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ESPECIALIZAÇÃO EM AQUACULURA E PESCAS

NOVEL LIVE FEEDING, WEANING STRATEGIES, AND FUNCTIONAL MICRO FEEDS FOR COD EARLY STAGES: EFFECTS IN HEALTH CONDITION

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Abstract

One of the biggest bottlenecks in Atlantic cod (*Gadus morhua*) farming is guaranteeing larvae and juvenile quality. Nutrition can be a key factor in ensuring larvae's performance. Therefore, the present work aimed to study the implementation of an innovative early feeding protocol, combining live cryopreserved plankton with improved digestibility and novel dry microfeeds. It is hypothesized that such an approach will strengthen larvae during the critical weaning stage, reduce mortalities and stress effects, and improve the overall quality and growth of cod larvae and juveniles while lowering production costs. In this study, 2 different experimental diets (dry micro feeds), differing on the phospholipids source, plant-based or marine, were compared against a commercial diet (COM). For this purpose, 40,000 larvae were transferred to an experimental unit with an aquaculture system composed of 9 tanks with 400 L of seawater. Larvae were fed in a co-feeding regime, live prey, rotifers, and cryo-plankton until 46 days post-hatching (dph). Dry microfeeds were introduced at 22 dph until the end of the trial at 67 dph. Growth performance, survival, and oxidative stress response in whole larvae homogenates were measured. Results revealed stronger potential in the diet with marine phospholipids (MARINE). Cod larvae fed this diet showed higher superoxide dismutase activity and better overall growth performance compared to larvae fed diet CTRL. In contrast, catalase activity and lipids peroxidation did not show any significant differences between dietary treatments. The survival rate of cod larvae fed the experimental microfeeds was relatively low compared to those fed the control diet, which suggests that further investigation in phospholipids-rich diets is still necessary.

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Keywords: Atlantic cod (*Gadus morhua*), larvae, phospholipids, live feeds, growth, oxidative stress

Resumo

Um dos maiores problemas da produção do Bacalhau do Atlântico (*Gadus morhua*), é garantir qualidade das larvas e dos juvenis. A nutrição pode ser um fator chave no desempenho larvar. Sendo assim, o presente trabalho tem como objetivo estudar a implementação de protocolos inovadores de alimentação precoce, combinando com plâncton crio preservado com melhor digestibilidade e novas micro rações secas. Hipotetiza-se que tal abordagem fortalecerá as larvas durante a fase crítica de desmame, reduzindo mortalidades assim como efeitos do stress, e melhorando a qualidade e o crescimento das larvas e juvenis de bacalhau, com custos de produção mais reduzidos. Neste estudo, foram comparadas 2 dietas experimentais diferentes (micro rações secas), que diferem na fonte de fosfolípidos, de origem vegetal ou marinha, em comparação com uma dieta comercial (controlo). Para este fim, 40 000 larvas foram transferidas para uma unidade experimental com um sistema de aquicultura composto por 9 tanques com 400 L de água salgada. As larvas foram alimentadas num regime de co-alimentação, com presas vivas, rotíferos e crio-plâncton até aos 46 dias pós-eclosão (dpe). As micro dietas secas foram introduzidas aos 22 dpe até ao final do ensaio aos 67 dpe. Foram medidos o desempenho de crescimento, a sobrevivência e a resposta ao stress oxidativo em homogeneizados de larvas inteiras. Os resultados revelaram um potencial mais forte na dieta com fosfolípidos marinhos (MARINE). As larvas de bacalhau alimentadas com esta dieta apresentaram uma maior atividade da superóxido dismutase e melhor desempenho de crescimento em comparação com as larvas alimentadas com a dieta CTRL. Em contraste, a atividade da catalase e a peroxidação lipídica não mostraram diferenças significativas entre os tratamentos dietéticos. A taxa de sobrevivência das larvas de bacalhau alimentadas com as micro rações experimentais foi relativamente baixa em comparação com as alimentadas com a dieta de controlo, o que sugere que é necessária uma investigação mais aprofundada em dietas ricas em fosfolípidos.

Palavras-chaves: Bacalhau do Atlântico (*Gadus morhua*), larvas, fosfolípidos, rações vivas, crescimento, stress oxidativo

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Abbreviation list

ACTH- Adrenocorticotropin

ALA- Alpha-linolenic acid

CAT- Catalase

CNS- Central nervous system

CRH- Corticotropin-releasing hormone

Cryo-µ- Blue mussel eggs

Cryo-l- Large Cryo plankton

Cryo-s- Small Cryo plankton

DHA- Docosahexaenoic acid

DNA- Deoxyribonucleic acid

DPH- Days post-hatching

DW- Dry weight

DWSGR- Dry weight-specific growth rate

EFA- Essential fatty acids

EPA- Eicosapentaenoic acid

GSH- Glutathione

GSSG- Glutathione disulfide

GSx- Glutamine synthetase

HPI- hypothalamic-pituitary-interrenal

HUFA- Highly unsaturated fatty acids

LA- Linolenic acid

LPO- Lipid peroxidation

NADPH- Nicotinamide adenine dinucleotide phosphate hydrogen

PC- Phosphatidylcholine

PE- Phosphatidylethanolamine

PI- Phosphatidylinositol

PL- Phospholipids

PS- Phosphatidylserine

PUFA- Polyunsaturated fatty acids

RBA- Respiratory burst activity

ROS- Reactive oxygen species

SD- Standard deviation

SGR- Specific growth rate

SL- Standard length

SLSGR- Standard length specific growth rate

SOD- Superoxide dismutase

TAG- Triacylglycerols

TBARS- Thiobarbituric acid reactive substances

1. Introduction

1.1 Global Aquaculture State

In the last decade, aquatic animal production has been consolidated as the main source of aquatic protein for human consumption, surpassing the fisheries sector with 51 percent of the global output (94.4 million in 2022). Worldwide aquatic animal food consumption has been increasing at an average annual rate of 3.0%, following a global population increase, which is reflected in a rise in consumption per capita (20.6 kg in 2021) (Figure 1). Notice that countries like Portugal and Norway have much higher numbers per capita, above 50 kg (FAO, 2024).

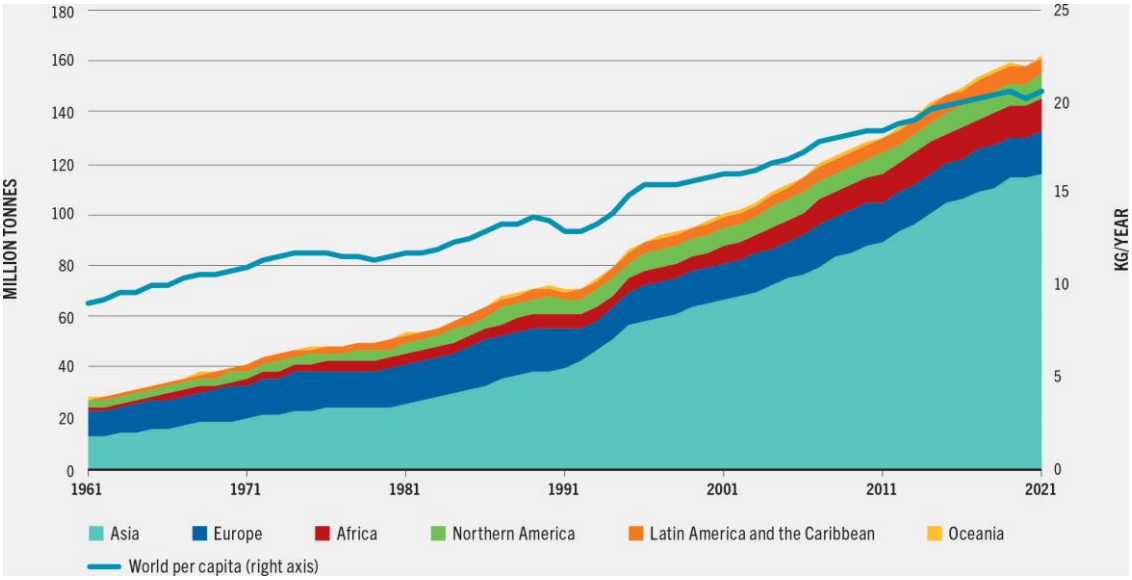

Figure 1 Estimated worldwide consumption of aquatic animal foods from 1961 to 2021 (FAO, 2024), open access.

Aquatic animal products, especially fish, offer plenty of nutritional benefits for humans. They are an excellent source of digestible protein, contributing close to 20% of global animal protein. Fatty fish, like salmon and tuna, hold great nutritional value and are an important source of omega-3 fatty acids for the global population, as well as vitamins (D and B12) and minerals (Fe, I, Zn) (FAO, 2024).

Finfish farming is the most diverse aquaculture sector with 368 species being produced globally. China continues to be the biggest exporter of aquatic animal products (in value), followed by Norway in second place (Figure 2), a reference in European production, which mostly exports salmonids (4th globally most produced group), and other species, like Atlantic cod, Herring, and Mackerel (FAO, 2024).

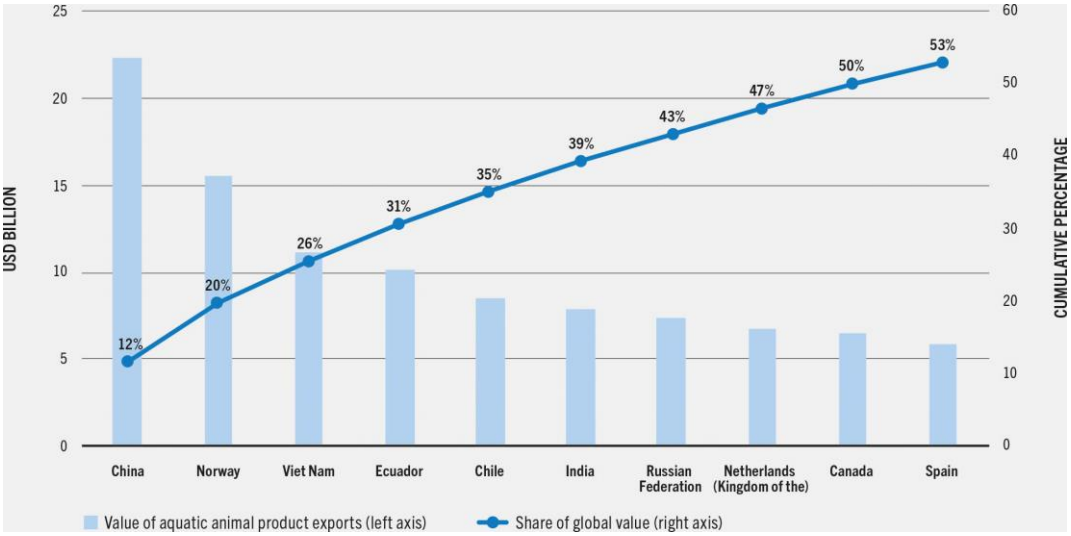

Figure 2 Top exporter countries in value of aquatic animal products, (FAO, 2024), Open access

Due to continuous advances in the industry, aquatic animal production is projected (until 2032) to reach 205 million tons. From 2022 to 2032, an average annual increase of 1.6 percent is predicted, a lower number than the annual 4.0 percent rate of the 2012-2022 period (FAO, 2024).

Recent developments in the sector highlighted the prominence of fish welfare and stress management, so it is essential to consider these factors to achieve a more sustainable aquaculture (Ashley, 2007; Conte, 2004)

1.2 Atlantic cod

The Atlantic cod (*Gadus morhua*) (Figure 3) is a teleost fish from the *Gadidae* family (Cohen et al., 1990). In terms of geographical distribution, it varies across the North Atlantic arc (Lilly et al., 2008). This species is considered demersal, although, depending on prey distribution, and spawning season, it can become pelagic. The continental shelf (around 200 m in depth) is the most common habitat, juveniles prefer more shallow environments, in sublittoral waters, and adults prefer deeper, colder waters. Regarding water parameters, Atlantic cod can be found in various temperatures, from 20 degrees to freezing point, and in salinities between 30 and 35 psu. During the day, the formation of schools occurs, dispersing at nighttime for feeding (Cohen et al., 1990). Atlantic cod is primarily carnivorous species, feeding on, invertebrates, small fish, and even young cod, larvae feed on plankton (Cohen et al., 1990; Uzars & Plikshs, 2000).

The spawning period (once a year) on the Norwegian coast, starts in the winter, during February, and lasts until spring, but can be from December to June, depending on the stock (Cohen et al., 1990). Females can release around 20 batches of eggs around that period (Kjesbu et al., 1996; Petersen et al., 2016). Normally, Atlantic cod spawns at or near the bottom (50 to 200 m depth), where temperatures range from 0 to 12 degrees (Cohen et al., 1990). In the wild, adult individuals' maturity can vary within different areas, around 2-8 years (Brander, 2005; Cohen et al., 1990). Early maturation of the species can be connected to compensatory growth, a consequence of fishing pressure, which reduces competition for food (Brander, 2005).

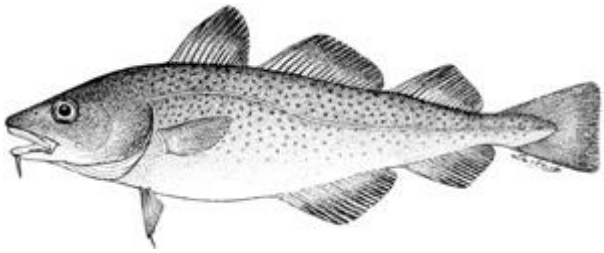

Figure 3 Atlantic cod (*Gadus morhua*) FAO, Editorial use only

Environmental conditions and different areas influence the fecundity range, reaching around 2.5 million eggs per individual to 9 million eggs in a larger female. At first eggs and larvae up to 75 days post-hatching are pelagic, after the larvae phase, juveniles start to settle in the bottom (Cohen et al., 1990). The larval stage is crucial for aquaculture and fish ecology because it is a phase with considerably high mortality. Prey concentration and predation are the main reasons for the high mortality rate of larvae in the wild, in an aquaculture environment, feeding-related constraints and poor water quality are the main sources of mortality (Puvanendran et al., 2022; Puvanendran et al., 2023).

1.2.1 Atlantic Cod aquaculture

Historically, the Atlantic cod is one of the most important species for fisheries in the North Atlantic Ocean, along both coasts. The consumption of this fish species is very settled in the cultures of different nations. Traditional cod fisheries are considered a seasonal activity, considerable oscillations in the quotas over the years due to wild stock reduction contribute to the unpredictability of this food source. Atlantic cod farming surge as an alternative to overcome the challenges of the diminished wild cod stocks (Puvanendran et al., 2022).

The first steps for commercial farming of Atlantic cod happened around 1980 in Norway, as a way of selling cod outside of the harvest season (Puvanendran et al., 2022). Contrary to other captured-base aquaculture, this method does not catch juveniles, instead, it catches adults more than 4 years old, which are kept alive for about 6 to 8 months before slaughter (Dreyer et al., 2008). Around that time, the production of cod fry in lagoons (extensive method) started, and about 75 000 juveniles were harvested in Norway, which resulted in a perfect boost for the industry (Nardi et al., 2021; Øiestad et al., 1985).

Although that initial trigger during the 1980s, profitability was low, and the industry started to collapse, due to limited juvenile production capacity, early maturation that led to a lack of growth, and reduced optimization of formulated feeds (Nardi et al., 2021; Svåsand et al., 2004).

At the start of the millennium, the interest in cod farming revived in several countries around the North Atlantic, mainly in Norway. Advances in juvenile production technology (land-based system) associated with larger hatcheries allowed the industry to develop and peak in cod production around 2010 with 21,240 tons (Norway) (Puvanendran et al., 2022). The second boom of the Atlantic cod industry faced some problems around the year 2011, coinciding with the 2008 world economic crisis, eventually collapsing once again (Nardi et al., 2021).

There are several reasons for the 2000s failure of cod farming, including, the poor volume of quality juvenile production to support commercial cod farming (Puvanendran et al., 2022); the use of Artemia and rotifers (less nutritious) as a live feed, instead of the use of copepod nauplii (Busch et al., 2011; Koedijk et al., 2010; Puvanendran et al., 2022); High larval mortality, related to prey quality, prey concentration, temperature, light regime, diets, and cannibalism (Hansen et al., 2011; Koedijk et al., 2010; Puvanendran & Brown, 2002; Puvanendran & Brown, 1999; Puvanendran et al., 2022); Juvenile deformities, due to rearing conditions and egg incubation (Fitzsimmons & Perutz, 2006; Puvanendran et al., 2022); Escapes from sea cages (Moe et al., 2007); Early sexual maturation that can result

in reduced growth (Puvanendran et al., 2022); Economic downfall lead to the increase of the total allowable catch (Puvanendran et al., 2022).

Currently, Norway is the only active country in the production of cod as an industry (Puvanendran et al., 2022). Advances in technology and cod biology, formulated feeds, as well as overall investment, will allow cod farming to evolve and prosper (Nardi et al., 2021; Puvanendran et al., 2022).

1.2.2 Production cycle

Atlantic cod farming is going in the direction of an intensive model of production (Brown et al., 2003; Puvanendran et al., 2022). Recent hatcheries have their broodstock or get eggs from other production facilities. An appropriate diet and gamete quality, as well as improved handling techniques to reduce stress, are essential for egg quality, which impacts larvae survivability (Puvanendran et al., 2022). During spawning, females in good condition can produce 1-1.5l eggs/kg body weight (Moksness et al., 2008). Following fertilization, the eggs are incubated at around 6-8ºC, until they hatch (Puvanendran & Brown, 1999). After hatching, cod larvae have a yolk sack, from one to two weeks (Herbing, 2001; Moksness et al., 2008; Skreslet, 2018), and then they are introduced to live feed (first to enriched rotifers and then to *Artemia*) (Brown et al., 2003; Howell, 1984). Weaning feeds are introduced gradually in a co-feeding strategy, during the metamorphosis stage (Figure 4) (Brown et al., 2003; Puvanendran et al., 2022).

Figure 4 Atlantic cod larvae, metamorphosis process, from egg to larvae with the yolk sac (3 DPH), and metamorphosed larvae (47 DPH) with full fin development. Photo by: Monteiro, Manuel, Norway, 2024

Frequently, juveniles are kept in tanks until they reach a suitable size for going into sea cages. Juvenile quality is a key factor for the Atlantic cod production and adjusting feeding protocols (prey concentration, feeding frequency, and water flow) is crucial (Figure 5) (Puvanendran et al., 2023).

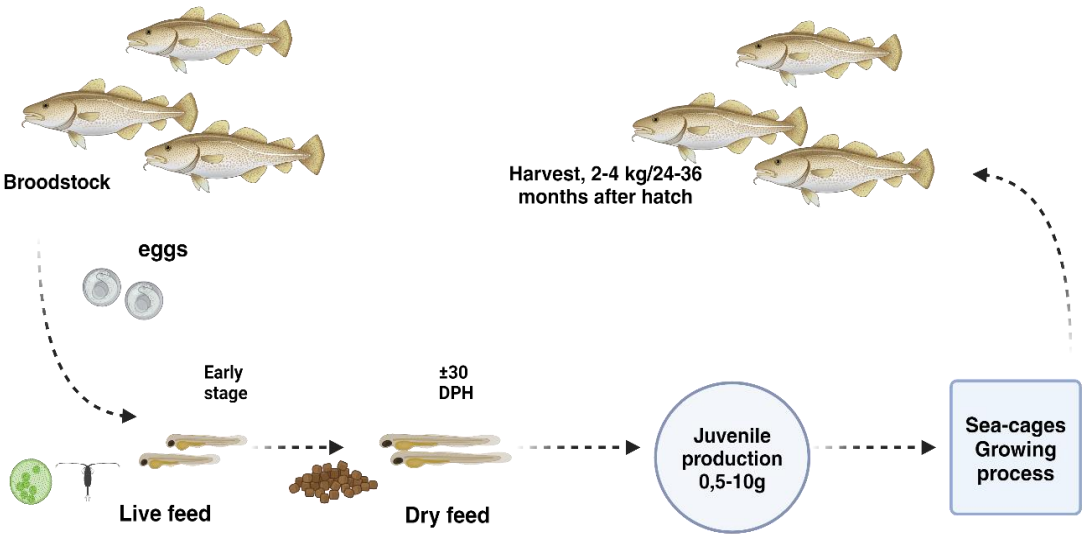

Figure 5 Schematic view of intensive production of Atlantic cod (*Gadus morhua*), based on (FAO 2024). Created in BioRender.com

1.3 Larvae nutrition

In Aquaculture conditions, fish larvae have specific nutritional demands essential for growth, performance, and survivability (Hamre et al., 2013). Nutrition plays a huge part in the success of nurseries. A susceptible stage of larvae development, a representative case, is the use of high concentrations of vitamin C (ascorbic acid) and E (a-tocopherol), to promote immunity and stress resistance (Hamre, 2011).

During the first weeks, after hatching, marine fish larvae, including Atlantic cod larvae, require live feed as prey. Rotifers (*Brachionus* spp.) and *Artemia* are the most used live feeds in marine fish farming (Busch et al., 2011). Although they are not a natural food for marine fish larvae, the straightforward way of production and easy manipulation of nutrient content makes them a great alternative source of live feed. Zooplankton-like copepods are the frequent prey in the wild for marine fish larvae, but intensive production of copepods is not commercially viable yet, although studies showed that cod larvae fed with zooplankton instead of rotifers experienced higher growth and lower skeleton deformities, copepods, and *Artemia* also have differences between lipid content and fatty acid composition due to higher amounts of n-3 HUFA present in copepods (Evjemo et al., 2003; Imsland et al., 2006; Øie et al., 2011).

For optimal larvae growth, it is essential to ensure nutritional composition quality as well as prey concentration (Rosenlund & Halldórsson, 2007). Higher prey concentration (4000 prey/liter) brings advantages for survivability and growth in cod larvae. Lower concentrations (≤ 2000 prey/liter), result in lower feeding opportunities leading to higher mortality rates (Puvanendran & Brown, 1999).

The formulation of micro diets is necessary to complement live feeds and enhance larvae development to optimize the rearing process. A common approach is co-feeding, where formulated diets and live feeds are provided to fish larvae simultaneously. This strategy helps larvae to adapt gradually to the diets, preparing them for a dry feed plan (Figure 6) after weaning (C. Cahu et al., 2003; Kolkovski, 2001; Kolkovski, 2013).

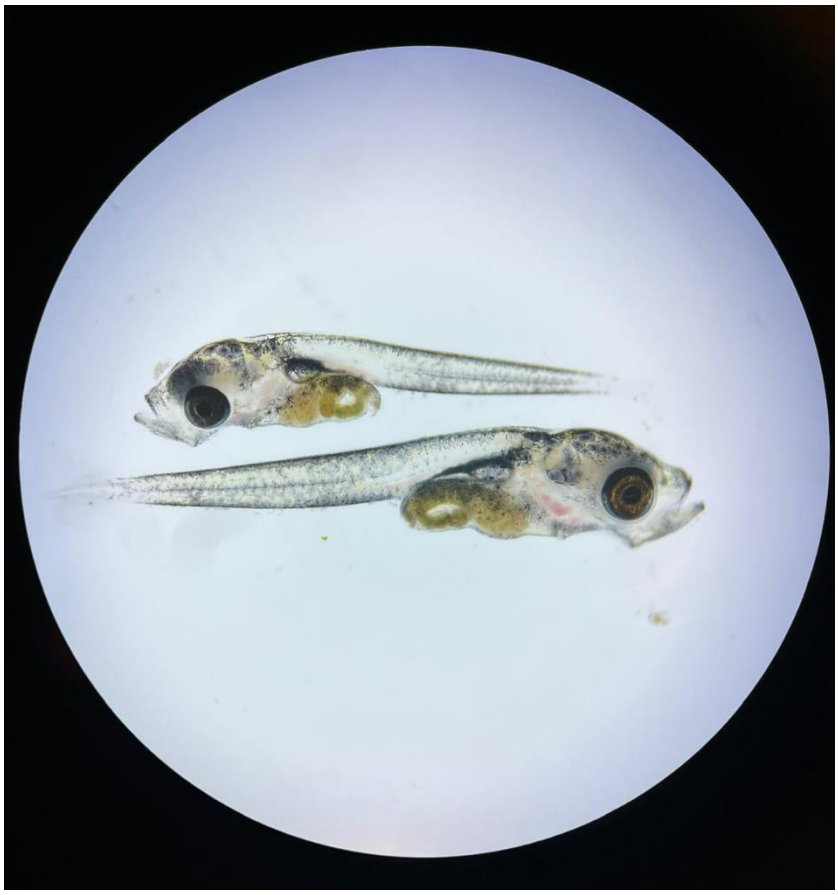

Figure 6 Atlantic cod larvae with 25 dph, co-feeding with cryo plankton and dry feed. Photo by: Pedro, Joana, Norway, 2024

1.3.1 Role of Phospholipids in larvae development

Overall, lipids have numerous functions in fish larvae' nutrition. They are an important source of metabolic energy, which plays an essential role in the functional integrity of cell membranes (Tocher, 2003).

The role of Phospholipids (PL) in marine fish larvae is crucial for their development and growth and therefore for the quality of juveniles and adult individuals (Izquierdo & Koven, 2011; Li et al., 2016). Phospholipids, also called Phosphoglycerids, a more correct term, consist of two fatty acids (positions sn-1 and sn-2), a glycerol backbone, and a phosphate group. The vital phosphoglycerides present in fish tissue are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) (Tocher et al., 2008).

Phospholipids (PL) have hydrophobic and hydrophilic properties that make them important in various cellular membrane functions, cell signaling, absorption, digestion, and transport of neutral lipids. Due to its nature, PL are selectively permeable. Also, they can be broken down into smaller molecules acting as signaling messengers, regulating numerous cell activities. The lipoprotein outer layer contains phospholipids responsible for the transportation of fatty acids that have been absorbed in the small intestines into the bloodstream and distributed all over the body (Jaxion-Harm, 2021).

Although Triacylglycerols (TAG) are the primary source of energy and lipid storage, phospholipids can provide energy to the fish in certain conditions, including the early larvae development stage (Tocher et al., 2008). Some marine fish larvae eggs, like *Gadus morhua* eggs, have low levels (<15% of total lipid) of neutral lipids (e.g. TAG), compensating with high levels of phospholipids, mostly PC (Fraser, 1988; Salze et al., 2005; Tocher et al., 2008; Wiegand, 1996).

Phospholipids in formulated feeds can be from marine or plant-based sources. Krill oil, copepod oil (marine sources), and soybean lecithin oil have been effectively utilized because of their positive impact on the development of fish (Martins et al., 2020). Plant-based sources of PL are a good alternative to formulated feeds because they are more sustainable and cost-effective than marine sources. Significant structural differences between marine and plant-based PL in long-chain polyunsaturated fatty acids (PUFA), exist and can impact fish performance. Soybean lecithin oil is higher in n-6 PUFA (Linoleic acid (LA)) but has lower n-3 PUFA (Alpha-linolenic acid (ALA)), on the other hand, marine PL sources have both types of PUFA present in their composition. Some studies showed that due to the presence of n-3 PUFA (eicosapentaenoic acid (EPA) and docosahexaenoic acid

(DHA)) in marine PL sources, marine species had increased overall performance (Izquierdo & Koven, 2011; Martins et al., 2020; Tocher et al., 2008).

1.4 Fish welfare in aquaculture

As introduced before, the discussion about fish welfare in the aquaculture industry has become a major research topic in recent years (Ashley, 2007). Good welfare can be interpreted in various ways. Huntingford (2006) defined it as the way the animals express their natural behavior, be free of negative experiences like fear and pain, and be promoted with good experiences like social interactions (Huntingford et al., 2006). Aquaculture practices, like daily routines (e.g., handling, transport), water parameters, and stock density, are common factors that affect farmed fish welfare and stress (Ashley, 2007; Conte, 2004).

Although stress responses are crucial to fish for reestablishing their homeostasis state, physiological responses are a good indicator of welfare. Tertiary responses which are associated with behavioral changes and chronic stress are the most substantial concerns about fish welfare (Ashley, 2007; Barton, 2002). ´

Poor farming conditions translate into diminished fish welfare, reducing overall health performance. Susceptibility to diseases caused by poor immunity as well as, reduced growth due to the energy cost of stress responses, are huge concerns in terms of fish welfare and economic losses in aquaculture (Segner et al., 2012).

1.4.1 Stress response

The concept of stress does not have a consensual definition in the scientific community (Wendelaar Bonga, 1997). In teleost fish, stress can be defined as a disruption of the organism's dynamic equilibrium called the homeostasis state (Chrousos & Gold, 1992). For a better understanding of fish health and welfare, it is crucial, to determine the differences between existing stressors and analyze the pathways of the stress response, which are a set of coordinated changes, either physiological or behavioral, to adapt and overcome a potential threat (Wendelaar Bonga, 1997).

Physiological responses to various external stressors can be categorized into different stages, primary, secondary, and tertiary stage, in which behavioral responses take place (Barton, 2002). The primary response, after perception of the threat in the central nervous system (CNS) is associated with an endocrine cascade of events, that starts with the release of catecholamines (e.g., adrenaline and non-adrenaline) from the chromaffin tissue,

located in the head kidney (Barton, 2002; Randall & Ferry, 1992; Reid et al., 1998), which induces, a higher branchial blood flow and oxygen diffusing capacity, increasing oxygen transport in the blood (Randall & Ferry, 1992; Wendelaar Bonga, 1997). The stimulation of the hypothalamic-pituitary-interrenal (HPI) axis, causes the secretion of corticotropin-releasing hormone (CRH), which promotes the activation of corticotrophic cells of the anterior pituitary resulting in the production of adrenocorticotropin hormone (ACTH). Interrenal cells from the head kidney start to release corticosteroid hormones (e.g., cortisol) into the bloodstream in response to ACTH (Figure 7) (Barton, 2002; Wendelaar Bonga, 1997).

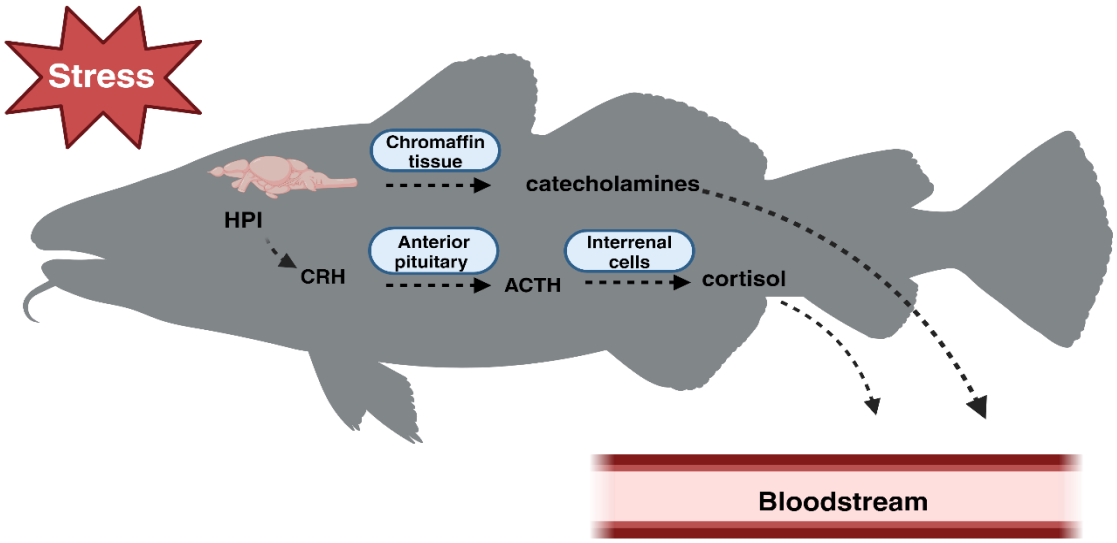

Figure 7 Schematic overview of primary stress response in fish, adapted from (Schreck & Tort, 2016)

Secondary stress response starts once stress hormones are in circulation. At this stage, physiological changes occur in the organism. Mainly metabolic adjustments, like activation of liver glycogenesis and gluconeogenesis and inhibition of glycolysis, osmoregulation, and immune system alterations, are coping mechanisms to avoid potential damage (Barton, 2002; Randall & Ferry, 1992; Segner et al., 2012).

Stressful situations associated with the aquaculture environment can reduce performance and overall health (Segner et al., 2012). A tertiary stage in the stress response leads to behavioral changes in fish. Alterations in swimming patterns, decreased growth, disease resistance, and feeding activity, all resemble consequences of exposure to chronic stress (Barton, 2002; Wendelaar Bonga, 1997).

1.4.2 Oxidative stress

Respiratory burst activity (RBA) is defined as the release of reactive oxygen species (ROS), mainly from neutrophils and macrophages to eliminate potential pathogens threats to the organism (Fridovich, 1998; Hampton et al., 2020).

Oxidative stress is associated with ROS that at an intracellular level can provoke damage to lipids, proteins, cell membranes, and DNA as well as induce cell apoptosis (Ashrafizadeh et al., 2020; Hoseinifar et al., 2020; Martínez-Álvarez et al., 2005; Schieber & Chandel, 2014). When the ROS generation rate is higher than the removal rate, an imbalance between ROS and their enzymatic and non-enzymatic antioxidants occurs and oxidative stress acts (Sies, 1986; Valko et al., 2007). ROS are produced via aerobic metabolism (Schieber & Chandel, 2014). In an oxygen-rich environment, oxidative stress is an unpreventable result. Oxygen enters a dual role, a paradox, which can be vital and harmful simultaneously (Davies, 1995)

Reactive oxygen species in particular, the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxy radical ($OH^\cdot$) are involved in the oxidative stress response (Martínez-Álvarez et al., 2005; Schieber & Chandel, 2014).

To prevent the harmful effects of ROS, organisms developed a strong and complex antioxidant defense mechanism (Jomova et al., 2024). Enzymes are part of the antioxidant defense system, especially catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione S-transferase (GST), but also non-enzymatic compounds like glutathione (GSH), which exist in two forms inside the cell, as reduced GSH and oxidized, glutathione disulfide (GSSG) (Slaninova et al., 2009; Valko et al., 2007).

ROS have specific pathways for their formation, hydrogen peroxides can react with transition metals (eg. Fe^{2+} and Cu^+) via Fenton reactions, originating highly unstable free radicals, namely hydroxyl radicals ($^\cdot OH$) $(Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-)$ (Liochev & Fridovich, 1994; Valko et al., 2007). Additionally, the superoxide anion radical (O_2^-) is formed by the reduction of the oxygen molecule moderated by NAD(P)H oxidases and xanthine oxidase. Redox-reactive compounds of the mitochondrial electron transport chain can also format superoxide radicals, $O_2 + e^- \rightarrow O_2^-$(Hayyan et al., 2016; Jomova et al., 2024; Valko et al., 2004; Valko et al., 2007). To counteract the superoxide radical formation, superoxide dismutase (SOD) dismutates O_2^- to hydrogen peroxide (H_2O_2) which, is further reduced to molecular O_2 and water by glutathione peroxidase (GPx), requiring GSH as an electron donor. During this process, GSH is oxidized and NADPH is used as a substrate to recycle GSSG back to GSH by the enzyme glutathione reductase (GSR) (Valko et al.,

2007). Catalase (CAT) also has a significant role in H_2O_2 detoxification, due to its high turnover rate, converting high numbers of H_2O_2 molecules into oxygen and water per unit of time (Nandi et al., 2019; Von Ossowski et al., 1993) (Figure 8). Accumulation of OH radicals can initiate lipid peroxidation; OH, can take a hydrogen atom from PUFA C-H double bound, creating a lipid radical (Valko et al., 2007).

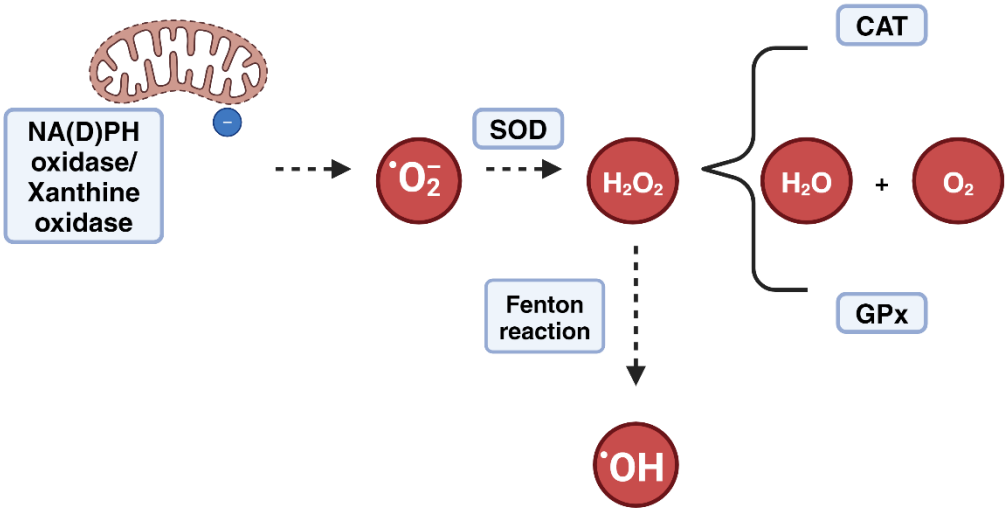

Figure 8 A simplified schematic view of the Oxidative stress pathway, based on (Valko et al., 2007) Created in BioRender.com

1.5 Objective

The present proposal is part of project EarlyCOD, an international consortium of companies and academia to develop a new range of aquafeeds for improved performance, increased survival, and enhanced quality of cod larvae and juveniles.

The main objective of this proposal is to implement an innovative early feeding protocol, combining live cryopreserved plankton with improved digestibility and novel dry microfeeds. It is hypothesized that such an approach will strengthen Atlantic cod larvae during the critical weaning stage, reduce mortalities as well as stress effects, and improve the overall quality and growth of cod larvae and juveniles while reducing production costs.

2. Material and methods

2.1 Feeds

The dry feed consisted of one commercial diet (COM), from Skretting, Norway, and two formulated feeds provided by Sparos Lda, a company that develops feeds and nutritional innovations for aquaculture in Olhão, Portugal, CTRL, and MARINE. Each feed has 3 different pellet sizes, 150µm, 300 µm, and 500 µm, to satisfy larvae development during the trial. The transition between pellet sizes was performed in a co-feeding regime for easier adaptation of the larvae.

Table 1 Formulated feeds from Sparos Lda, proximate composition. Main ingredients; CTRL: Squid meal, Crustacean meal, Fish solubles, wheat gluten, **Soybean lecithin**, Algal oil, Vitamin and mineral premix; MARINE: Squid meal, Crustacean meal, Fish solubles, wheat gluten, Soybean lecithin, Algal oil, **Marine phospholipids oil**, Vitamin and mineral premix; Skretting diet composition: Fish meal, hydrolyzed aquatic invertebrates, **lecithin**, wheat gluten, algae, **fish oi**l, maize starch

Proximate composition	***CTRL***	***MARINE***	**COM**
Protein (%DM)	**68.9**	**66.8**	**59**
Fat (%DM)	**12.8**	**14.1**	**14**
Ash (%DM)	**12.6**	**14.1**	**13**

Formulated feeds, CTRL and MARINE, contain krill meal, squid meal, hydrolysate, and wheat gluten as the main ingredients. There were different inclusions of ingredients as a source of phospholipids. CTRL contains a higher inclusion of plant-based sources of PL (Soybean lecithin and wheat gluten), while MARINE contains a higher inclusion of marine sources of PL (Table 1).

2.2 Experimental design

Atlantic cod eggs were collected from a natural spawning broodstock population kept at the National Cod Breeding Center (Nofima) in Tromsø (Norway), transferred to Ode hatchery facilities in Stadsbygd (Norway) and placed on incubators with a capacity of 600 L for 10 days at 6.0 ± 0.5 Cº. At 2 dph, 40,000 larvae were transferred to each of nine 400 L start-feeding tanks. This pilot scale feeding trial was run in triplicate and started on 18/04/2024 (0 dph) with a duration of 67 days. Each tank was randomly assigned to each dietary treatment. The live feed was the same for each treatment, starting with Cryo-m (Blue mussel

eggs) from 3 dph to 10 dph, enriched rotifers from 3 dph to 24 dph, Cryo-s (1st stage offspring (nauplius) from *Balanus crenatus*. Length 200µm/Width 100µm) was introduced at 10 dph, to 22 dph, finally, Cryo-L (1st stage offspring (nauplius) from *Semibalanus balanoides*. Length 320µm/Width 150µm) start at 18 dph to 45 dph. All live feeds followed a co-feeding strategy for minimizing harsh transitions. Cryo-plankton circulated in the tanks supported by a pump. Formulated feeds, a control diet (CTRL) from Sparos, and two other diets, COM from Gemma Micro, Skretting, Norway, and MARINE, also from Sparos, were introduced at 22 days post-hatching in a co-feeding regime. The pellet size gradually increased to fulfill larvae needs. The daily dry feed increased gradually in grams from 2 to 40 grams and was provided by hand-feeding and automatic feeders (Figure 9).

The water parameters were measured daily, maintaining oxygen saturation above 90 %, salinity at 35 psu, and temperature at 8.03 ± 0.17 Cº from 2 dph until 13 dph, followed by an increase, at 10.1 ± 0.3 Cº from 14 to 67 dph. A photoperiod of 24L:0D was present throughout the trial, with algae paste (*Nanochloropsis gaditana*, AlgaSpring, The Netherlands) additions from 2 to 45 dph.

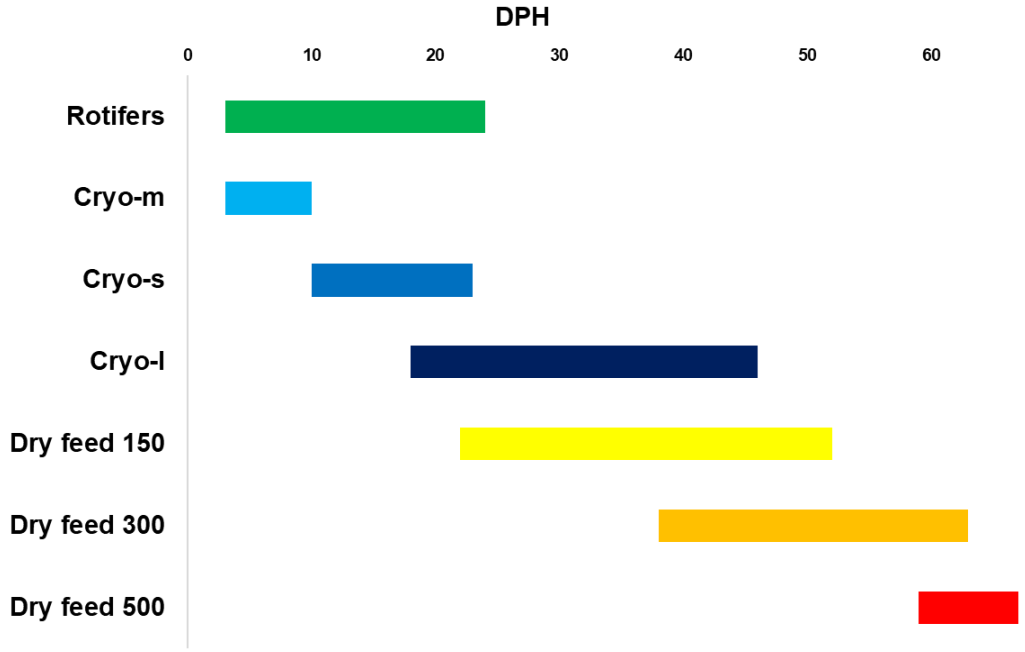

Figure 9 Schematic representation of feed plan and co-feeding interactions timeline.

2.3 Sampling

A total of 8 sampling points were taken throughout the trial. For each sampling procedure, larvae were selected randomly from each tank with the assistance of a filter or beaker, posteriorly larvae were euthanized with an overdose of Tricaine methanesulfonate (MS-222). Sampling was carried out in the Ode facilities laboratory.

The first sampling (S1) at 3 dph, was to evaluate zootechnical parameters, dry weight (DW, mg), and standard length (SL, mm), 20 larvae from each tank were collected, for a total of 180 larvae. Larvae were measured in millimetric paper during sampling, washed in distilled water for dry weight, stored at -20ºC, and later put in a freeze dryer for 24-48h to remove the moisture.

In the second (S2) and third (S3) samplings at 10 dph and 17 dph, respectively, 20 larvae were collected from each tank in total. The same biometric methods of S1 were applied.

At 25 dph, a fourth sampling (S4) was performed, the same biometric methods as before were used for measuring the standard length and dry weight. Then, 30 larvae from each tank were collected for standard length and 20 larvae per tank for dry weight, a total of 270 larvae.

In the fifth sampling point (S5) at 30 days post-hatching, 30 larvae from each tank were collected for measuring standard length, for a total of 270 larvae. The sixth sampling at 35 dph (S6) followed the same principles as S4. The seventh sampling (S7) at 48 dph, was for measuring standard length, using the same strategy as S5.

The final sample (S8) was at 67 dph, larvae were collected for zootechnical parameters, DW, and SL. For oxidative stress analysis, 8 larvae per replicate were collected and euthanized by anesthetic overdose, Thereafter, whole larvae were placed in a 2 ml cryotube, snap-freeze in liquid nitrogen, and stored at -80 °C. Oxidative stress analyses were carried out in the Aquatic Animal Health laboratory of CIIMAR (Interdisciplinary Centre of Marine and Environmental Research), Matosinhos, Portugal.

2.4 Zootechnical Calculations

DW (mg) and mean SL (mm) were measured for zootechnical parameters.

SGR % d^{-1} = (Ln (Final DW)-Ln (Initial DW)) x 100 / Δt

Survival rate % = (Dead count/Tank count) x 100

2.5 Oxidative stress analysis

2.5.1 Larvae Homogenization

Full larvae were previously weighted (mg) in a precision scale and then homogenized (1:10) in 0.1 M phosphate buffer (pH 7.4) using T 25 digital ULTRA TURRAX-IKA

One aliquot of larvae homogenate was used to determine the extent of endogenous lipid peroxidation (LPO) by measuring thiobarbituric acid-reactive species (TBARS) as suggested by (Bird & Draper, 1984). Moreover, 4 % BHT (2,6-Di-tert-butyl-4-methylphenol) in methanol was added to the aliquot to prevent artifactual lipid peroxidation.

The remaining larvae homogenate was centrifuged for 20 min at 10,000 g (4ºC). All samples were immediately frozen at -80ºC until used.

2.5.2 Total Protein

Total protein concentration (TP) was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). The method uses colorimetry to measure total protein (TP) in a sample by mixing it with a working reagent and comparing the result against a bovine serum albumin (BSA) standard with a known protein concentration.

Prepared samples were diluted in 0.1 M phosphate buffer (pH 7.4) in a proportion of 1:10. Using 96-well plates, 25 µl of samples and standards were pipetted to the wells in triplicate, followed by 200 µl of working reagent using a multichannel pipette, the plate was mix thoroughly on a plate shaker for 30 seconds. Once mixed, the cover plate was incubated at room temperature (RT), 25ºC. Absorbance was read at 562 nm using a Synergy HT microplate reader (Biotek).

2.5.3 Catalase activity

Catalase (CAT) activity was determined in larvae homogenate by measuring substrate consumption (H_2O_2) at 240 nm according to (Claiborne, 2018), adapted to microplates. Based on TP results, samples were diluted with 0.1 M phosphate buffer (pH 7.4) to 0.7 mg mL^{-1}.

Using a UV microplate, 10 µl of diluted samples and blanks were pipette in triplicate, followed by 140 µl of 0.05 M phosphate buffer (pH 7.0), and 150 µl of reaction buffer (performed quickly). Absorbance was read at 240 nm for 2 minutes (1 read every 15 sec. interval) using a Synergy HT microplate reader (Biotek). CAT activity was expressed as µmol of substrate broken down per minute per milligram of protein (U/mg^{-1})

2.4.4 Superoxide dismutase activity

Superoxide dismutase (SOD) activity was measured according to (Flohé & Otting, 1984), and adapted for microplate by (Lima et al., 2007) with some modifications.

Based on TP results, samples were diluted with 0.1 M phosphate buffer (pH 7.4) to 0.3 mg mL^{-1} and added 50 µl in triplicate to a 96 microplate, blanks and standards included.

Two solutions were prepared, the reaction solution, containing 0.7mM-xanthine, 0.03 mM of cytochrome, and a 50 mM phosphate buffer (pH 7.8) solution; the xanthine oxidase solution 0.03 U mL^{-1}.

After the samples were plated, 200 µl of the reaction solution, followed by 50 µl the xanthine oxidase solution (this step must be performed quickly). Absorbance was read at 550 nm for 2 minutes (1 read every 20 sec. interval) using a Synergy HT microplate reader (Biotek). SOD activity was expressed as µmol of substrate broken down per minute per milligram of protein (U/mg^{-1}).

2.4.5 Lipid peroxidation

This method is based on the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) with the production of a pink pigment with a 535 nm absorption maximum (Bird & Draper, 1984).

LPO was measured, according to (Almeida et al., 2010), with few adjustments. The previous aliquots for LPO analysis were mixed with 100 µl of 100 %TCA (6.1 M), followed by 1 mL of a solution containing 0.73% TBA (5 mM), Tris-HCL (60 mM) and DTPA (0.1 mM).

After mixing the samples, they were incubated for 1 h at 100ºC in a water bath, and the centrifuge for 5 min at 11500 rpm (RT). 200 µl of the supernatant were pipetted into a 96-microplate in triplicate. Absorbance was single read at 535 nm using Synergy HT microplate reader (Biotek), and results were expressed in TBARS (nmol/g wt)

2.6 Statistical Analysis

The acquired data was statistically analyzed using the IBM SPSS Statistics software, version 29.0.0.0 (241). The data was tested for normality using Shapiro-Wilk (Sample size<50) or Kolmogorov-Smirnov (>50) and for homogeneity of variance using Levene´s test. Descriptive data was presented as mean value ± standard deviation.

Whenever required, outliers were removed and data was logarithmically transformed. One-way ANOVA was performed to investigate the effects of each treatment (independent variable) on oxidative stress responses and to compare zootechnical data between different diets. A Tukey HSD test was conducted when significant differences were detected to

identify which groups differed significantly. Non-parametric tests, Kruskal Wallis, were performed when assumptions were not fulfilled (Zootechnical Parameters), and “Adg. Sig” was used to ensure the reliability and robustness of the pairwise test.

For all statistics tests, a 95% confidence interval was used, with a level of significance of $p \leq 0.05$.

3. Results

3.1 Zootechnical Parameters

Mean Standard length (mm) was measured throughout the days post-hatching, at 3 dph, there was no significant difference between diet groups (p=1.0).

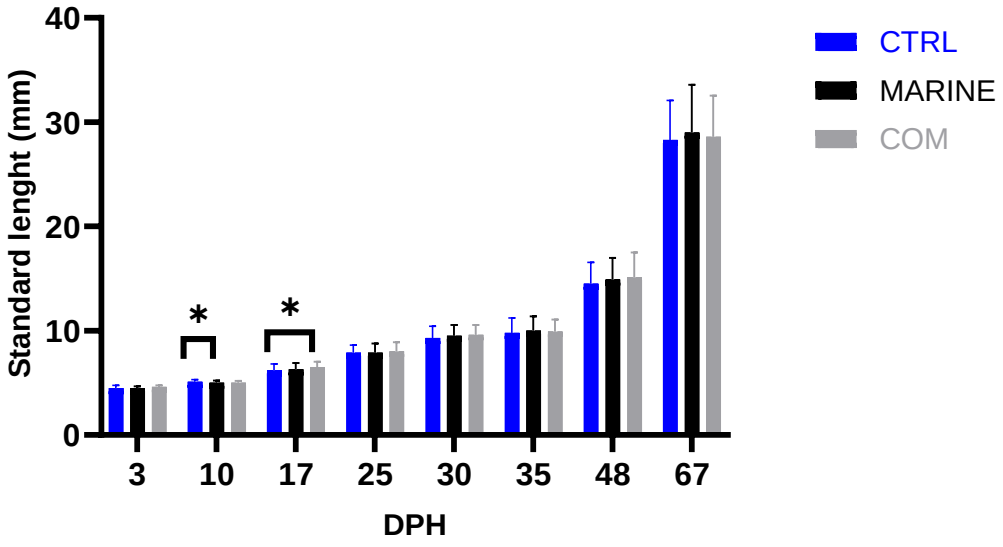

Figure 10 Mean Standard length (mm) of cod larvae throughout the days post-hatching. Asterisk (*) denotes significant differences between dietary groups. Bars represent the mean value ± sd

Larvae, at 10 dph, from group CTRL (5.1 ± 0.2 mm) have significant differences regarding mean SL (mm) in comparison with the MARINE group (5.0 ± 0.2 mm), (p=0.03). The control diet (5.0 ± 0.2 mm) had no significant differences in the experimental feed groups. At 17 dph, the COM diet (6.5 ± 0.5 mm) shows significantly higher mean SL (mm) in comparison to the CTRL group (6.2 ± 0.6 mm), (p=0.03). In the next sampling points at 25, 30, 35, 48, and 67 days post-hatching a specific diet did not have a significant impact on larvae, standard length (mm) (p>0.05). In the 67 dph, larvae reached an average SL (mm) of 28.6 ± 3.9 mm (COM), 28.3 ± 3.8 mm (CTRL), and 29.0 ± 4.6 mm (MARINE) (Figure 10)

The initial DW (mg) of larvae showed no significant differences between dietary groups. CTRL group had a mean DW of (0.058 ± 0.001 mg), the MARINE group had a mean DW of (0.058 ± 0.001 mg), and the COM diet larvae weighed (0.0579 ± 0.0011 mg). At 35 days post-hatching, there were significant differences between dietary groups DW (p<0.001 between all groups). The CTRL group had the lowest mean DW, (88.0 ± 0.01 mg) at mid-trial (35 dph), MARINE (0.91 ± 0.01 mg), and the Commercial group had (0.90 ± 0.01 mg).

In the last sampling, 67 dph, although there were no relevant differences between dietary groups. CTRL (34.35 ± 13.60 mg) showed a lower mean DW in comparison to MARINE (39.14 ± 18.96 mg) and COM (39.53 ± 16.42 mg) (Figure 11).

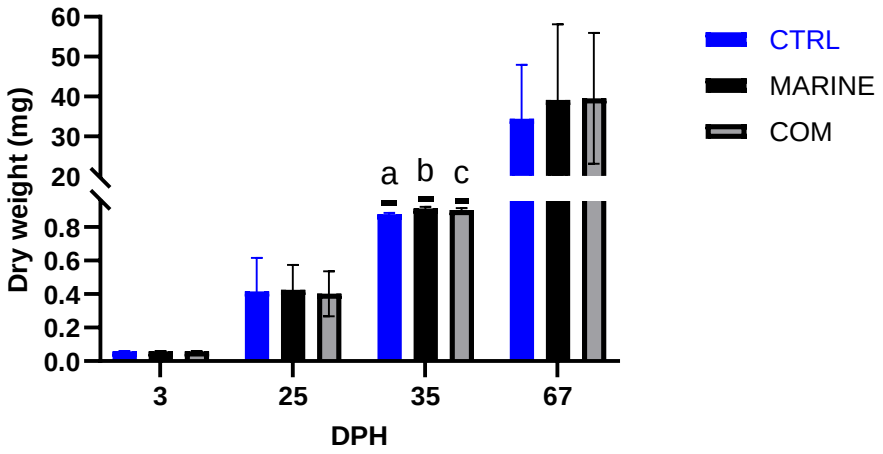

Figure 11 Mean Dry weight (mg) of cod larvae during the trial length, different lowercase letters showed significant differences between dietary groups. Bars represent the mean value ± standard deviation

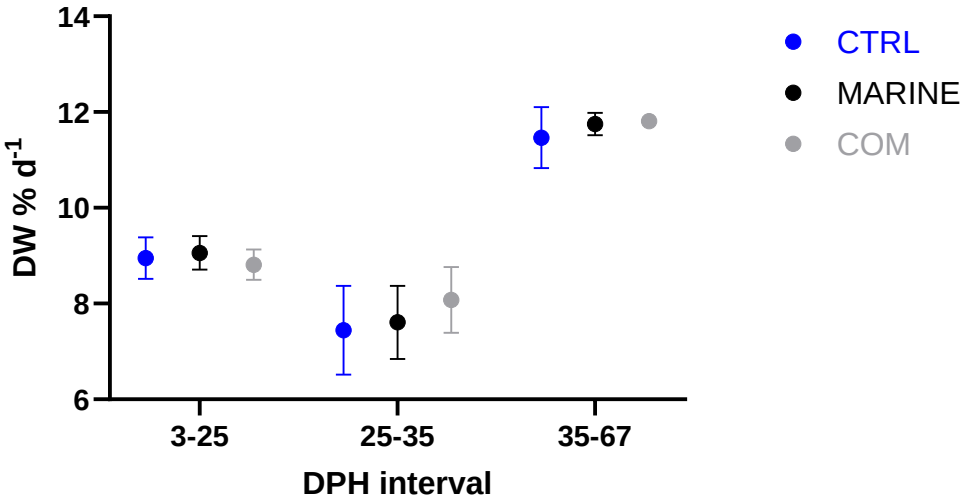

Figure 12 Mean DW SGR % d-1 of cod larvae from different diet groups, throughout time (dph). Points represent mean ± sd

Larvae did not show significant differences between dietary groups, regarding DW % d^{-1}. During 25-35 (dph) intervals, mean values decreased, coinciding with a reduction in SL % d^{-1} at 30 to 35 intervals (dph) (Figures 12 and 13).

Specific growth rate % (SL) was used between sampling points, where Δt was the difference between days-posting hatching. There was a significant difference between CTRL and COM in the 17-25 period (p=0.017).

At 25-30 dph, SGR was considerably high in all diet groups, peaking since day 3, from 30 to 35 dph, larvae' SGR dropped in all groups. SGR continuously increases from 35 to 48 days, and 48 to 67 days (Figure 13).

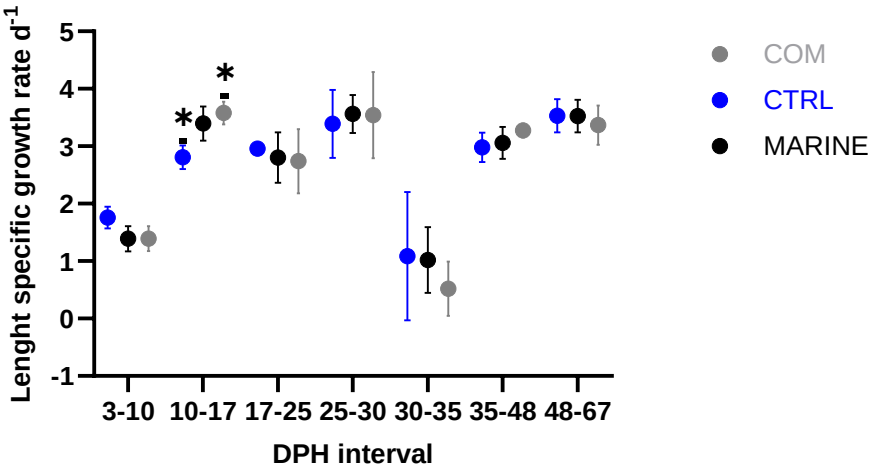

Figure 13 Length-specific growth rate in d-1, daily growth percentage, of cod larvae from 3 different group diets. Asterisk (*) represents a significant difference between CTRL and COM. All points represent the mean ± sd

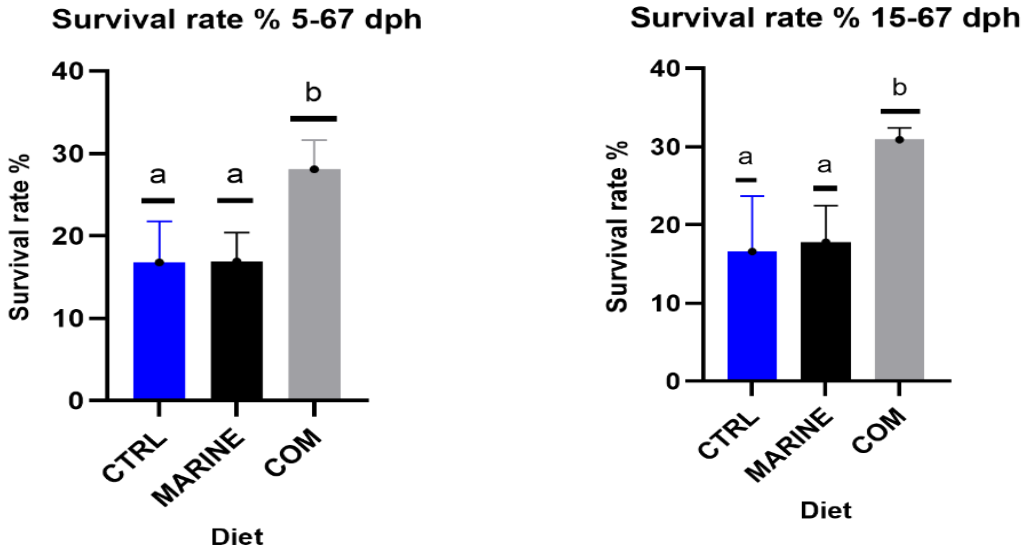

Figure 14 Survival rate %, of cod larvae, from two periods of time (DPH), lowercase letters represent significant differences between diet groups. Bars represent mean ± sd

In terms of survivability, two periods were analyzed, 5 to 67 dph, and 15 to 67 days. In both periods (dph), the COM diet seems to impact survivability throughout the trial (Figure 14) positively. Significant differences were registered between the commercial and the other dietary groups from 5 to 67 days or 15 to 67 days (Table 3). In 5-67 (dph), a comparison between CTRL and COM resulted in a p-value=0.01, MARINE, and COM p-value=0.025. In 15-67 (dph) CTRL and COM had a p-value of 0.01 and between MARINE and COM a p-value of 0.04.

3.2 Oxidative Stress Parameters

Overall Catalase activity showed no significant differences among diet groups, (p=0.121). LPO activity also revealed no significant differences between diets, (p=0.738). Although not significant, the control diet presents a higher average in both parameters (12.1 ± 4.9; 29.33 ± 7.04) respectively (Figure 15).

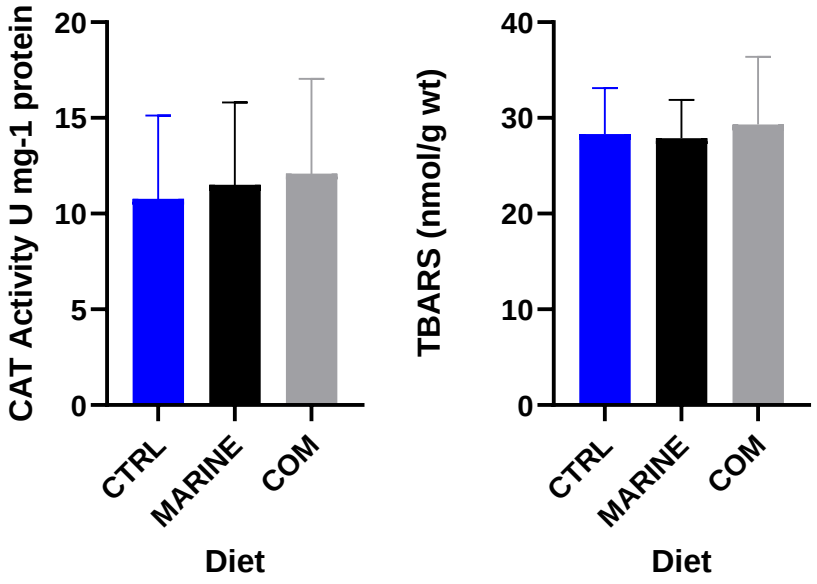

Figure 15 Average Catalase Activity (U mg-1 protein), and average TBARS (nmol/g wt) (LPO), in cod larvae with 67 dph between different diets. There was no significant difference between the groups. Bars represent the mean value ± standard deviation.

Superoxide dismutase activity showed significant differences (p=0.01) between the experimental diet MARINE (102.2 ± 44.5 U mg^{-1} protein) and the commercial diet from Skretting (60.27 ± 16.6 U mg^{-1} protein) (Figure 16).

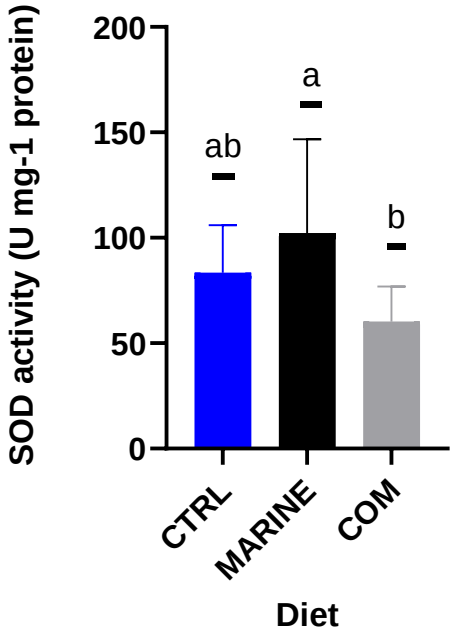

Figure 16 Mean SOD Activity (U mg^{-1} protein) in cod larvae with 67 dph between different diets. Different lowercase letters indicate significant differences between diet groups. Bars represent the mean value ± standard deviation

4. Discussion

4.1 Zootechnical parameters

Phospholipids (PL) are essential for the growth performance and survivability of marine fish larvae. There have been studies on other marine fish species that demonstrate that PL in diets enhances length, weight, and survivability (C. L. Cahu et al., 2003). Experimental feeds from the present study differed in PL sources, CTRL diet included PL almost exclusively from plant-based ingredients whereas MARINE contained a mix of marine and plant-based PL. Growth performance was evaluated to compare these feeds against a commercial diet.

First, zootechnical observations did not show the effects of formulated feeds, because, at the initial stages, only live feeds were delivered to the tanks. In terms of standard length (SL), the larvae from the CTRL group presented a higher mean SL compared to the larvae in the MARINE group at 10 dph, but that trend changed quickly at 17 dph, where the CTRL group presented the lowest mean in SL compared to the group COM. Although not significantly different, larvae from the MARINE group also showed a higher mean in SL at 17 dph. That can be explained by the higher SGR observed in larvae from the COM group compared to those from the CTRL group. A possible explanation for these slight differences in growth can be related to individual and egg variability that can influence larvae size from the early stages, also noted that size variability, in the beginning, was not accounted (Browman et al., 2003). For the rest of the trial, larvae grew as expected in terms of SL, similar to that reported in other studies with cod larvae (Wold et al., 2007). At the end of the feeding period (67 dph, larvae fed MARINE dietary treatment, and COM showed a tendency to have higher SL.

Regarding dry weight, cod larvae had a daily increase of around 9 % from 3 to 25 dph, which strongly matches a former study (Kjørsvik et al., 2009) done with PL experimental diets fed to Atlantic cod larvae. From 35 to 67 dph, the period where dry feeds were more influential, larvae had an increased growth rate, around 11 %, but no significant differences between diets were reported. At 35 days dph, CTRL mean dry weight, was significantly lower than that from MARINE and COM groups. Moreover, cod larvae fed the MARINE diet had significantly higher dry weight compared to the COM diet. MARINE was formulated to contain a higher inclusion of marine PL compared with the CTRL group, which can explain these differences. A study by Wold et al., (2007) on cod larvae, which included 3 dietary groups with 3 groups of diets, 2 of them being PL-rich diets, demonstrated significant findings related to weight at 45 dph, where PL-rich diets showed higher dry weight than a

Neutral lipid diet. Despite not being significant, DW results at 67 dph, also follow the trend, where the MARINE and COM groups presented higher weight in comparison with a more Plant-based inclusion diet (CTRL).

Both SL SGR and DW SGR showed a decrease at 30 to 35 dph and from 25 to 35 dph, respectively. Visual observations and overall size, around 12 mm, indicated that larvae were entering the metamorphosis phase. Since in this stage larvae tend to allocate energy due to physiological changes, like muscle and organ development, it is expected a reduced growth rate (Di Pane et al., 2019; Pedersen & Falk-Petersen, 1992; Vo et al., 2016). Formulated feeds were introduced at 22 dph, whereas cryo-s, as well as rotifers supply, ended at 23 and 24 dph, respectively, which can be connected to the reduced SGR.

Survival is a crucial factor in larvae rearing and ecology (Puvanendran & Brown, 1999). A diet formulated with DHA and EPA incorporating PL may increase growth performance and survival, in agreement with that reported by Wold et al., (2007). In the present study, Larvae fed the COM diet presented higher survival in comparison with cod larvae fed CTRL and MARINE experimental feeds. Larvae in the COM group, from 15 to 67 days had a 30.9 % survival rate, whereas those fed CTRL and MARINE showed 16.6 and 17.8 %, respectively. The COM diet was conceived for optimal growth and maximal survival and is commercially available. An important factor in consideration when evaluating differences in survival percentage is the total lipids content (C. L. Cahu et al., 2003), which was higher in COM than CTRL feed from the present study. In addition, the COM diet also contains a considerable amount of marine lipid sources such as PL and fish oil. Although MARINE has also a substantial amount of marine PL and overall fat, cod larvae fed this dietary treatment showed a lower survival rate than those fed the COM diet. A possible explanation could be related to the fact that MARINE lacks fish oil. DHA and EPA are essential fatty acids present in fish oil which have great advantages for larvae development. Different studies testing fatty acid-rich diets, in Atlantic cod and Pacific cod larvae, showed that the presence of DHA and EPA enhances the growth and survival of the larvae (Copeman 2010; Park et al., 2006). In the present study, it can be hypothesized that the COM diet presented a similar effect and therefore higher survival rate.

Overall, in terms of zootechnical performance COM and MARINE diets had the best growth performance compared to CTRL. The survival rate in cod larvae fed COM was higher compared to the CTRL and MARINE feeds. Indeed, MARINE dietary treatment showed good potential in terms of growth performance, and the survival rate is acceptable compared to previous studies (Kjørsvik et al., 2009). Having this in mind, in future studies the inclusion of different PL levels in the MARINE diet could be a good option for optimizing growth and

survival due to the efficiency of lipids transport and absorption, helping deliver fatty acids to tissues, including the essential role of DHA and EPA in larvae survival (Tocher et al., 2008).

4.2 Oxidative stress

The oxidative stress response is a critical concern to the health and development of marine fish (Tocher et al., 2008). The current study is also intended to evaluate the influence of different dietary groups on several oxidative stress parameters.

Cod larvae fed the MARINE diet increased SOD activity compared to those fed COM diet and tended to be higher than those fed with CTRL diet. Despite all groups presenting a clear antioxidant response, data from the present study suggest that marine PL from the MARINE diet could improve antioxidant ability. Studies in other fish species also presented results regarding the influence of dietary PL on the antioxidant capacity, and SOD activity values were in agreement with this study (Cai et al., 2016; Huang et al., 2021). Marine PL, due to its high composition of omega-3 fatty acids, EPA, and DHA, may improve antioxidant capacity. These two types of n-3 PUFA play an important role in cell membrane integrity, protecting cellular membranes against oxidative damage during early sensitive developmental stages in fish larvae (Tocher, 2010; Tocher et al., 2008).

Catalase activity and lipid peroxidation did not show significant differences between dietary groups. Catalase is responsible for the degradation of hydrogen peroxide to water and oxygen, completing the antioxidation process initiated by SOD. Catalase is a highly efficient enzyme that can break millions of H_2O_2 molecules in seconds (Ighodaro & Akinloye, 2018). This antioxidant interaction can help explain the lower concentration of catalase concerning SOD enzyme activity, not implying oxidative stress. Dietary influence was not shown in lipid peroxidation activity.

5. Conclusion

This study aimed to evaluate the impact of different PL sources on Atlantic cod larvae' growth performance, survival, and oxidative stress response. By comparing formulated micro feeds with distinct inclusions of plant-based and marine PL, against a commercial feed, the results showed that the diets with marine PL inclusion have better potential than the diet with plant-based PL inclusion. However, the survival rate in larvae fed the MARINE diet (rich in marine PL) decreased compared to those fed the commercial feed, suggesting that further understanding of the nutritional requirements and optimization of PL diet composition are necessary. Incorporating high-quality feeds, rich in essential fatty acids and PL, is crucial for improving the growth and quality of larvae, which can help cod aquaculture to prosper once more. Further research should aim at nutritional strategies, and improvements in larvae and juvenile quality, opting for sustainability and welfare practices minimizing stress, and ensuring overall health.

References

Almeida, J. R., Oliveira, C., Gravato, C., & Guilhermino, L. (2010). Linking behavioural alterations with biomarkers responses in the European seabass *Dicentrarchus labrax L*. exposed to the organophosphate pesticide fenitrothion. *Ecotoxicology*, *19*(8), 1369-1381.

Ashley, P. J. (2007). Fish welfare: Current issues in aquaculture. *Applied Animal Behaviour Science*, *104*(3), 199-235.

Ashrafizadeh, M., Ahmadi, Z., Samarghandian, S., Mohammadinejad, R., Yaribeygi, H., Sathyapalan, T., & Sahebkar, A. (2020). MicroRNA-mediated regulation of Nrf2 signaling pathway: Implications in disease therapy and protection against oxidative stress. *Life Sciences*, *244*, 117329.

Barton, B. A. (2002). Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integr Comp Biol*, *42*(3), 517-525.

Bird, R. P., & Draper, H. H. (1984). Comparative studies on different methods of malonaldehyde determination. *Methods Enzymol*, *105*, 299-305.

Brander, K. (2005). Spawning and life history information for North Atlantic cod stocks. *ICES Cooperative Research Reports (CRR)*.

Browman, H., St-Pierre, J.-F., Skiftesvik, A., & Racca, R. (2003). Behaviour of Atlantic cod (*Gadus morhua*) larvae: an attempt to link maternal condition with larval quality. *The Big Fish Bang Proceedings of the 26 th Annual Larval Fish Conference. Bergen, Norway*, 72-94.

Brown, J. A., Minkoff, G., & Puvanendran, V. (2003). Larviculture of Atlantic cod (*Gadus morhua*): progress, protocols and problems. *Aquaculture*, *227*(1), 357-372.

Busch, K. E. T., Peruzzi, S., Tonning, F., & Falk-Petersen, I.-B. (2011). Effect of prey type and size on the growth, survival and pigmentation of cod (*Gadus morhua L.*) larvae. *Aquaculture Nutrition*, *17*(2), e595-e603.

Cahu, C., Zambonino Infante, J., & Takeuchi, T. (2003). Nutritional components affecting skeletal development in fish larvae. *Aquaculture*, *227*(1), 245-258.

Cahu, C. L., Zambonino Infante, J. L., & Barbosa, V. (2003). Effect of dietary phospholipid level and phospholipid: neutral lipid value on the development of sea bass (*Dicentrarchus labrax*) larvae fed a compound diet. *Br J Nutr*, *90*(1), 21-28.

Cai, Z., Feng, S., Xiang, X., Mai, K., & Ai, Q. (2016). Effects of dietary phospholipid on lipase activity, antioxidant capacity and lipid metabolism-related gene expression in large yellow croaker larvae (*Larimichthys crocea*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *201*, 46-52.

Chrousos, G. P., & Gold, P. W. (1992). The Concepts of Stress and Stress System Disorders: Overview of Physical and Behavioral Homeostasis. *JAMA*, *267*(9), 1244-1252.

Claiborne, A. (2018). Catalase activity. In *Handbook methods for oxygen radical research* (pp. 283-284). CRC press.

Cohen, D. M., Inada, T., Iwamoto, T., & Scialabba, N. (1990). FAO species catalogue. Vol. 10. Gadiform fishes of the world (Order Gadiformes). An annotated and illustrated catalogue of cods, hakes, grenadiers and other gadiform fishes known to date.

Conte, F. S. (2004). Stress and the welfare of cultured fish. *Applied Animal Behaviour Science*, *86*(3), 205-223.

Davies, K. J. A. (1995). Oxidative stress: the paradox of aerobic life. *Biochemical Society Symposia*, *61*, 1-31.

Di Pane, J., Joly, L., Koubbi, P., Giraldo, C., Monchy, S., Tavernier, E., Marchal, P., & Loots, C. (2019). Ontogenetic shift in the energy allocation strategy and physiological condition of larval plaice (*Pleuronectes platessa*). *PLoS One*, *14*(9), e0222261.

Dreyer, B. M., Nøstvold, B. H., Midling, K. Ø., & Hermansen, Ø. (2008). Capture-based aquaculture of cod. *FAO (Food and Agriculture Organization of the United Nations) Fisheries Technical Paper*, *508*, 183-198.

Evjemo, J. O., Reitan, K. I., & Olsen, Y. (2003). Copepods as live food organisms in the larval rearing of halibut larvae (*Hippoglossus hippoglossus L.*) with special emphasis on the nutritional value. *Aquaculture*, *227*(1), 191-210.

© FAO 2024. *Gadus morhua*. Cultured Aquatic Species Information Programme. Text by Håkon Otterå. In: Fisheries and Aquaculture. Rome. Updated 2010-11-26 [Cited Thursday, September 19th 2024]. https://www.fao.org/fishery/en/culturedspecies/gadus_morhua/en

FAO. 2024. The State of World Fisheries and Aquaculture 2024 – Blue Transformation in action. Rome.

Fitzsimmons, S. D., & Perutz, M. (2006). Effects of egg incubation temperature on survival, prevalence and types of malformations in vertebral column of Atlantic Cod (*Gadus morhua*) larvae. *Bull. Eur. Ass. Fish Pathol*, *26*.

Flohé, L., & Otting, F. (1984). Superoxide dismutase assays. *Methods Enzymol*, *105*, 93-104.

Fraser, A. J. (1988). Changes in lipid content, lipid class composition and fatty acid composition of developing eggs and unfed larvae of cod *(Gadus morhua) (Vol. 99). Springer Science and Business Media LLC.*

Fridovich, I. (1998). Oxygen Toxicity: A Radical Explanation. *Journal of Experimental Biology*, *201*(8), 1203-1209.

Hampton, L. M. T., Jeffries, M. K. S., & Venables, B. J. (2020). A practical guide for assessing respiratory burst and phagocytic cell activity in the fathead minnow, an emerging model for immunotoxicity. *MethodsX*, *7*, 100992.

Hamre, K. (2011). Metabolism, interactions, requirements and functions of vitamin E in fish. *Aquaculture Nutrition*, *17*(1), 98-115.

Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L. E. C., & Izquierdo, M. (2013). Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing. *Reviews in Aquaculture*, *5*(s1), S26-S58.

Hansen, Ø. J., Puvanendran, V., Jøstensen, J. P., & Ous, C. (2011). Effects of dietary levels and ratio of phosphatidylcholine and phosphatidylinositol on the growth, survival and deformity levels of Atlantic cod larvae and early juveniles. *Aquaculture Research*, *42*(7), 1026-1033.

Hayyan, M., Hashim, M. A., & AlNashef, I. M. (2016). Superoxide Ion: Generation and Chemical Implications. *Chemical Reviews*, *116*(5), 3029-3085.

Herbing, I. H. v. (2001). Development of feeding structures in larval fish with different life histories: winter flounder and Atlantic cod. *Journal of Fish Biology*, *59*(4), 767-782.

Hoseinifar, S. H., Yousefi, S., Van Doan, H., Ashouri, G., Gioacchini, G., Maradonna, F., & Carnevali, O. (2020). Oxidative Stress and Antioxidant Defense in Fish: The Implications of Probiotic, Prebiotic, and Synbiotics. *Reviews in Fisheries Science & Aquaculture*, *29*(2), 198-217.

Howell, B. (1984). The intensive rearing of juvenile cod, *Gadus morhua L. Flødevigen Rapportserie*, *1*, 657-675.

Huang, Y., Xu, J., Sheng, Z., Chen, N., & Li, S. (2021). Integrated response of growth performance, fatty acid composition, antioxidant responses and lipid metabolism to dietary phospholipids in hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) larvae. *Aquaculture*, *541*, 736728.

Huntingford, F. A., Adams, C., Braithwaite, V. A., Kadri, S., Pottinger, T. G., Sandøe, P., & Turnbull, J. F. (2006). Current issues in fish welfare. *Journal of Fish Biology*, *68*(2), 332-372.

Ighodaro, O. M., & Akinloye, O. A. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine*, *54*(4), 287-293.

Imsland, A. K., Foss, A., Koedijk, R., Folkvord, A., Stefansson, S. O., & Jonassen, T. M. (2006). Short- and long-term differences in growth, feed conversion efficiency and deformities in juvenile Atlantic cod (*Gadus morhua*) start fed on rotifers or zooplankton. *Aquaculture Research*, *37*(10), 1015-1027.

Izquierdo, M., & Koven, W. (2011). Lipids. In *Larval Fish Nutrition* (pp. 47-81).

Jaxion-Harm, J. (2021). Effects of dietary phospholipids on early stage Atlantic Salmon (*Salmo salar*) performance: A comparison among phospholipid sources. *Aquaculture*, *544*, 737055.

Jomova, K., Alomar, S. Y., Alwasel, S. H., Nepovimova, E., Kuca, K., & Valko, M. (2024). Several lines of antioxidant defense against oxidative stress: antioxidant enzymes, nanomaterials with multiple enzyme-mimicking activities, and low-molecular-weight antioxidants. *Archives of Toxicology*, *98*(5), 1323-1367.

Kjesbu, O. S., Solemdal, P., Bratland, P., & Fonn, M. (1996). Variation in annual egg production in individual captive Atlantic cod (*Gadus morhua*). *Canadian Journal of Fisheries and Aquatic Sciences*, *53*(3), 610-620.

Kjørsvik, E., Olsen, C., Wold, P.-A., Hoehne-Reitan, K., Cahu, C. L., Rainuzzo, J., Olsen, A. I., Øie, G., & Olsen, Y. (2009). Comparison of dietary phospholipids and neutral lipids on skeletal development and fatty acid composition in Atlantic cod (*Gadus morhua*). *Aquaculture*, *294*(3), 246-255.

Koedijk, R. M., Folkvord, A., Foss, A., Pittman, K., Stefansson, S. O., Handeland, S., & Imsland, A. K. (2010). The influence of first-feeding diet on the Atlantic cod *Gadus morhua* phenotype: survival, development and long-term consequences for growth. *Journal of Fish Biology*, *77*(1), 1-19.

Kolkovski, S. (2001). Digestive enzymes in fish larvae and juveniles—implications and applications to formulated diets. *Aquaculture*, *200*(1), 181-201.

Kolkovski, S. (2013). 6 - Microdiets as alternatives to live feeds for fish larvae in aquaculture: improving the efficiency of feed particle utilization. In G. Allan & G. Burnell (Eds.), *Advances in Aquaculture Hatchery Technology* (pp. 203-222). Woodhead Publishing.

Li, K., Olsen, R. E., Østensen, M.-A., Altin, D., Kjørsvik, E., & Olsen, Y. (2016). Atlantic cod (*Gadus morhua*) larvae can biosynthesis phospholipid de novo from 2-oleoyl-glycerol and glycerol precursors. *Fish Physiology and Biochemistry*, *42*(1), 137-147.

Lilly, G. R., Wieland, K., Rothschild, B. J., Sundby, S., Drinkwater, K., Brander, K., Ottersen, G., Carscadden, J. E., Stenson, G. B., & Chouinard, G. A. (2008). Decline and recovery of Atlantic cod (*Gadus morhua*) stocks throughout the North Atlantic.

Lima, I., Moreira, S. M., Osten, J. R., Soares, A. M., & Guilhermino, L. (2007). Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. *Chemosphere*, *66*(7), 1230-1242.

Liochev, S. I., & Fridovich, I. (1994). The role of O2.− in the production of HO.: in vitro and in vivo. *Free Radical Biology and Medicine*, *16*(1), 29-33.

Martínez-Álvarez, R. M., Morales, A. E., & Sanz, A. (2005). Antioxidant Defenses in Fish: Biotic and Abiotic Factors. *Reviews in Fish Biology and Fisheries*, *15*(1), 75-88.

Martins, G., Diogo, P., Santos, T., Cabrita, E., Pinto, W., Dias, J., & Gavaia, P. J. (2020). Microdiet Formulation with Phospholipid Modulate Zebrafish Skeletal Development and Reproduction. *Zebrafish*, *17*(1), 27-37.

Moe, H., Dempster, T., Sunde, L. M., Winther, U., & Fredheim, A. (2007). Technological solutions and operational measures to prevent escapes of Atlantic cod (*Gadus morhua*) from sea cages. *Aquaculture Research*, *38*(1), 91-99.

Moksness, E., Kjorsvik, E., & Olsen, Y. (2008). *Culture of cold-water marine fish*. John Wiley & Sons.

Nandi, A., Yan, L. J., Jana, C. K., & Das, N. (2019). Role of Catalase in Oxidative Stress- and Age Associated Degenerative Diseases. *Oxid Med Cell Longev*, *2019*, 9613090.

Nardi, G., Prickett, R., van der Meeren, T., Boyce, D., & Moir, J. (2021). Atlantic cod aquaculture: Boom, bust, and rebirth? *Journal of the World Aquaculture Society*, *52*(3), 672-690.

Øie, G., Reitan, K. I., Evjemo, J. O., Støttrup, J., & Olsen, Y. (2011). Live Feeds. In *Larval Fish Nutrition* (pp. 307-334).

Øiestad, V., Kvenseth, P., & Folkvord, A. (1985). Mass Production of Atlantic Cod Juveniles *Gadus morhua* in a Norwegian Saltwater Pond. *Transactions of the American Fisheries Society*, *114*, 590-595.

Pedersen, T., & Falk-Petersen, I. B. (1992). Morphological changes during metamorphosis in cod (*Gadus morhua L.*), with particular reference to the development of the stomach and pyloric caeca. *Journal of Fish Biology*, *41*(3), 449-461.

Petersen, P. E., Penman, D. J., Dahle, G., Patursson, Ø., & Taggart, J. B. (2016). Differential Survival among Batches of Atlantic Cod (*Gadus morhua L.*) from Fertilisation through to Post-Metamorphosis. *PLoS One*, *11*(6), e0158091.

Puvanendran, V., & Brown, J. (2002). Foraging, growth and survival of Atlantic cod larvae reared in different light intensities and photoperiods. *Aquaculture*, *214*, 131-151.

Puvanendran, V., & Brown, J. A. (1999). Foraging, growth and survival of Atlantic cod larvae reared in different prey concentrations. *Aquaculture*, *175*(1), 77-92.

Puvanendran, V., Mortensen, A., Johansen, L.-H., Kettunen, A., Hansen, Ø. J., Henriksen, E., & Heide, M. (2022). Development of cod farming in Norway: Past and current biological and market status and future prospects and directions. *Reviews in Aquaculture*, *14*(1), 308-342.

Puvanendran, V., Swain, T., Tveiten, H., Hansen, Ø. J., & Mortensen, A. (2023). Optimizing intensive culture protocols for Atlantic cod (*Gadus morhua*) larvae. *Aquaculture International*, *31*(6), 3457-3472.

Randall, D. J., & Ferry, S. F. (1992). 4 Catecholamines. In W. S. Hoar, D. J. Randall, & A. P. Farrell (Eds.), *Fish Physiology* (Vol. 12, pp. 255-300). Academic Press.

Reid, S. G., Bernier, N. J., & Perry, S. F. (1998). The adrenergic stress response in fish: control of catecholamine storage and release1Communicated by Dr P.W. Hochachka, Editor.1. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, *120*(1), 1-27.

Rosenlund, G., & Halldórsson, Ó. (2007). Cod juvenile production: Research and commercial developments. *Aquaculture*, *268*(1), 188-194.

Salze, G., Tocher, D. R., Roy, W. J., & Robertson, D. A. (2005). Egg quality determinants in cod (*Gadus morhua L.*): egg performance and lipids in eggs from farmed and wild broodstock. *Aquaculture Research*, *36*(15), 1488-1499.

Schieber, M., & Chandel, N. S. (2014). ROS function in redox signaling and oxidative stress. *Curr Biol*, *24*(10), R453-462.

Schreck, C. B., & Tort, L. (2016). 1 - The Concept of Stress in Fish. In C. B. Schreck, L. Tort, A. P. Farrell, & C. J. Brauner (Eds.), *Fish Physiology* (Vol. 35, pp. 1-34). Academic Press.

Segner, H., Sundh, H., Buchmann, K., Douxfils, J., Sundell, K. S., Mathieu, C., Ruane, N., Jutfelt, F., Toften, H., & Vaughan, L. (2012). Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. *Fish Physiology and Biochemistry*, *38*(1), 85-105.

Sies, H. (1986). Biochemistry of Oxidative Stress. *Angewandte Chemie International Edition in English*, *25*(12), 1058-1071.

Skreslet, S. (2018). Ecology of Planktonic Atlantic Cod (*Gadus morhua*). InTech.

Slaninova, A., Smutna, M., Modra, H., & Svobodova, Z. (2009). A review: oxidative stress in fish induced by pesticides. *Neuro Endocrinol Lett*, *30 Suppl 1*, 2-12.

Svåsand, T., Otterå, H. M., Taranger, G. L., Litvak, M., Skiftesvik, A. B., Bjelland, R. M., Pavlov, D. A., Holm, J. C., Harboe, T., Mangor-Jensen, A., Norberg, B., & Howell, B. (2004). The Status and Perspectives for the Species. In *Culture of Cold-Water Marine Fish* (pp. 433-474).

Tocher, D. (2010). Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquaculture Research*, *41*.

Tocher, D. R. (2003). Metabolism and Functions of Lipids and Fatty Acids in Teleost Fish. *Reviews in Fisheries Science*, *11*(2), 107-184.

Tocher, D. R., Bendiksen, E. Å., Campbell, P. J., & Bell, J. G. (2008). The role of phospholipids in nutrition and metabolism of teleost fish. *Aquaculture*, *280*(1), 21-34.

Uzars, D., & Plikshs, M. (2000). Cod (*Gadus morhua L.*) cannibalism in the Central Baltic: interannual variability and influence of recruit abundance and distribution. *ICES Journal of Marine Science*, *57*(2), 324-329.

Valko, M., Izakovic, M., Mazur, M., Rhodes, C. J., & Telser, J. (2004). Role of oxygen radicals in DNA damage and cancer incidence. *Molecular and Cellular Biochemistry*, *266*(1), 37-56.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology*, *39*(1), 44-84.

Vo, T. A., Galloway, T. F., Bardal, T., Halseth, C. K., Øie, G., & Kjørsvik, E. (2016). Skeletal muscle growth dynamics and the influence of first-feeding diet in Atlantic cod larvae (*Gadus morhua L.*). *Biol Open*, *5*(11), 1575-1584.

Von Ossowski, I., Hausner, G., & Loewen, P. C. (1993). Molecular evolutionary analysis based on the amino acid sequence of catalase. *Journal of Molecular Evolution*, *37*(1), 71-76.

Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiol Rev*, *77*(3), 591-625.

Wiegand, M. D. (1996). Composition, accumulation and utilization of yolk lipids in teleost fish. *Reviews in Fish Biology and Fisheries*, *6*(3), 259-286.

Wold, P.-A., Hoehne-Reitan, K., Cahu, C. L., Infante, J. Z., Rainuzzo, J., & Kjørsvik, E. (2007). Phospholipids vs. neutral lipids: Effects on digestive enzymes in Atlantic cod (*Gadus morhua*) larvae. *Aquaculture*, *272*(1), 502-513.