamium cartilagineum*: Chemistry and antioxidant potential. *Food Chemistry*, *119*(4), 1359–1368. https://doi.org/10.1016/j.foodchem.2009.09.015

Valko, M., Jomova, K., Rhodes, C. J., Kuča, K., & Musílek, K. (2016). Redox- and non-redox-metal-induced formation of free radicals and their role in human disease. *Archives of Toxicology*, *90*(1), 1–37. https://doi.org/10.1007/s00204-015-1579-5

Vanderlei, E., de Araújo, I. W. F., Quinderé, A. L. G., Fontes, B. P., Eloy, Y. R. G., Rodrigues, J. A. G., Silva, A. A. R. e., Chaves, H. V., Jorge, R. J. B., de Menezes, D. B., Evangelista, J. S. A. M., Bezerra, M. M., & Benevides, N. M. B. (2011). The involvement of the HO-1 pathway in the anti-inflammatory action of a sulfated polysaccharide isolated from the red seaweed *Gracilaria birdiae*. *Inflammation Research*, *60*(12), 1121–1130. https://doi.org/10.1007/s00011-011-0376-8

Vázquez, J. A., Rodríguez-Amado, I., Sotelo, C. G., Sanz, N., Pérez-Martín, R. I., & Valcárcel, J. (2020). Production, Characterization, and Bioactivity of Fish Protein Hydrolysates from Aquaculture Turbot (*Scophthalmus maximus*) Wastes. *Biomolecules*, *10*(2), Article 2. https://doi.org/10.3390/biom10020310

Volkoff, H., & London, S. (2018). *Nutrition and Reproduction in Fish*. https://doi.org/10.1016/B978-0-12-809633-8.20624-9

Wan, A. H. L., Davies, S. J., Soler-Vila, A., Fitzgerald, R., & Johnson, M. P. (2019). Macroalgae as a sustainable aquafeed ingredient. *Reviews in Aquaculture*, *11*(3), 458–492. https://doi.org/10.1111/raq.12241

Wang, L., Zhou, H., He, R., Xu, W., Mai, K., & He, G. (2016). Effects of soybean meal fermentation by *Lactobacillus plantarum* P8 on growth, immune responses, and intestinal morphology in juvenile turbot (*Scophthalmus maximus L.*). *Aquaculture*, *464*, 87–94. https://doi.org/10.1016/j.aquaculture.2016.06.026

Wu, Q., Liu, L., Miron, A., Klímová, B., Wan, D., & Kuča, K. (2016). The antioxidant, immunomodulatory, and anti-inflammatory activities of Spirulina: An overview. *Archives of Toxicology*, *90*(8), 1817–1840. https://doi.org/10.1007/s00204-016-1744-5

Wu, Y., Cheng, Y., Qian, S., Zhang, W., Huang, M., Yang, S., & Fei, H. (2023). An Evaluation of Laminarin Additive in the Diets of Juvenile Largemouth Bass (*Micropterus* *salmoides*): Growth, Antioxidant Capacity, Immune Response and Intestinal Microbiota. *Animals*, *13*(3), Article 3. https://doi.org/10.3390/ani13030459

Xu, D.-P., Li, Y., Meng, X., Zhou, T., Zhou, Y., Zheng, J., Zhang, J.-J., & Li, H.-B. (2017). Natural Antioxidants in Foods and Medicinal Plants: Extraction, Assessment and Resources. *International Journal of Molecular Sciences*, *18*(1), Article 1. https://doi.org/10.3390/ijms18010096

Xu, H., Huang, L., Liang, M., Zheng, K., & Wang, X. (2015). Effect of dietary vitamin E on the sperm quality of turbot (*Scophthalmus maximus*). *Journal of Ocean University of China*, *14*(4), 695–702. https://doi.org/10.1007/s11802-015-2555-7

Yin, G., Li, W., Lin, Q., Lin, X., Lin, J., Zhu, Q., Jiang, H., & Huang, Z. (2014). Dietary administration of laminarin improves the growth performance and immune responses in *Epinephelus coioides*. *Fish & Shellfish Immunology*, *41*(2), 402–406. https://doi.org/10.1016/j.fsi.2014.09.027