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How can natural compounds supplemented in eggs improve digestive efficiency in fish larvae?

In ovo supplementation of spermine as promoter of early digestive system maturation

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In ovo supplementation of spermine as promoter of early digestive system maturation

Master's degree in Aquaculture & Fisheries

(Field of Aquaculture)

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Declaração de autoria de trabalho

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

Universidade do Algarve, 30 de setembro de 2020

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Agradecimentos

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Abstract

The world Human population is growing, and it is expected to reach almost 10 billion until 2050. The dietary protein trends in developing countries are changing from vegetable and cereal to animal sources. The superimposition of these anthropogenic factors will, if solutions are not found, increase the pressure on the already fragilized natural resources. Aquaculture is a growing animal production sector that may be the one of the solutions for supplying the world with food, specifically marine products, while reducing the pressure on natural stocks and having the minimum impact on the environment. Nonetheless fish production has some major bottlenecks concerning, for example, sustainable feed production and fish juveniles' supply. This is mostly due to the need to replace the typically provided live feeds with microdiets, specifically tailored to meet fish larvae´s nutritional needs, but still with constraints derived from fish larvae digestive inability to digest and assimilate the nutrients provided in these diets. For this reason, the objective of this study was to test the viability of early metabolic programing in *Solea seneganelensis*, a marine fish species with high commercial value, by supplementing eggs with three different amounts of spermine. Spermine, a polyamine shown to promote the maturation of the gut, was delivered through a novel technique for *in ovo* supplementation named sonophoresis. Each treatment was done in triplicate from egg stage until 35 days after hatching (DAH) larvae. Results demonstrate the short- and long-term safety of both, sonophoresis technique and *in ovo* supplementation with spermine. Regarding gut maturation, some degree of improvement in terms of lipolytic capacity were observed at 14 DAH in Sole from LOW and MED treatments. In the future, sonophoresis may become a keystone in early metabolic programing and the supplementation of spermine may generate more pronounced results if trials are conducted through longer periods and/or spermine supplementation is done through the dietary route.

Key words: digestive efficiency, early programming, Senegalese sole, spermine, sustainable aquaculture.

Resumo

A população humana mundial continua em elevado crescimento, é esperado que atinja perto de 10 bilhões até ao ano de 2050. Para além disso, nos países em desenvolvimento é constatada uma modificação dos padrões de consumo de proteína, designadamente no que diz respeito à sua proveniência, verificando-se uma substituição de fontes vegetais por fontes animais. A conjugação destes fatores antropogénicos irá no futuro, caso não sejam encontradas soluções viáveis, incrementar a já existente pressão imputada sobre os recursos naturais. A aquacultura é um dos sectores de produção animal que apresenta um maior crescimento e que poderá ser parte da muito necessária resposta ao fornecimento de alimento à escala mundial, particularmente de peixe e de marisco, e que promova a redução de pressão sob os stocks de pesca e minimize o impacto ambiental. Não obstante, a produção de pescado está sujeita a condicionantes, como a necessidade de uma elaboração mais sustentável de ração e a produção estável de juvenis. Esta última deve-se em parte, à necessidade de substituição de alimento vivo por alimento inerte especificamente formulados, que se adequem às necessidades nutricionais das larvas de peixes marinhos. Todavia, persistem ainda algumas limitações resultantes da incapacidade das larvas de peixes marinhos em digerir e assimilar os nutrientes presentes nestes alimentos. Por esta razão, o objetivo do presente estudo foi verificar a viabilidade da aplicação do conceito de "programação metabólica precoce" em linguado (*Solea senegalensis*), uma espécie de elevado interesse comercial, através da suplementação de distintas concentrações de espermina no estádio de ovo. A espermina é uma poliamina com resultados validados na promoção do desenvolvimento precoce do sistema digestivo em animais, tendo a sua aplicação sido efetivada com recurso a uma técnica recentemente desenvolvida (sonoforese), que permite suplementação de compostos através de canais criados na membrana do ovo. A suplementação foi realizada com três concentrações diferentes de espermina, sendo que para cada tratamento o cultivo foi feito em triplicado até ao trigésimo quinto dia após eclosão (DAE). Os resultados obtidos comprovam a segurança a curto e longo prazo tanto da utilização de sonoforese como da suplementação de espermina. No que diz respeito à maturação do sistema digestivo, foi verificado um incremento na capacidade lipolítica das larvas dos tratamentos LOW e MED aos 14 DAE. No futuro, a sonoforese poderá tornar-se um dos pontos-chave no contexto de "programação metabólica precoce" e a suplementação com espermina poderá gerar efeitos mais pronunciados em ensaios com uma duração superior e/ou cuja suplementação seja realizada pela via alimentar.

Palavras-chave: aquacultura sustentável, eficiência digestiva, espermina, programação metabólica, Linguado.

Abreviattions

ANOVA - analysis of variance

BHT - 2,6-Di-tert-butyl4-methylphenol

CTRL - control

DAH – days after hatching

DMSO – dimethylsulfoxide

DNA - deoxyribonucleic acid

DTNB - 5,5'-dithiobis-(2-nitrobenzoic acid)

g – grams

GC-MS - gas chromatography–mass spectrometry

GR - glutathione reductase

GSH – reduced glutathione

h - hours

HPF - hours post-fertilization

HUFA - highly unsaturated fatty acids

Hz – Hertz

L – liters

LPO – lipid peroxidation

m – meter

MED - medium

mg – milligrams

min – minutes

mL - milliliters

mM - millimolar

mM - millimolar

MSTFA - N-Methyl-N-(trimethylsilyl)trifluoroacetamide

mV – millivolts

n – number

nmol - nanomol

NADPH - Nicotinamide adenine dinucleotide phosphate

nm- nanometers

RFU - relative fluorescence units

RNA - ribonucleic acid

ROS - reactive oxygen species

SD – Standard deviation of the mean

sec – seconds

t – tons

TBA – 2-thiobarbituric acid

TCA - trichloroacetic acid

TCA cycle - tricarboxylic acid cycle

US$ - United States of America dollars

UV – ultraviolet

µg - micrograms

µL – microliters

µM - micromolar

Contents

1. Introduction

1.1 World aquaculture

The world Human population is growing. It is expected that by the year 2050 it will reach almost 10 billion. This increase will mean that, if not acted upon, the problematics of food security and nutrition will persist and increase. Nonetheless, satisfying the world's need for food cannot be done in a way that generates further toll on already fragilized ecosystems, increases greenhouse emissions or promotes further environmental degradation (FAO, 2017). Furthermore, socio-economic changes such as rising incomes, increased urbanization, and aging populations are generating a shift in food consumption patterns, resulting in an increased demand in animal-derived protein in developing countries (Henchion et al., 2017).

Aquaculture is the fastest growing food-producing sector in the world. Its global trend tends to expansion, intensification, and further development. Since the beginning of the 60s' until 2016 the average annual increase in global fish consumption grew at a faster pace than the world population production (Herrero et al., 2017) and by 2018, aquaculture production covered almost half of the world's fishery (figure 1) (FAO, 2020).

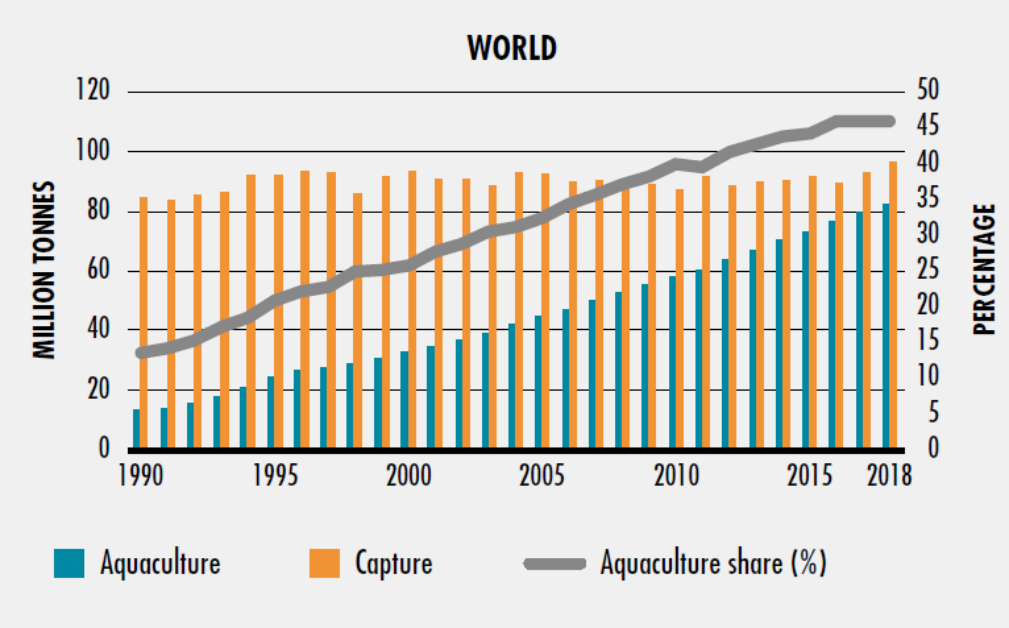

Figure 1 – Aquaculture share on the world's fisheries, source: FAO, 2020

Additionally, fish is on the top of food commodities traded around the world (FAO, 2016). The path leading to a sector that can provide food for millions of people, while trying to be as profitable and environmentally friendly as possible still has some challenges that need to be looked upon. One of the long-lasting goals for the aquaculture industry, and consequently for fish nutritionists, has been the reduction of feeding costs since in this sector more than half of the production costs has been and are spent on feed (Arru et al., 2019; Henry et al., 2015; Rana et al., 2009). Nonetheless, this cost reduction must be done while advocating for animal welfare and sustainability and, logically for final product quality. Feed production is, in fact, a sector that is in continuous development and where the cost volatility of the raw materials used, specifically protein sources, is leading to the search for alternative ingredients. Juveniles fish diets are generally composed by 20% to 55% of crude protein, depending on the fish species and nowadays the main protein source used is fish meal, although increasing prices and the decline of supplies are leading to less expensive sources such as animal and fisheries byproducts, and bacterial and plant proteins (Ayadi et al., 2012).

In Europe the aquaculture industry has grown over the last decades, but with a limited number of species contributing to this expansion, resulting in a need for the diversification of the cultured species, specifically in the south of Europe (Borges et al., 2009). European aquaculture assumes many different shapes and methods, each one adapted to the conditions of the farm and the geographical conditions of the coastal region where it is inserted, encompassing from Norway coast to the Mediterranean Sea. The uniqueness of the Mediterranean sea gives it a niche position within world aquaculture (Massa et al., 2017). The Mediterranean is a semi-enclosed sea, with specificities regarding its environmental, social and economic conditions. Its climate conditions are described by mild winters and warm to hot summers (Lionello et al., 2006), which are considered good conditions for the settlement of the aquaculture industry. The social and economic prospects are highly variable although, in a broad way, it has been estimated to a value of US$5.6 trillion (Randone et al., 2017).

Mediterranean aquaculture is not a novel sector, in fact, aquaculture in this area has been done for centuries (Beveridge and Little, 2007). Traditionally the setup for fish aquaculture in this region consisted of the use of excavated earth ponds, which is still one of the currently used ways of production. Nonetheless, offshore sea cages are being more commonly used due to the increasing production, the diversification of the cultured species and the improvement of the production

technologies. Currently, the most valued species in Europe are salmon and trout, representing 25 and 14 % of the of the value of total aquaculture output (Eurostat, 2020).

One of the challenges incurring from the increasing productivity of the sector is a higher demand for juvenile fish, which will be only satisfied if high-quality larvae and juveniles are produced in a sustainable way.

1.2 Larviculture challenges

Recently, no significant progress in what regards the increase of the survival rate of cultured marine fish larvae has been obtained when fed live preys, thus, offspring viability and predictability still represents a factor of the utmost importance for finfish production (Ueberschär et al., 2018). The fact is that still today, there is a bottleneck that occurs in the transition between yolk absorption and exogenous feeding, when a high mortality is usually recorded in marine fish larvae (Turingan et al., 2007). This means that, providing increasing number of juveniles to the aquaculture sector will probably, in the future, be dependent on increasing survival rates, promoting faster development and replacing live prey with optimally tailored microdiets. Larval growth is dependent on feeding, and feed particle utilization is dependent on a multitude of factors ranging from particle size and composition to the digestion and assimilation process (figure 2).

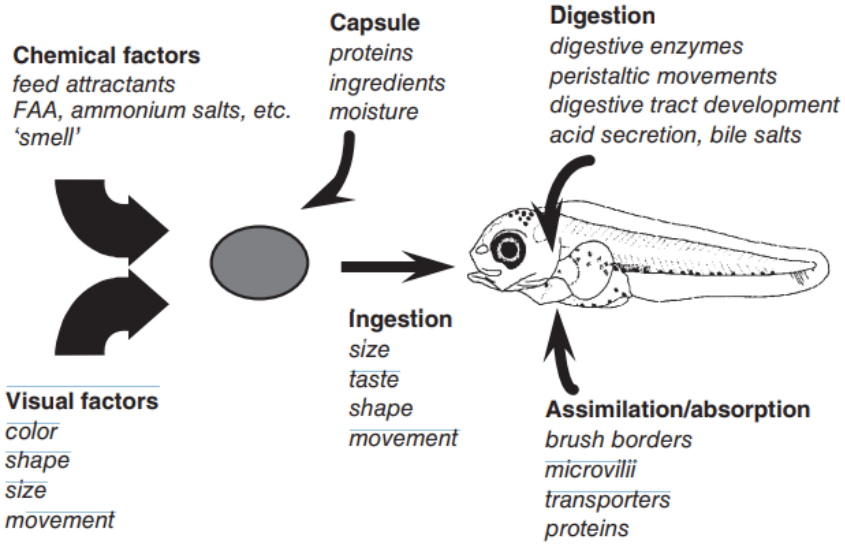

Figure 2 - Factors affecting food particle utilization, Source: Kolkovski et al., 2009

Furthermore a thorough knowledge in the biologic and physiologic characteristics of the species of interest is required for an optimization of the culture techniques (Martínez-Lagos et al., 2014). Moreover, these challenges still require extensive studies on the zootechnical, nutritional, genetic, along with many others, for a successful industry development. Specifically, it is needed to fully understand how the process of fish larvae development happens and how it affects its culture requirements such as hydrodynamics, light patterns, and diel feed intake.

In the last decades, the ontogeny of marine fish larvae has been the focus of several studies, with particular interest on the digestive aspects. These studies had the objective of complementing the needs of commercial hatcheries in the mitigation of the bottlenecks faced in fish larvae culture and nutrition. As described by Rønnestad et al. (2013), nutritional requirements knowledge is dependent on comprehension of the feeding biology and digestive physiology of larvae through growth. Regarding the developmental stage of fish at hatching, it can be divided into two main groups, the precocial and altricial larvae. In fact, most of the species used in Mediterranean aquaculture are what is known as an altricial larvae, characterized by a low level of ontogenetic development at the onset of exogenous feeding. On the other hand, precocial larvae have, in the first stages, characteristics much similar to adults, like Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). The dietary modulation of digestive enzymes, as well as the histological description of the main organs, has been broadly studied in marine fish larvae (Conceição et al., 2007; Martínez et al., 1999; Ribeiro et al., 1999b; Sarasquete et al., 1993; Zambonino Infante and Cahu, 2001). Dietary needs and digestive physiology knowledge needs to be in tune with the changing daily patterns of feed intake during ontogenesis, as well as the optimal dietary composition (Canada et al., 2019; Navarro-Guillén et al., 2015). Clearly, feeding behavior and digestive physiology play a major role in the optimization of the rearing protocols, leading to most needed improvements in larvae growth and survival (Rønnestad et al., 2013).

1.2.1 Larval Feeding

Larval rearing protocols typically contemplate the use of live feed approximately during the first month of age in most marine fish larvae. This, even though costly and sometimes unreliable, since it normally depends on several accessory cultures (such as microalgae, rotifers and *Artemia*), is done for the simple fact that there are some gaps on the knowledge of nutritional requirements

of marine fish larvae (Hamre et al. 2013), making difficult the formulation of a suitable microdiet. The onset of exogenous feeding is a particularly fragile cornerstone (Hamre et al., 2013) and at this time it is imperative that not only the larvae has properly developed all the organs needed for feed uptake, digestion and assimilation but also that he appropriate feed is provided (Yúfera and Darias, 2007). Developing inert feeds for fish larvae depends on a thorough knowledge on the nutritional requirements, diet digestibility and attractability potential. Meeting this will replace live feed with inert diets. Several trials have been done, on the use of co-feeding protocols (use of live and microfeed simultaneously), with varying results such as Rosenlund et al. (1997) that achieved improved growth and survival in co- fed Atlantic halibut larvae (*Hippoglossus hippoglossus*) while in turbot (*Scophthalmus maximus*) the growth rate did not seem to be altered by the use of co-feeding, however mortality was slightly higher. More recently in Gilthead seabream (*Sparus aurata)* fed in co-feeding with soybean meal demonstrated a limited ability of larvae to deal with this protein source at first feeding (Perera and Yúfera, 2017) while in red seabream (*Pagrus major*) and Ripon barbel (*Barbus altianalis*) growth was promoted with the use of co-feeding strategies (Duy Khoa et al., 2020; Aruho et al., 2020). In Senegalese sole (*Solea senegalensis*) co-fed from mouth opening, larvae were smaller at 20 DAH, however in the long-term, at 68 DAH, the same fish were larger than the fish exclusively fed with live feeds from mouth opening (Engrola et al. 2009). Nonetheless, in this species, results have shown that co-feeding with high levels of inert diet since mouth opening leads to reduced protein utilization (Engrola et al., 2010), but generates no compromise in terms of lipid metabolic utilization (Mai et al., 2009). Optimally, in the future, proper feed formulation and breakthroughs in the promotion of digestive system development will allow for the complete replacement of the use of live prey in altricial fish larvae species. For this, concepts like early nutritional programing can be extremely useful in the time to come.

1.3 Early programing

Early programming proposes that, a nutritional stimulus acting during critical periods of development may permanently alter the structure and functioning of the organism (Lucas, 1998). In nutrition, this gives the possibility of tailoring specific metabolic pathways in larval fish, such as the improvement of digestive efficiency as a result of a precocial intestinal maturation. In fish,

this concept has been applied in several species with different stimulus. For example, in European seabass (*Dicentrarchus labrax*) it was proven to be possible to modulate the metabolism of juveniles, making them more capable to adapt to a diet with low highly unsaturated fatty acids (HUFA) by exposing the fish in the larval stage to a HUFA deprived diet (Vagner et al., 2007). Also it has been demonstrated that supplementation of dietary spermine promoted a an enzymatic improvement in larvae (Pères et al., 1997). In zebrafish by enriching the eggs with glucose, a long-term modulation of carbohydrate metabolism was observed at later developmental stage (Rocha et al. 2014), while short-term exposure of first-feeding rainbow trout (*Oncorhynchus mykiss*) fry to plant based feeds generated improved growth, feed intake and feed utilization when the same fish were exposed to plant based diets at juvenile stage. The ability to improve utilization of a vegetable-based diet in Atlantic salmon through an early nutritional stimulus has been also tested by Clarkson et al. (2017) and additional results showed an up regulation of key pathways of intermediary metabolism, including oxidative phosphorylation, pyruvate metabolism, TCA cycle, glycolysis and fatty acid metabolism in Atlantic salmon (Vera et al. 2017).

Spermine is a biogenic polyamine formed from spermidine. A variety of organisms and tissues contain it and is an essential factor in for eukaryotic cell growth (NCBI, 2020). Polyamines (putrescine, spermidine, and spermine) are naturally occurring polycationic substances that are essential for cell proliferation and differentiation and in which the primary and secondary amino groups are protonated at physiological pH in cells. The synthesis of these three polyamines, in eukaryotic cells, is done by a series of enzyme reactions, with L-Arginine and L-Methionine as precursors (Wallace et al., 2003). The role of these molecules in processes such replication, transcription, translation, posttranslational modification, ion channel gating, and membrane stability is due to is interaction with negatively charged molecules such as DNA, RNA, proteins, and phospholipids (Igarashi and Kashiwagi, 2010). The interaction of these polyamines are closely linked to the regulation of cellular proliferation (Pegg and Casero, 2011; Tabor and Tabor, 1984). In mammals they are synthesized from arginine- or proline-derived ornithine (Wu et al. 2008). Several studies have been conducted, in rats and it has been confirmed that spermine supplementation in young rats leads to a precocious maturation of the intestinal tract, with structural and biochemical improvements such as villus height and crypt depth increase and enhanced antioxidant capacity (Buts et al., 1993; Dufour et al., *1988*; Elginaid Osman et al., 1998; Fang et al., 2016a; Harada et al., 1994) and more recently in suckling piglets in which it was

registered a premature gut maturation as well as an enhancement of the antioxidant status (Fang et al., 2016b). In the case of fish, it has been proven that, providing dietary spermine to European seabass larvae had an increasing effect on pancreatic enzymes as well as trypsin, chymotrypsin and amylase during larvae development (Pères et al. 1997). Taking into account the results of the already conducted researches on the topic it can be stated, that the branch of early metabolic programing has the potential to be of the upmost relevance within the aquaculture industry but in which a copious amount of research is still needed.

1.4 Sonophoresis

The introduction of exogenous compounds in fish is usually done through methods such as incorporation in feed, injection (intramuscular, intraperitoneal or intravenous), immersion or power spray (Bart et al., 2001). Nowadays, the direct injection of compounds represents one of the most effective way of administration in juvenile fish (Johnson and Amend, 1983; Palm Landolt et al., 1998). However, this method presents various disadvantages such as the need to handle individual fish, making it labor intensive, stressful to fish and difficult to apply in large populations. Those factors make it especially unviable when another factor is introduced into the equation: fish size during early developmental stages. Therefore a non-lethal and non-stressful method without compromising fish survival and fitness is needed in order to effectively deliver a specific compound during early stages of development (Bart et al., 2001), including the egg stage.

Some preliminary studies have been conducted to study the use of low-frequency ultrasounds to promote transport of compounds through fish skin and gills (Zohar et al. 1991; Frenkel et al. 1999). The fact that this method does not cause damage to fish health or wellbeing makes it a viable alternative for compound delivery. Sonophoresis consists of acoustic cavitation (acoustically induced bubble phenomena). During this process, adjacent bubbles merge to form larger bubbles that continue to enlarge until bursting, creating channels in the cell membranes. These channels grow with time allowing for molecules to transverse the entire thickness of the membrane, leading to the formation of new internal transport routes (Stewart et al., 2018).

Aside from the feeding pathway, the incorporation of specific nutrients in fish eggs through these new channels in the embryo membrane might be one the cornerstone of early programming in fish nutrition research. Some preliminary trials have been done with the use of sonophoresis

(low-frequency ultrasounds-based technique) with promising results shown by Engrola et al. (2014) that managed develop a method for *in ovo* delivery of nutrients, using a sonophoresis protocol and successfully supplement fish eggs with aspartate, having achieved almost direct dose-response, and Lopes (2016) that was able to supplement gilthead seabream eggs with methionine through sonophoresis, promoting growth in the long-term.

1.5 Senegalese sole

Senegalese sole is a marine fish belonging to the order of Pleuronectiformes. Its natural habitat is comprised by the Mediterranean and the south Atlantic coast. It has a high commercial interest being commonly reared in Spain and Portugal (Boglino, 2013; Villalta et al., 2005), with an amount of 100 t produced between Portugal and Spain in the year 2012 (Morais et al., 2016). This species is particularly relevant if the aquaculture production in Europe increase from 68 to 1457 t in the period of 2007 to 2015 is considered (FEAP, 2016).

The ontogenesis of Senegalese sole has been well described and it is characterized by a pronounced metamorphosis that occurs during the transition from larva to juvenile, associated with morphological, physiological, hormonal, behavioral and ecological changes (Geffen et al. 2007). In aquaculture this species bottleneck is related the difficulty on obtaining eggs from captive breeders (Sánchez et al., 2019). A high effort and interest in this species have led to, in the last decades, the gathering of knowledge regarding nutritional and zootechnical needs. However, weaning is still one of the most challenging rearing phases in Sole production (Pinto et al., 2018) but where massive breakthrough have been done in the last decades regarding the weaning protocols (figure 3).

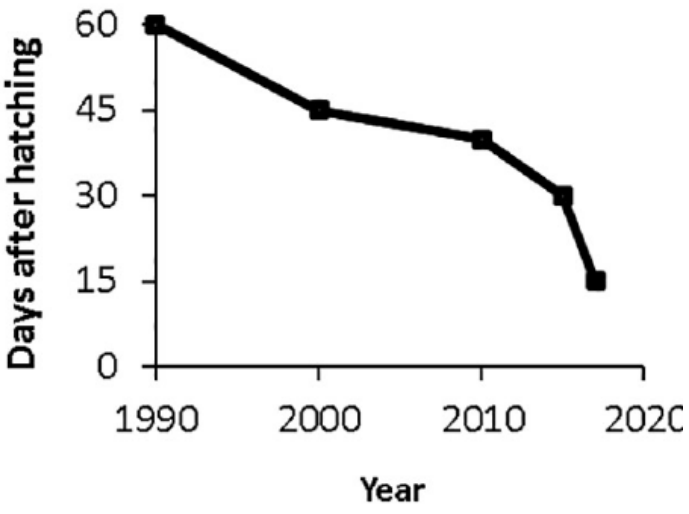

Figure 3 – Evolution on Senegalese sole (*Solea senegalensis*) age at the onset of weaning, Source: Pinto et al. (2018)

Senegalese sole undergoes a strong metamorphic process, as it happens in all flatfish, they hatch with the typical bilaterally symmetrical present in fish that than proceeds to acquire a lateralized swim posture after undergoing metamorphosis, a process characterized in part by the migration of the left eye (Xing et al., 2020), to the opposite side of the head and the transition to a lateralized swim posture (Schreiber, 2013). Such alteration is linked to the adaptation to the benthic habitat, which also comprises a change in dietary habits and therefore is accompanied by changes in digestive physiology (Tanaka et al., 1996)

In early stages, the absence of a functional stomach at the onset of exogenous feeding, similarly to other marine fish species, instigates a digestion based on pancreatic enzymes such as trypsin, lipase and amylase (Ribeiro et al., 1999a). During the yolk absorption phase, the digestive tract is a straight, undifferentiated tube. This then changes, at the onset of first feeding giving origin to a system divided into buccopharynx, foregut, midgut and hindgut. Acid digestion in the case of this species is never reached, there is, in fact a gastric pH decrease with the development but it never reaches values lower than 6 (Yúfera and Darías, 2007). Gastric gland development timing is still dabatable, with authors reporting its apearence at 18 DAH (Fehri-Bedoui et al., 2000), 27 DAH (Ribeiro et al., 1999a) and even between 35 and 40 DAH (Sarasquete et al., 2009).

In spite of the fact that, the rearing protocols for Senegalese sole are today standardized, several studies are still focused on the early stages, as nutritional improvements are a key factor on improving the quantity and quality of the juveniles so needed for the expansion of the production of this species.

2. Objectives

The main objective of this MSc thesis was to assess if spermine supplementation through sonophoresis technique in Senegalese sole (*Solea senegalensis*) eggs would promote a precocious digestive system maturation during larval stage. The supplementation efficacy was done by assessing key performance indicators, digestive enzymatic capacity and oxidative stress status in eggs and larvae.

3. Material and methods

3.1 *Solea senegalensis* egg supplementation

Senegalese sole eggs were obtained from natural spawn of a broodstock at - Estação Piloto de Piscicultura de Olhão (EPPO) – IPMA, and transported to Ramalhete Field Station facilities (Universidae do Algarve-CCMAR). Viable eggs selection was performed by buoyancy testing, where non-buoyant eggs were rejected. Subsequently the viable eggs were equally divided into four treatments to which a sonophoresis protocol for the supplementation of spermine was applied.

3.1.1 Sonophoresis Protocol

The sonophoresis prototype system was developed by AQUAGROUP (CCMAR) and previously used by Engrola et al. (2014) and Lopes (2016). The system is comprised of a signal generator, a signal amplifier, and an ultrasound immersion transducer (figure 4). Definition of the of the signal intensity, frequency and duration was done through a software. The protocol consisted in two pulses, each one with 150 sec of duration (5 sec of interval between pulses), with a frequency 80 000 Hz, and amplitude of 150 mV, following the protocol described by Engrola et al. (2014) and Lopes (2016)

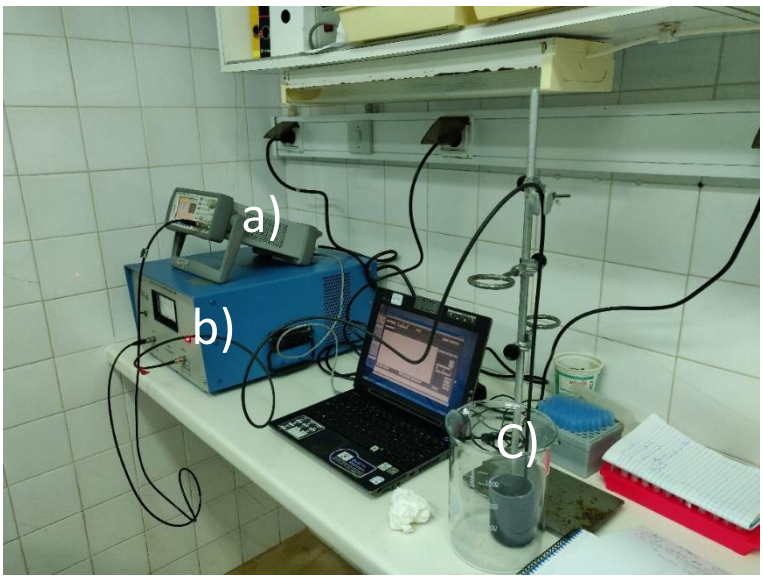

Figure 4 - Sonophoresis equipment: a) Signal generator; b) Signal amplifier; c) ultrasound immersion transducer

Approximately 30 hours post-fertilization (HPF) Senegalese sole eggs were submitted to four different treatments in triplicate (*n*=5000 eggs per replicate); Control (CTRL, no supplementation); Low (LOW; low spermine supplementation), Medium (MED, medium spermine supplementation) and High (HIGH; high spermine supplementation) in Ringer solution for teleost fish (204.4 g/L NaCl, 8 g/L KCl, 2.25 g/L CaCl2, 3.65 g/L MgCl2 + 6H2O, 2.25 g/L NaHCO3, pH 8.2) (Young, 1933). Spermine concentrations were selected based on preliminary trials done on incorporation efficiency (unpublished data). After sonophoresis, the eggs were maintained for one hour in seawater, to allow egg membrane stabilization. After that period 50 eggs per replicate were sampled for incorporation efficiency analysis, and the remaining eggs were equally divided per treatment in triplicate into 100L cylindroconical tanks for larval rearing.

3.1.2 Assessing the effects on hatching rate

To evaluate the effects of the use of the sonophoresis protocol, as well as the effect of the spermine supplementation in the eggs hatching rate, after sonophoresis 150 eggs per treatment were collected and placed in incubation chambers in triplicate (*n*=50 eggs per replicate) (figure 5) as well as 150 eggs (*n*=50 per replicate) not subjected to the sonophoresis protocol. The incubation chambers, with a bottom of a fine mesh, were placed floating in a rearing tank with recirculated

seawater at 18ºC and aeration and incubated for 24 h until hatching. The hatching rate was calculated by counting the number of larvae and applying the following formula:

$$Hatching\ rate\ (\%) = \frac{larvae\ (n)}{eggs\ (\mathrm{n})} \times 100$$

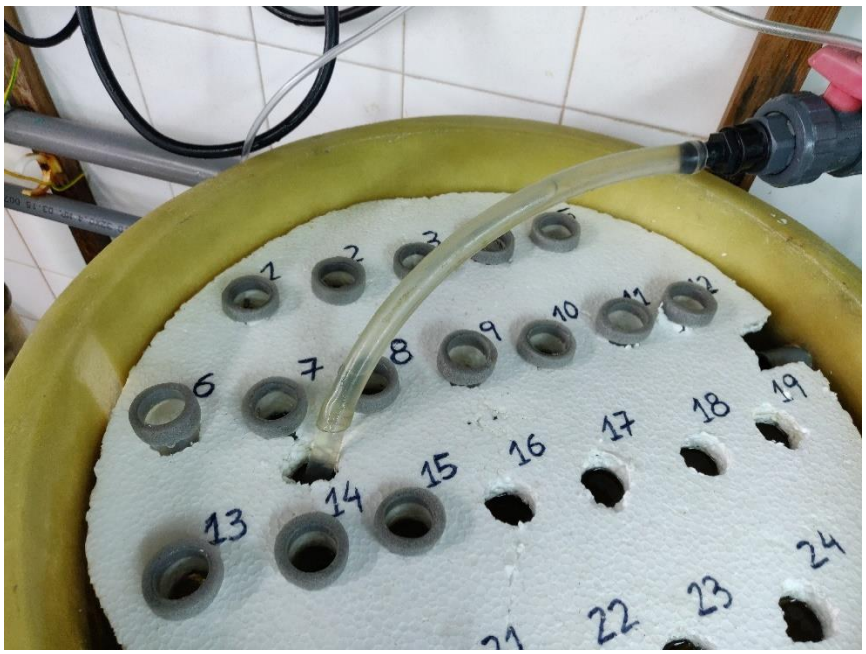

Figure 5 – Incubation chambers used to assess the egg hatching rate for the different treatments

3.2 Larval Rearing

Larvae rearing was done in two distinct systems, used for the pre- and post-metamorphic stage. The first one, comprising the first 19 DAH, when sole larvae are in the pelagic phase, was conducted at the Ramalhete Field Station. The second rearing phase, from 19 to 35 DAH, was conducted at CCMAR facilities at University of Algarve. The system used for the first 19 days, considered the pelagic behavior of the larvae, while in the second part of the trial , when larvae already undergone metamorphosis and adopted a benthic life-style, the system needed to accommodate the new habitat. The experiment was carried out in compliance with the Guidelines of the European Union Council (2010/63/EU) on the protection of animals used for scientific purposes.

3.2.1 Pelagic Phase

Larval rearing in the pre-metamorphic phase was done in 100L-cylindroconical tanks at an initial density of 50 larvae/L, in a temperature-controlled room with a seawater temperature of 18.9±1.0 ºC, dissolved oxygen in water was 93.5±5.9% of saturation and a salinity of 36.2±0.2 ‰. Photoperiod was adjusted to 10L: 14D (09:30h to 19:30h), with the light cycle starting on the 2 DAH. Water flow was maintained by individual internal circulation in each tank; and, external circulation, as part of a recirculation system, that included mechanical and a biological filter, UV filters, a protein skimmer and a seawater inlet automatically activated to compensate for seawater lost due to daily cleaning routines.

3.2.2 Benthic Phase

At the end of the metamorphic process Senegalese sole post-larvae were transferred to the second rearing system. This system was comprised by 3L-flat bottom tanks, a biological and mechanical filter, a protein skimmer and a UV filter. Larvae were distributed with an initial density of 235larvae / tank which represented a density of around 3100 larvae/m^2. Abiotic parameters were kept as stable as possible. Photoperiod was kept, as in the previous phase, at 10L: 14D (09:30h to 19:30h). Temperature was controlled and kept at 19.3±1.3 ºC, oxygen in water was maintained at 92.6±3.4% saturation and salinity 31.0±2.8 ‰.

3.3 Feeding

For the purpose of assessing the effect of *in ovo* spermine supplementation as a promoter of digestive system maturation a nutritional challenge was purposed. This challenge consisted on the use of early co-feeding protocol, with a provided amount of live prey 20% lower than the usual feeding protocols, together with an early weaning, meaning that larvae had less live prey for a shorter period, promoting a progressive adaptation to inert diet ingestion and digestion. The objective was to persuade larvae to depend on the ingestion of inert diet from mouth opening. Therefore, live prey was progressive reduced concomitantly with an increase of the inert diet, from mouth opening (2 DAH) until weaning (21 DAH). Live prey was supplied 4 times a day (50% at 10.30h, 10% at 12.00h, 10% at 14:30h and the remaining 30% at 16:50h) while inert diet was provided continuously during the day using automatic feeders. Live prey, rotifers (*Brachionus*

spp.), *Artemia* nauplii and metanauplii (*Artemia sp.*), were provided according to the following protocol: rotifers from mouth opening (2 DAH) until 5 DAH, *Artemia nauplii* from 5 to 13 DAH and *Artemia metanauplii* from 13 to 21 DAH, from 22 DAH until the end of the experiment larvae were fed only with inert diet. The inert diet used consisted of WinFast until the 19th day and WinFlat, commercial feeds produced by SPAROS Lda (Olhão, Portugal), from then onwards. These feeds were supplied in two different sizes (100–200 and 200–400 μm.), according to the larvae size.

Both, rotifers and *Artemia* metanauplii were enriched with commercial products. In the case of rotifers, two meals of Easy DHA SELCO (INVE, Belgium) were provided overnight (2 x 0.05g/L). In the case of *Artemia* metanauplii, two meals of the enrichment emulsion were provided overnight, each one composed by 0.2 g/L of Easy DHA SELCO (INVE, Belgium) and 0.2 g/L of Micronorse®. Green water technique was used during the pre-metamorphic phase by adding, before the 1st and last feeding, 5 g of algae divided by the 12 tanks. This technique has been used for several years and it is recognized to improve the larvae survival and growth rates for several marine fish species (Faulk and Holt, 2005; Gulbrandsen et al., 1996; Naas et al., 1996; Reitan et al., 1997; Rocha et al., 2008).

3.4 Sampling

To evaluate treatment's effects, four different developmental stages were considered, two before metamorphosis and two after metamorphosis: A) mouth opening (3 DAH); B) start of the metamorphosis (14 DAH); C) almost at the end of metamorphosis (end of the pelagic phase, 19 DAH); and D) benthonic post-larval phase (35 DAH) (Table 1). The samples collected were always washed twice in distilled water with the purpose of reducing the amount of salt, after were snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

TABLE 1 - -SAMPLING POINTS

STAGE	MOUTH OPENING	START METAMORPHOSIS	END PELAGIC	END
Code	**MO**	**SM**	**EP**	**END**
Days After Hatching (DAH)	**3**	**14**	**19**	**35**

3.5 Key performance indicators

For the determination of individual dry weight larvae were sampled at 3 DAH (*n*= 3 pools of 30 larvae per treatment), 14 DAH (*n*=15 individual larvae per replicate), 19 DAH (*n*=15 individual larvae per replicate) and 35 DAH (*n*=20 individual larvae per replicate), freeze-dried and weighted in a precision scale (±0.001 mg, Sartorius MSA365-000-DH). Larval total length at the end of the experiment was determined by image analysis using a stereomicroscope (Leica S9) with camera and ImageJ software. Survival rate was calculated for each developmental stage (benthic and pelagic) by using the following formula:

$$Survival\ rate\ (\%) = \frac{final\ number\ of\ individuals}{initial\ number\ of\ individuals} \times 100$$

3.6 Biochemical Analysis

For the purpose of evaluating the digestive maturation induced by spermine supplementation several biochemical analyses were conducted. Since *in ovo* spermine supplementation it had never done before and was one of the fundamental results arising from this trial, supplementation efficiency was determined by measuring the spermine content in samples of eggs for each treatment. The analysis of digestive enzyme activities such as lipase, trypsin, chymotrypsin, amylase, alkaline phosphatase, and aminopeptidase was used as direct indicator of larval digestive capacity (Engrola, 2008; Navarro-Guillén, 2016).

3.6.1 *In ovo* spermine incorporation efficiency

For the evaluation of spermine incorporation into the eggs, 3 pools of 50 eggs per treatment were snap frozen at the time of sampling and kept at -80°C. For the analysis the samples were thawed and homogenized in 1 mL 5% trichloroacetic acid (TCA), followed by 10 min centrifugation at 12500 g's and 4°C, by the end of which the supernatant was collected and mixed with 1 mL of 5% TCA. A second centrifuge cycle with the same characteristics as the first was

performed. Supernatant was collected after the second centrifuge cycle and mixed with a solution of 28 % NH_4 until pH 11 was achieved. After this the sample was loaded into a Strata-X 33u Polymeric Reversed Phase Cartridge extractor previously conditioned with 4 mL methanol + 4 mL milli Q water using a vacuum system. 2 mL of the sample were passed through the cartridges, followed by a rinse with 2 mL of 5% methanol. Cartridges were, then, dried under vacuum for 5 min, to remove excess of water. Analytes were eluted from the Strata-X sorbents with 2 + 2 mL of a mixture methanol/acetic acid (99:1, v/v) (Sagratini et al., 2012). The eluting solutions were dried with nitrogen gas and derivatized with N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA). Sample processing was done until this step and kept at -20ºC, but the finalizing step for spermine levels measurement, a gas chromatography–mass spectrometry (GC-MS) protocol (Chen et al.2009), was not applied due to equipment malfunction. Samples will be analyzed, and the results published as soon as the process is finalized.

3.6.2 Enzymatic analysis

Enzymatic analyses were done at 14 DAH (n=3 pools of 2-3 larvae per replicate), 19 DAH (n=3 pools of 2-3 larvae per replicate) and 35 DAH (n=10 pools of 2 larvae per replicate). Before the enzymatic analysis the pools were freeze dried and subsequently homogenized in distilled water (220µL for 14 DAH, 400µL for 19 DAH and 500 µL for 35 DAH). Freeze drying was performed since previous studies have determined that hydrated freeze dried samples maintained the original enzymatic functions (Lau et al., 2013; Spigno et al., 2007; Sundari and Adholeya, 2000). Following this homogenization, the samples were centrifuged at 12500 g's for 5 min at 4°C and the supernatant collected. Samples were maintained in ice during the homogenization process and later analysis to prevent sample degradation. The supernatant (enzyme extract) was stored at -20°C until the analyses were performed. The digestive enzymes were analyzed using a fluorescent subtract specific for each enzyme. Trypsin analysis was performed using Boc-Gln-Ala-Arg-7-methylcoumarin hydrochloride (BOC-SIGMA B4153). Chymotrypsin analysis was performed using N-Succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin (SIGMA S9761). Both substrates were diluted in dimethylsulfoxide (DMSO), to a final concentration of 20 µM. For the analysis of the previously mentioned proteases 15 µL of sample homogenate, 5µL of the substrate and 190 µL of 50 mM Tris + $CaCl_2$ 10 mM (buffer, pH 8.5) were added to a microplate and fluoresce was measured at 355 nm (excitation) and 460 nm (emission). Aminopeptidase, was measured following

the protocol described by Sanz and Toldra (2002) using Nα-Benzoyl-L-arginine-7-amido-4-methylcoumarin hydrochloride (SIGMA B7260) diluted in DMSO, to a final concentration of 20 μM, as substrate. For the analysis 15 μL of sample homogenate, 5μL of the substrate and 190 μL of 50 mM Tris (buffer, pH 8.5) were added to a microplate and fluoresce was measured at 355 nm (excitation) and 460 nm (emission). Lipases, 4C- and 18C-like lipases, were measured using the reactive substrates 4-methylumbelliferyl butyrate (MU-Bu, SIGMA M19362) and 4-methylumbelliferyl oleate (SIGMA M75164), respectively, diluted to a final concentration of 0.4 mM in a phosphate buffer at pH 7. For the analysis 15 μL of sample homogenate were loaded into a microplate followed by 250 μL of substrate, following the protocol of Rotllant et al. (2008). Fluorescence was measured at 355 nm (excitation) and 460 nm (emission). Amylase analysis was done using Ultra Amylase Assay Kit (Invitrogen E33651), a commercially available kit based on starch derivate labeled with a fluorophore dye as substrate. Preparation of the substrate + buffer solution was done according to manufacturer's instructions. Reading was made by loading 15 μL of sample homogenate into a microplate and mixture with 50 μL of substrate. Fluoresce was measured at 485 nm (excitation) 538 (emission). Alkaline phosphatase was measured applying a modification to the protocol described by Fernley and Walker (1965). 4-methylumbelliferyl phosphate (MUP, SIGMA M8168) was used as reactive fluorescent substrate for alkaline phosphatase. Preparation of the substrate was executed by diluting the substrate to a concentration of 10 mM in borate buffer solution (pH=8.5). For the analysis 15 μL of sample homogenate were loaded into a microplate followed by 100 μL of MUP substrate and fluoresce was measured at 360 nm (excitation) 400 nm (emission).

All enzyme activities were expressed as relative fluorescence units (RFU) per mg of larva dry weight.

3.6.3 Oxidative stress indicators

As indicators for the oxidative stress status both glutathione and lipid peroxidation (LPO) were measured in whole fish larvae homogenates. Oxidative stress happens when there is an imbalance between the reactive oxygen species (ROS) and the ability of an organism to respond to it by using antioxidants, which results in oxidative stress and consequently damage in cellular components. (Asada, 1984; Fridovich, 1998; Halliwell and Gutteridge, 2015). Lipid peroxidation happens as an interaction between lipids and ROS and results in loss of cell's integrity (Dix and Aikens, 1993)

while glutathione is considered one of the most relevant cellular redox buffers (Gaucher et al., 2018), meaning it plays a role of the upmost importance regarding protection against oxidative stress.

For the analysis of LPO it was followed the protocol described by Bird and Draper (1984) Briefly, samples were homogenized in 1200 µl ultra-pure water, 200 µl of homogenate were separated and mixture with 4 µL of 2,6-Di-tert-butyl4-methylphenol (BHT) 4% in methanol and vortex to avoid the oxidation of lipids. 100µL of cold TCA 100% were added to the samples, followed by 1000 µl of 2-thiobarbituric acid (TBA) 73%. Samples were incubated at 100°C for 1h. After this period, samples were kept for up to 16 hours in the dark. Samples were centrifuged at 10350 g's for 5 min at 25 °C and 300 µl of resulting supernatant were loaded into a microplate and absorbance was read at 535 nm.

Total glutathione content was determined using a recycling reaction of reduced glutathione (GSH) with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase (GR) excess (Baker et al., 1990; Tietze, 1969) . For that, 50 µl of homogenate was loaded into a microplate and mixture with 250 µl of reaction buffer solution of GR, Nicotinamide adenine dinucleotide phosphate (NADPH) and DTNB on Na-K phosphate buffer, 0.2M; pH 8.0), and absorbance was read at 412 nm during 3 min.

3.7 Statistical analysis

Differences in growth performance, intestinal maturation parameters (trypsin, chymotrypsin, lipase, amylase and aminopeptidase activities), proximal composition and oxidative stress status due to treatments were evaluated using a one-way analysis of variance (ANOVA) after assessing equality of variances by a Levene's test. Post hoc multiple comparisons were carried out using Tukey's test. If equality of variances was not observed a Kruskal-Wallis non-parametric test was performed. Before analysis, all data was verified for outliers within each treatment by conducting a Grubbs' test. Percentual data, such as hatching and survival rate, were arcsine square root-transformed prior to analysis as described Pike et al. (1982). Statistical differences were considered significant at $p<0.05$. Analyses were performed with SPSS 26 software (IBM, New York, U.S.A.). Descriptive statistics are expressed as mean ± SD.

4. Results

4.1 Key performance indicators

4.1.1 Hatching rate

The spermine supplementation did not negatively affected the hatching rate (p-value=0.594). Overall average shows a hatching rate of 72.3±9.7 %. Analysis of the hatching rate demonstrates an inversely proportional trend between spermine supplementation and variance of treatment replicates (figure 6). It is also noticeable that all treatments with spermine present hatching rates with higher deviation than the control treatment. Furthermore, the second control treatment used, consisting of eggs not subjected to sonophoresis resulted in a hatching rate of 70±4%, and statistically equal to the treatments that were subjected to sonophoresis technique.

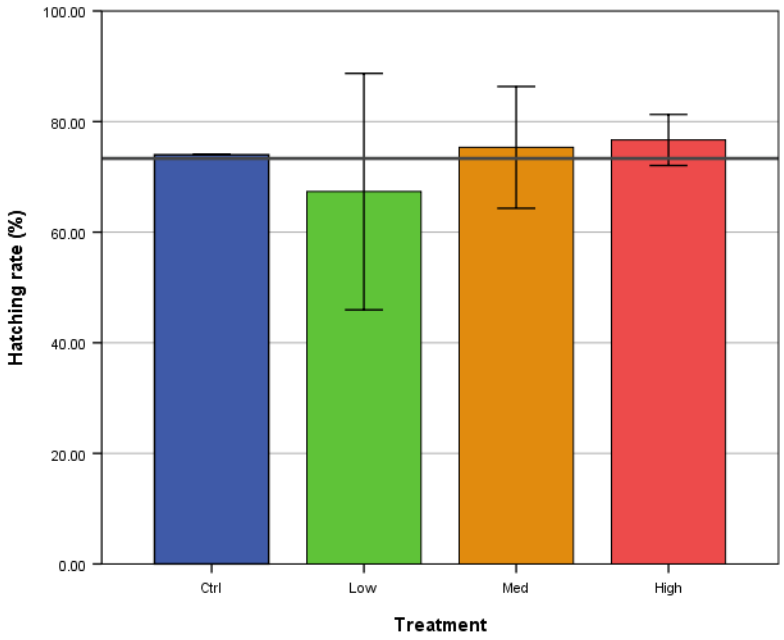

Figure 6 – Sole eggs hatching rate (%) between treatments. Values shown are means (±SD) of treatment replicates (n=3). A grey line marks the average between treatments. Absence of letters indicates no statistical differences (p-value≥0.05).

4.1.2 Survival rate

Survival rate, determined at 19 DAH (figure 7a), was similar among treatments (p-value=0.152). In relation to the post-metamorphic stage (figure7b), no differences between treatments were detected (p-value=0.975). Survival rate average in the pelagic and benthic phase were of 57.0±10.6% and 72.3±16.8%, respectively.

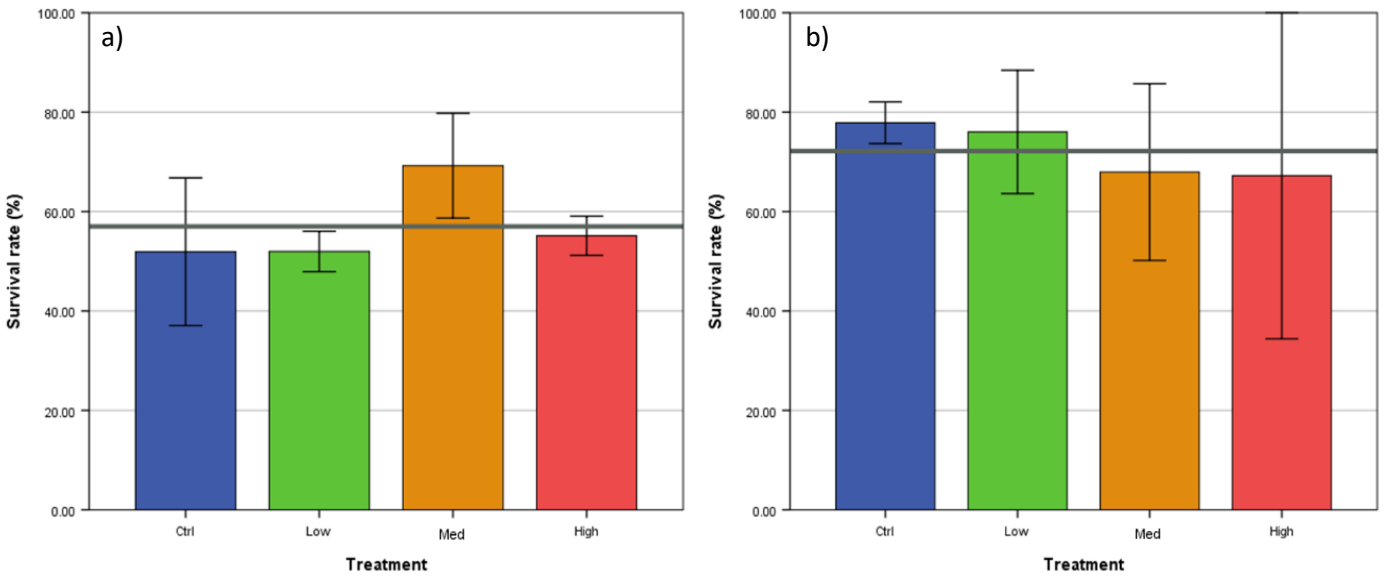

Figure 7 – Sole survival rate (%) in: a) the pelagic phase and b) benthic phase. Values shown are means (±SD) of treatment replicates (n=3). A grey line marks the average between treatments. Absence of letters indicates no statistical differences between treatments (p-value≥0.05).

4.2.3 Biometrics

Initial average dry weight (3 DAH) values ranged between 22.2 - 33.2µg for the treatments LOW and HIGH, respectively. Spermine supplementation had no effect on larvae dry weigh (p-value=0.690) (figure 8a). Individual dry weight was determined to be of 23.0±2.3µg. At 14 DAH (figure 8b) larvae from CTRL and MED treatments were statistically heavier than those from LOW and HIGH treatments (p-value=0.000). Individual dry weight averaged between 0.501 and 0.608 mg for treatments, LOW and MED, respectively. At 19 DAH (figure 8c) no statistical differences were found between larvae from the different treatments (p-value=0.239). Average weights ranged from 0.736 to 0.801 mg in treatments HIGH and MED, respectively. Overall, weight average was

of 0.758±0.134 mg. At the end of the trial (figure 8d), post-larvae presented similar weight (p-value=0.469). Averages ranged from 1.390 to 1.580 mg, for treatments LOW and CTRL, respectively. Average weigh at 35 DAH was 1.465 ±1.051 mg.

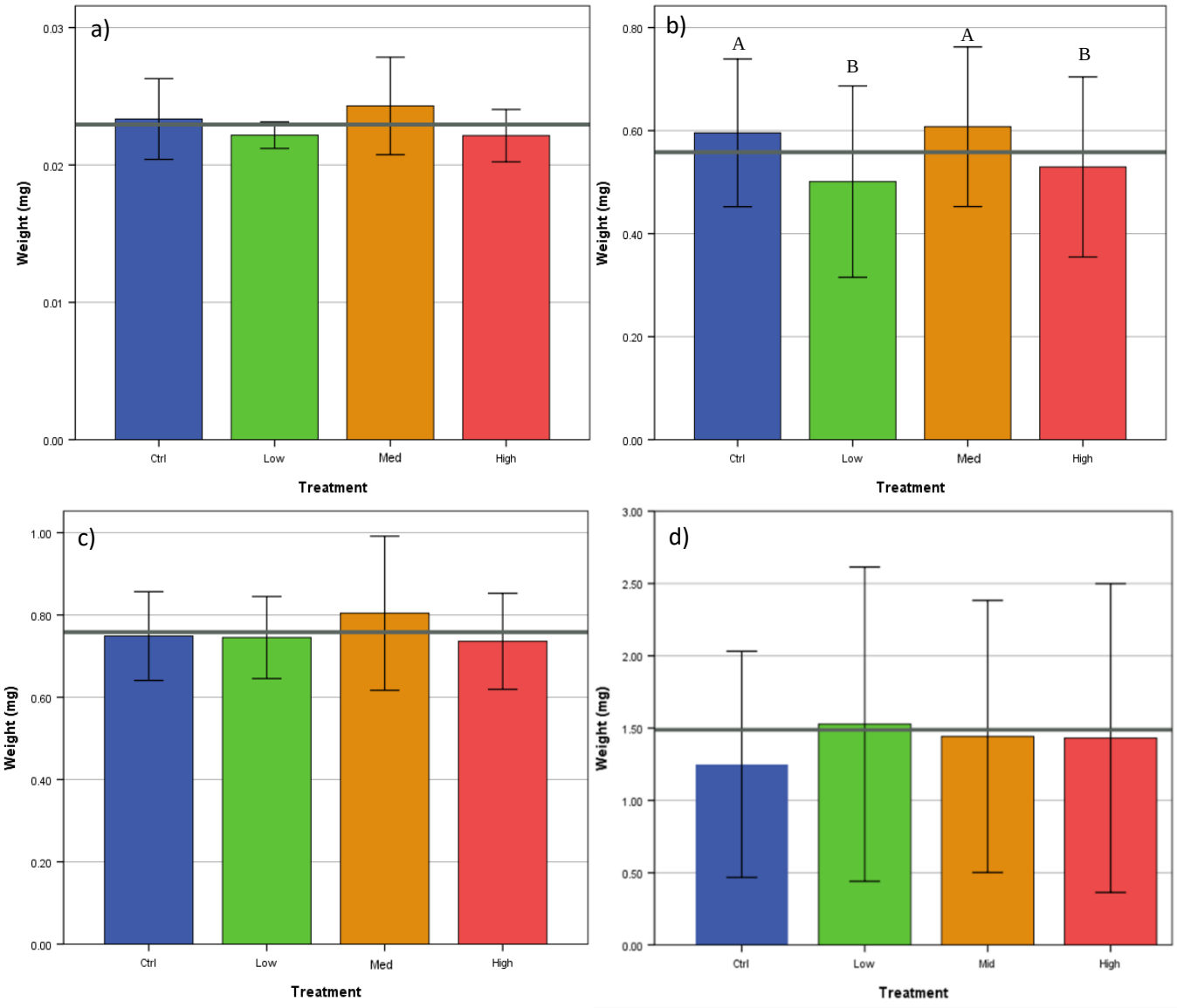

Figure 8 – Dry weight of larvae at: a) 3 DAH; b) 14 DAH; c) 19 DAH and d) 35 DAH. Mean values (±SD) of treatment (n=60) are shown. A grey line marks the average between treatments and letters mark statistical differences between treatments (p-value<0.05).

In relation to length, measured at 35 DAH, statistically significant differences were detected between treatments. These differences were observed between treatments LOW and HIGH (p-value=0.012) (figure 9).

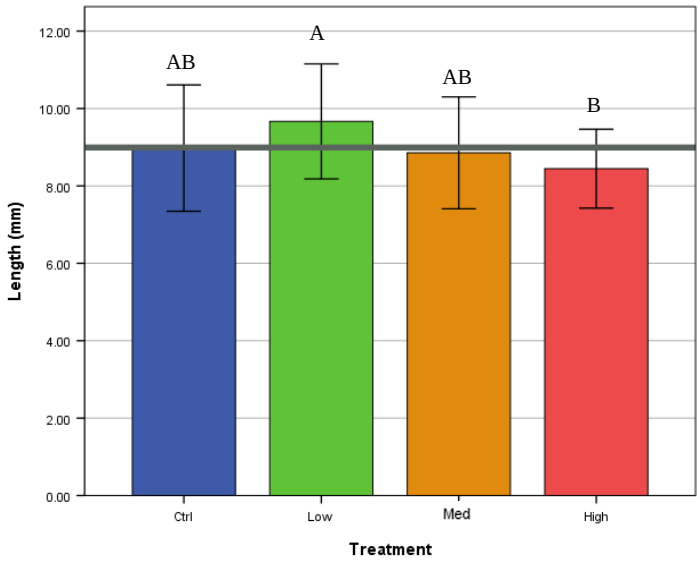

Figure 9 – Sole length at 35 DAH. Values are means (±SD) of treatment replicates (n=30). A grey line marks the average between treatments and letters represent statistical differences between treatments (p-value<0.05).

4.2 Digestive enzymes

For trypsin, despite no significant differences being detected in larvae in the first two sampling points, at 14 and 19 DAH (figure 10), with p-values of 0.173 and 0.678, respectively, significant differences were found between treatments at 35 DAH (figure 10). This difference was nonetheless between fish in treatments LOW and MED, meaning that no significant differences were generated in any treatment when compared with the control treatment. Regarding the temporal evolution, a similar pattern is verified in all treatments with an activity peak at 14 DAH, with an overall average of 15956±12998 RFUs/mg that decreases greatly to an average of 2201±764 at 19 DAH and posteriorly increases slightly to 3176±1458 RFUs/mg for fish at 35 DAH. Statistically the decrease in activity from 14 DAH to 19 DAH is significant for larvae from treatments CTRL, LOW and MED. Regarding the slight increase from 19 DAH to 35 DAH, it was only statistically significant for MED larvae (p-value=0.011).

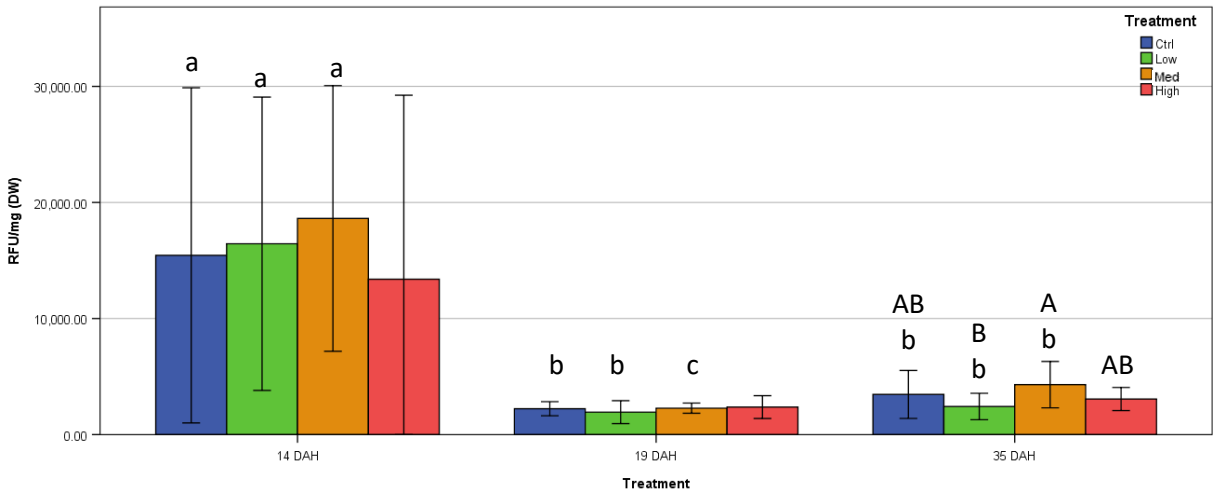

Figure 1 – Sole trypsin activity for the four treatments. Values shown represent means (±SD). Letters indicates the presence of statistical difference (p-value<0.05). Lower case letters (a, b, c) refer to enzymatic shifts during larval development (14, 19 and 35 DAH) within each spermine supplementation (CTRL, LOW, MED, HIGH) and (A, B) refer to differences between treatments at the same age.

Throughout the developmental period alkaline phosphatase activity (figure 11) demonstrated no significant differences between treatments, with p-values for 14, 19 and 35 DAH of 0.865, 0.347 and 0.400, respectively. A temporal pattern similar to the one detected for trypsin can be observed for this enzyme, with an overall average peak detected for the larvae sampled at 14 DAH of 24407±8101 RFUs/mg that steeply declines to 2334±804 RFUs/mg at 19 DAH and then increases to an average of 20609±6778 RFUs/mg. In the case of this enzyme, for larvae of all treatments, it is noticeable a significant decrease in activity at 19 DAH, returning to values statistically similar to the initial ones at 35 DAH (figure 11).

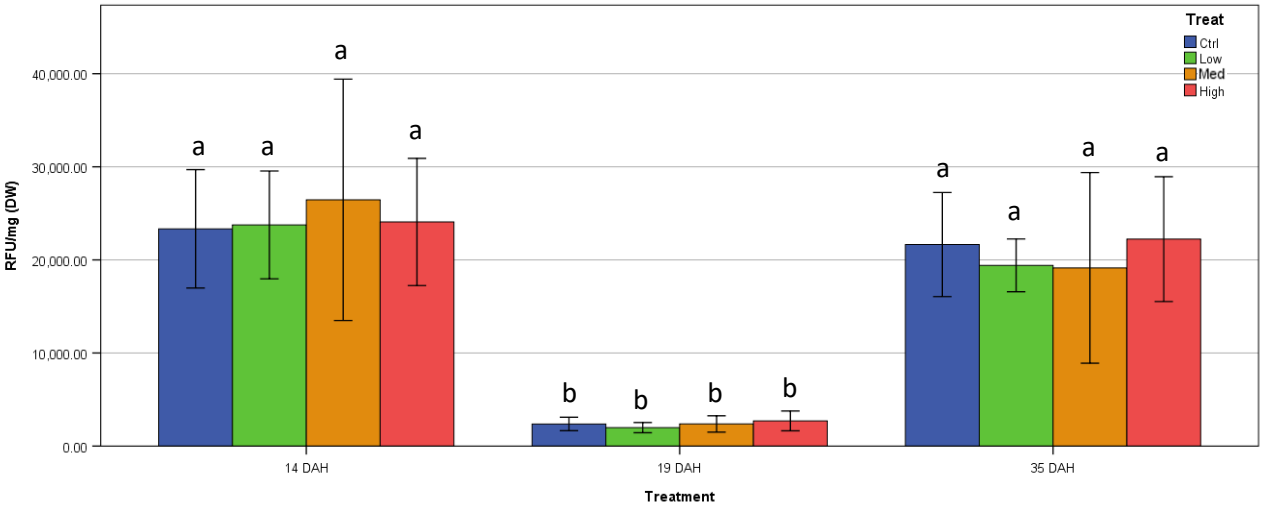

Figure 11 – Sole alkaline phosphatase activity for the four treatments. Values shown represent means (±SD). Letters indicates the presence of statistical difference ($p<0.05$). Lower case letters (a,b) refer to enzymatic shifts during larval development (14, 19 and 35 DAH) within each spermine supplementation (CTRL, LOW, MED, HIGH). Absence of capital letters indicate no differences between spermine supplementation at the same age.

For the short chain lipase (4C-like lipase) (figure 12), no statistically differences were detected between treatments in the first two sampling points, at 14 and 19 DAH, with p-values of 0.06 and 0.469, respectively. In the case of the activity for the fish of the last sampling point significant differences were detected between treatments (p-value=0.005), with significant differences recorded between treatments LOW/MED and HIGH. Alike what was verified for trypsin activity, a clear pattern is present, with an overall average peak of activity at 14 DAH of 2977±742 RFUs followed by a decline to an average of 150±150 RFUs followed and a slight increase to 505±138 RFUs of average. In this case, for all treatments the decrease in activity from 14 to 19 DAH is significant as well as the increase from 19 to 35 DAH, assuming values also significantly different to the initial ones.

The second analyzed lipolytic enzyme, 18C-like lipase (figure 13) presented significant differences between treatments (p-value=0.000) with larvae from LOW and HIGH showing statistically higher activity than CTRL larvae at 14 DAH. Associated with this disparity in the average activity values it is also noticeable a considerable increase in variance in treatment MED.

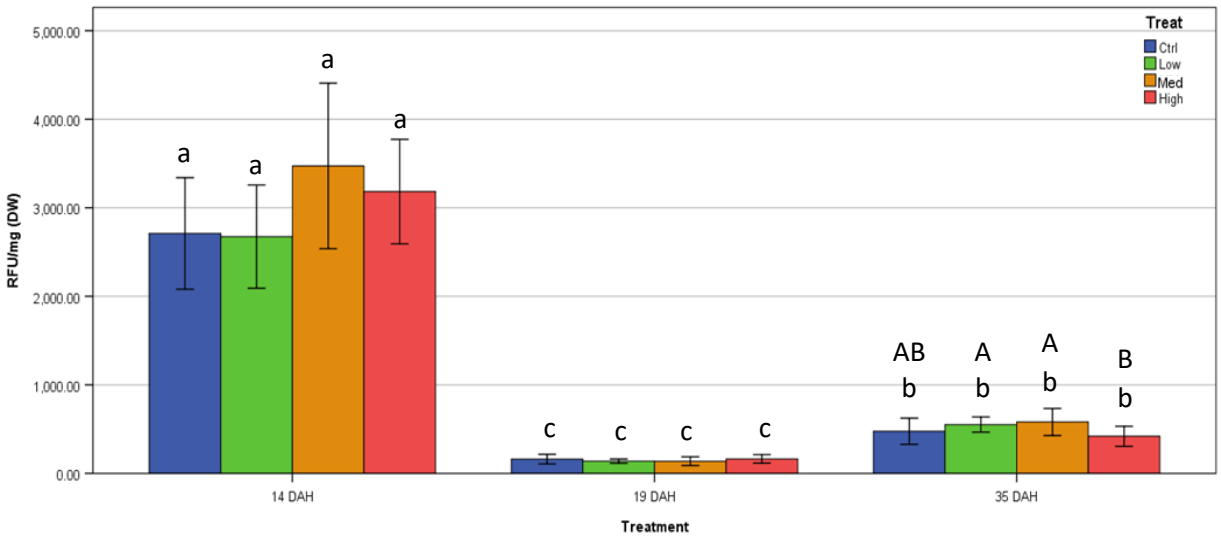

Figure 2 – Sole 4C like Lipase activity for the f Figure 11 - 4 C Lipase activity for the four treatments. Values shown represent means (±SD). Letters indicates the presence of statistical difference ($p<0.05$). Lower case letters (a, b, c) refer to enzymatic shifts during larval development (14, 19 and 35 DAH) within each spermine supplementation (CTRL, LOW, MED, HIGH). Capital letters (A, B) indicate differences between spermine supplementation at the same age.

For the second sampling point no 18C-like lipase activity was detected. For fish at 35 DAH (figure 13) no significant difference was detected. The same temporal pattern can be observed for all treatments with a statistically significant decrease at 35 DAH.

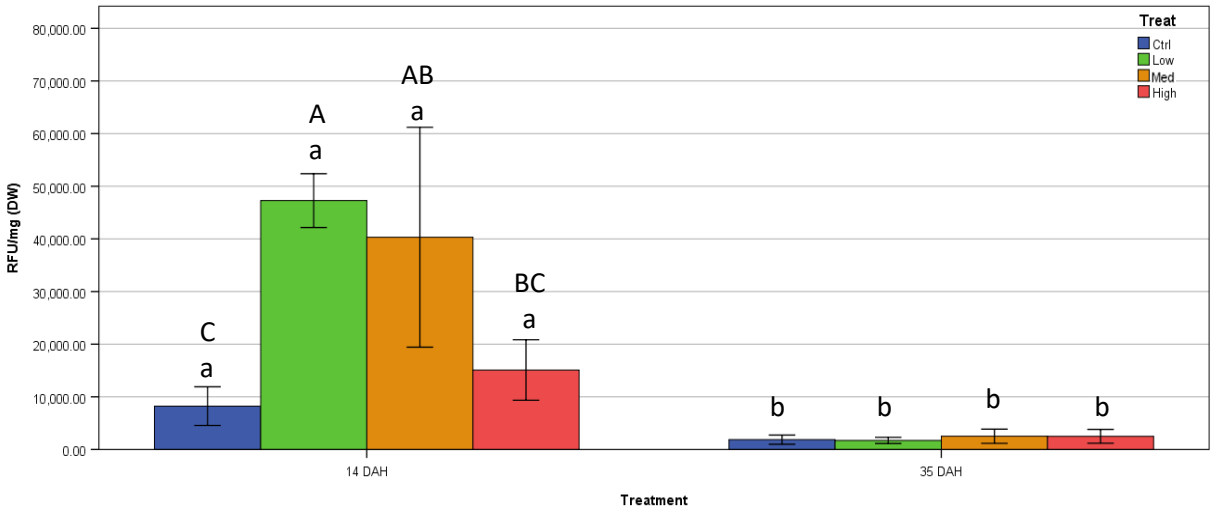

Figure 13 – Sole 18 C Lipase activity for the four treatments. Values shown represent means (±SD). Letters indicates the presence of statistical difference ($p<0.05$). Lower case letters (a, b) refer to enzymatic shifts during larval development (14 and 35 DAH) within each spermine supplementation (CTRL, LOW, MED, HIGH). Capital letters (A, B, C) indicate differences between spermine supplementation at the same age.

Lastly on the digestive enzymes, amylase (figure 14) did not show significant differences between treatments with p-values of 0.777, 0.144 and 0.614 for 14, 19 and 35 DAH, respectively. A pattern similar to that described for the other analyzed enzymes can be recognized, with an average of 6517±1499 RFU's at 14 DAH decreasing to 1822±841 RFU's at 19 DAH and increasing to 6627±3074 RFU's at 35 DAH. Values for 14 and 35 DAH were demonstrated to be statistically similar among all treatments.

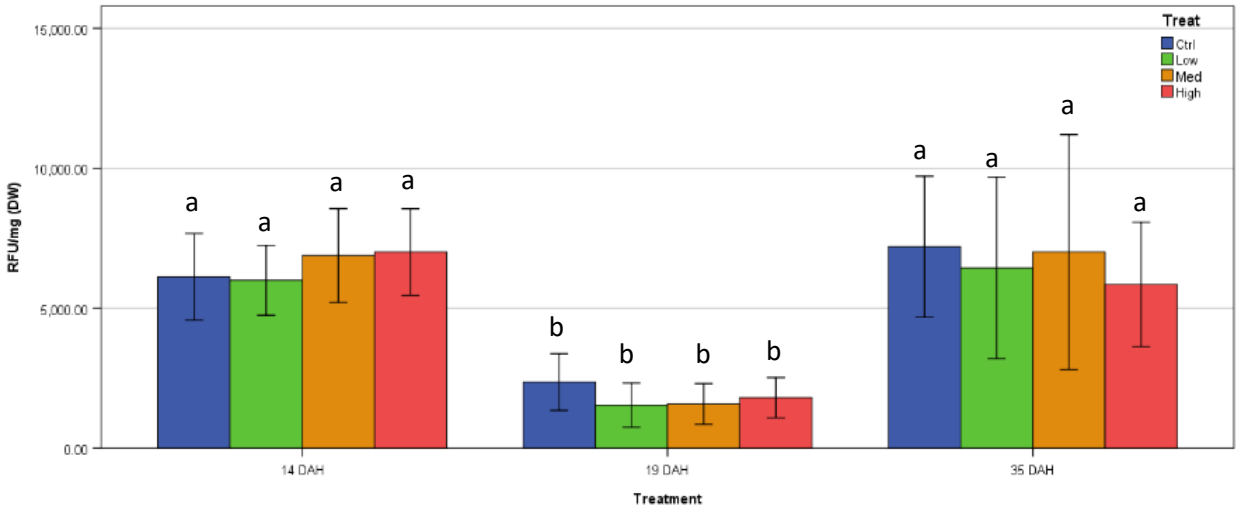

Figure 14 – Sole amylase activity for the four treatments. Values shown represent means (±SD). Letters indicates the presence of statistical difference ($p<0.05$). Lower case letters (a, b) refer to enzymatic shifts during larval development (14, 19 and 35 DAH) within each spermine supplementation (CTRL, LOW, MED, HIGH). Absence of capital letters indicate no differences between spermine supplementation at the same age.

For the two remaining tested enzymes, aminopeptidase and chymotrypsin, no activity was detected for none of the sampling points.

4.3 Oxidative stress indicators

Regarding the two analyzed indicators of oxidative stress, significant differences were detected for lipid peroxidation, between CTRL and MED treatments (p-value=0.028) with values ranging from 49.7 to 136.9 and 101.9 to 186.0 nmol per mg of protein for treatments CTRL and MED, respectively (figure 15a). For total glutathione content in full body homogenate a mean value of

26.3±9.2 µM per mg of protein was detected, with no statistically significant differences between treatments (p-value=0.133) (figure 15b).

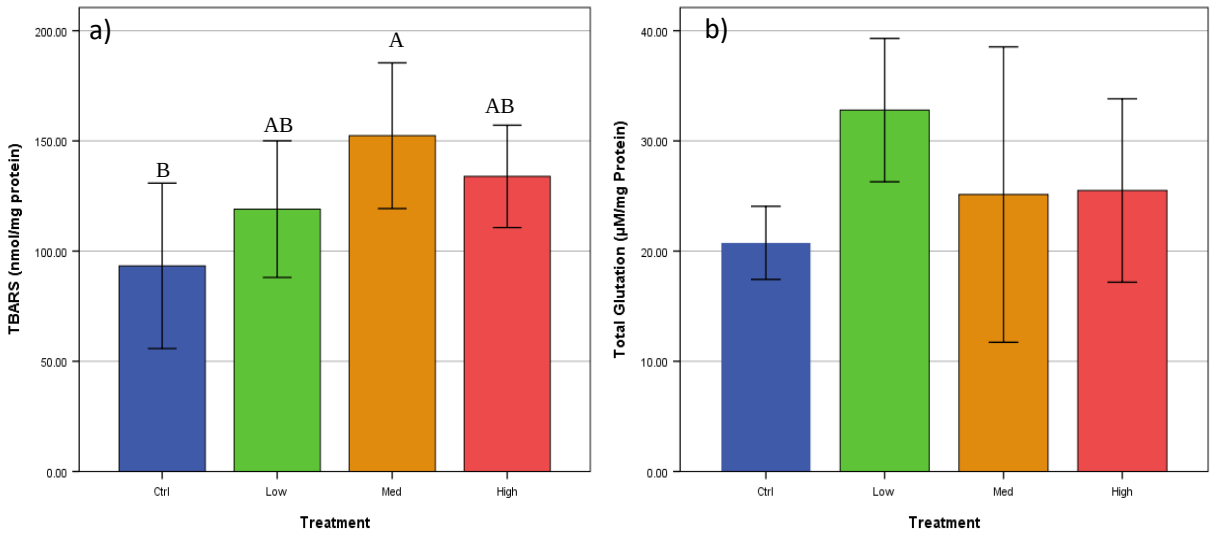

Figure 15 – Lipid peroxidation (a) and total glutatione in whole Sole body homogenate (b) for the four treatments. Values shown represent means (±SD). Letters indicates the presence of statistical difference (p-value<0.05).

5. Discussion

The hatching rate is traditionally used as an indicator of fish egg quality. In the present study, the average hatching rate observed was 73±10%, which was considered a positive result regarding egg batch quality and incubation protocol, since previous studies obtained hatching rates ranging from 49% to 64% (Martín et al., 2014). The results obtained in the present work indicate that: a) the novel *in ovo* modulation technique (sonophoresis) did not have a negative effect on the hatching rate of Senegalese sole, since values for the treatments subjected to this technique showed no differences in relation to the group not subjected to sonophoresis, and are in line with the ones described by Martín et al. (2014); b) the tested concentrations for *in ovo* spermine supplementation did not compromise eggs hatching rate, since no differences were detected in relation to the control treatment.

Senegalese sole larvae feeding protocols normally include a first period of live feed in the first weeks, followed by a period of co-feeding until reaching full weaned post-larvae (Pinto et al., 2018). Despite the efforts aimed at anticipating the weaning stage, which nowadays is around 22-24 DAH, the feeding regime used in the present study can be deemed risky. Although the complete transition to microdiet only happened at 21 DAH, a co-feeding regime from mouth opening together with a considerable reduction of the live prey provided can be considered a feeding challenge, designed with the objective of testing if and spermine supplementation could have affected larvae digestive capacity and resilience.

The absence of differences for survival rate at the end of the pelagic and benthic phases, might be interpreted as long-term safety regarding spermine supplementation. These results seem to indicate that no long-term adverse effect was generated. Comparing the 57.0±10.6% (average) in the present study with previous studies with values of 50% (Engrola et al 2009) or 30% (Engrola et al, 2010) at 20 DAH Sole using a more safe co-feeding protocol support the adequacy of the feeding protocol implemented in the present study. Even more, with the results obtained by Canada et al. (2016) of 23-24 % of survival for larvae reared exclusively on microdiets until 19DAH, it can be concluded that a compromise between the use of live prey and tailored feeds is still a much-needed reality. Overall, and by doing comparative analysis between the obtained results and the ones previously published, it seems that neither the use of the spermine supplementation through sonophoresis or the feeding protocol employed had adverse effect on larval survival.

No differences were found in dry weight at hatching (3 DAH) which implies that treatments had no effect on yolk sack absorption and assimilation. Dry weight differences recorded at 14 DAH suggest a growth impairment in treatments LOW and HIGH, compared to treatments MED and CTRL. However, the lack of differences in weight between treatments at 19 DAH means that since the weight differences verified at 14 DAH did not transpose to 19 DAH it can be stated the individuals in treatment LOW and HIGH were able to compensate the weight differences, suggesting a more efficient metabolism during the metamorphic phase, compared to treatments CTRL and MED. A slower growth, during metamorphosis, might be associated with the feeding behavior of Senegalese sole during metamorphosis that, despite of the increasing size, maintain an almost constant feeding intake at this stage (Navarro-Guillén et al., 2015) and also to a lower digestion capacity (Engrola et al., 2009b). Similarly, to what was found for the previous sampling points, at 35 DAH, no difference in dry weight between treatments was observed. The fact that,

the differences between treatments do not transpose onwards, may possibly mean that *in ovo* spermine supplementation only generated an effect at early developmental stages or during challenging situation, like metamorphosis climax. The combination of *in ovo* supplementation for an initial developmental boost, followed by spermine dietary supplementation might be an alternative to be tested to reinforce positive effects on early larval performance. Comparing the average dry weight results for 14, 19 and 35 DAH (figure 8), with the ones obtained by Martínez et al. (1999), that obtained 0.149, 0.707 and 6.300 mg for 13, 20 and 33 DAH respectively, using exclusively live prey, demonstrates the effectiveness of the co-feeding protocol used, in the first 19 days. The results obtained are also extremely coherent with the ones described by Engrola et al., (2010) that with a 58% replacement of *Artemia* with inert diet achieved an average weight of 0.74 ± 0.24mg at 20 DAH and with Navarro-Guillén et al. (2017), who obtained weights of around 0.6 mg and 1 mg for 20 and 32 DAH in a co-feeding regime from mouth opening. However a clear difference of almost 4.5 fold is noticeable between values obtained by the present study and the ones obtained at 33 DAH by Martínez et al. (1999). This is in agreement with what described by Engrola et al. (2009), where co-feeding generated post larvae at 20 DAH significantly smaller than the ones fed exclusively with live prey until weaning, but promoted growth in this same post-larvae, at 68 DAH, that achieved significantly higher sizes than larvae in all other feeding regimes. What this means is that post larvae subjected to co-feeding from mouth opening only achieve and surpass their live-fed until weaning homologous in a period posterior to 35 DAH. This corroborates what was demonstrated by Engrola et al. (2009), that reported that the post larvae subjected to co-feeding only at 40 DAH were able to equal the size of the of the other treatments and only at latter stages excelling in terms of weight. Stunted growth at this stage, due to the use of a highly demanding feeding plan are similar to the ones obtained with the use of co-feeding from mouth opening by Navarro-Guillén et al. (2017) that, at 32 DAH, registered larvae weight of around 1 mg. The use of the highly demanding feeding plan applied in this study may have reduced the growth potential at this stage and therefore not allow for differences between treatments become as pronounced as they might have been if growth had occurred as observed for a less demanding feeding plan. The fact that length at 35 DAH shows significant differences between treatments LOW and HIGH but no difference between these treatments and the control means that a slight improvement/impairment in growth might have been generated in LOW larvae related to HIGH larvae. These results might potentially become more evident and pronounced if the trial was

prolonged further in time. For this reason, it is recommended future trials comprising longer rearing periods, probably allowing for differences between treatments that are not yet evidenced to become more pronounced.

The sampling time can affect the results since diel enzymatic patterns can greatly vary (Navarro-Guillén et al., 2015). Furthermore, non-normalization of age to degrees-day makes comparison between trials and species difficult (Yúfera, 2018). The before mentioned reasons limits the ability to make direct comparison of the present study and others and only relative juxtapositions, such as the ones related to the developmental patterns can be made. Given the relevance of protein in the dietary requirements of most fish, trypsin and chymotrypsin are one of the most relevant indicator factors in the digestion process, and it is suggested that the ratio between trypsin and chymotrypsin might be a good indicator of the growth potential (Rønnestad et al., 2013). Relative to the proteolytic enzymes, the detection of trypsin and not chymotrypsin was as expected, since chymotrypsin activity was not detected, even at 32 DAH by Navarro-Guillén et al. (2015). Nonetheless chymotrypsin activity was detected with similar patterns to trypsin, at least until 8 DAH by Gamboa-Delgado et al. (2011), which can be justified by the use 100 to 300 pooled-larvae, whereas in this study 2- 3 pooled larvae homogenates were used. The fact that trypsin did not display significant differences between treatments might be due to an overall low tryptic activity at the moment of sample collection. Trypsin secretion is stimulated by feed ingestion and gut fullness. Navarro-Guillén et al. (2017) described a lower tryptic activity at the first light hours of the day in Senegalese sole larvae at 6 and 20 DAH, when larvae are still light-dependent feeders. This reduced activity may not highlight enzymatic activity differences that would be otherwise present and justify the variance present in all the treatments. The decreasing activity pattern of this enzyme during the metamorphosis process is similar to that observed by Ribeiro et al. (1999), which described a peak in trypsin activity at 5 DAH followed by a decrease until 18 DAH. In the present work, despite the difference of only 5 days between the first sampling point and the subsequent, done at 19 DAH, an abrupt decrease in enzyme activity can be observed in all treatments, possibly a snapshot of the already ongoing decreasing process, possibly linked to the reduced feed intake during this stage. At 35 DAH, the absence of differences on trypsin activity between spermine supplemented treatments and CTRL implies that no long-term improvement was achieved in the larvae protein digestive capacity. Even more, the juxtaposition of trypsin patterns with the smaller size recorded at the end of the experiment may

indicate a lower than normal feeding incidence, since it is suggested that trypsin activity levels may be used as a proxy for the nutritional status of sole under 5 mg (Engrola et al., 2007). Nonetheless, the fact that LOW treatment displayed significantly lower trypsin activity compared to MED without compromising growth performance might suggest a better digestive capacity and protein metabolism of LOW larvae, with the same protein accretion from lower digestive proteolytic activity. Therefore, further research needs to be conducted, with more samples collected and analyzed, in order to reduce variance and allow for more in-depth knowledge. Similarly to trypsin, the absence of differences in alkaline phosphatase, the catalyst for hydrolysis of phosphate monoesters (Sharma et al., 2014), between supplemented and CTRL larvae along the experiment confirmed the inexistence of differences in the brush border development between treatments. This enzyme follows a similar pattern to the one described by Ribeiro et al. (1999) with a global increase between day 3 and 9 followed by a decrease until 18 DAH. The observed increasing pattern in alkaline phosphatase activity at 35 DAH, after the reduction at 19 DAH, is indicative of progress in the digestive tract development after metamorphosis. The pattern of alkaline phosphatase matches the one described by Martínez et al. (1999) with a marked decrease with the end of metamorphosis, followed by an increase in activity levels. Curiously this pattern of decreased activity at the end of metamorphosis was also observed by Engrola et al. (2009) in the treatments using exclusively live feed, while in the treatment that closely resembles the one in the present study, with co-feeding from an early age, the alkaline phosphatase activity increased with the end of metamorphosis, probably as an indicator of higher digestion capacity. The fact that aminopeptidase was not detected at any of the sampling points was unexpected, since it has been described that with larval growth there is a sharp increase, around the third week after hatching, of other brush border enzymes such as aminopeptidase (Zambonino Infante and Cahu, 2001). For the detection of aminopeptidase a different approach might be used in future trials, probably increasing samples size, and applying the method developed for intestinal scrapping adapted to fish larvae by Cahu and Infante (1994) that allowed Ribeiro et al. (1999) to detect aminopeptidase in Senegalese sole between 2 and 18 DAH.

In the case of the two analyzed lipolytic enzymes, 4C and 18C-like lipase, that target short and long chain lipids, respectively, a significant difference was observed for 18C-like lipase in the first sampling point, with treatments LOW and MED showing values significantly higher that CTRL. These finds suggest that the previously mentioned treatments might have been in an advantageous

position in the metabolism of lipidic reserves, characteristic of the metamorphic stage that is in its starting process (Martínez et al., 1999). This increased lipolytic capacity, specifically in treatment LOW might be the reason why it was able to overcome the size reduction verified at 14 DAH. However, the increased lipolytic capacity in both treatments differentially affected the growth performance at that age, being only translated into a higher dry weight in MED larvae. Moroever it is remarkable that such pronounced increase is observed in the enzimes targeting long chain lipids while on the other the enzime targeting short chain lipids reamained inaltered. This might imply both, that these two enzimes are regulated by separate metabolic pathways, or just be a result of the lower specificity of 4C-like lipase analysis, since the signal generated migh be also a result of the presence of esterases. For the second sampling point, done at 19 DAH, a decrease in both 4C and 18C-like lipase activity levels was observed, being remarkable that this decline led the activity levels of 18C lipase below the detection limits, being in accordance with the previously detected at this stage by Navarro-Guillén et al. (2015), where 4C like lipases have higher activity than 18C-like lipase. These finds despite seemingly differing from the ones reported by Martínez et al. (1999), that noticed a decrease in lipase only from 23 DAH onwards may possibly imply that, despite smaller, the fish in the present study were imposed into a slightly advanced digestive maturity stage resulting from the co-feeding use from mouth opening until weaning. Finally, at 35 DAH a slight increase in the activity levels of both enzymes was observed. At this stage differences in 4C-like lipase activity between treatments LOW/MED and treatment HIGH suggest increased lipid digestion capacity for the lower and medium spermine levels compared with the highest spermine supplemented larvae. This higher lipolytic capacity in the treatments with lower levels of spermine supplementation but without improvement in in terms of overall fitness indicate a lower a digestive efficiency. The ontogeny patterns described in the present study for 4C- and 18C-like lipases are in line with those described by Navarro-Guillén et al., 2015, in which Senegalese sole´s ability to digest medium and long-chain lipids decreased while the ability to metabolize short-chain lipids was preserved along development. Furthermore the patterns observed for the lipolytic activity may be deemed as particularly relevant as they have been precedingly characterized as not being connected with the presence of food in the gut of larvae (Navarro-Guillén et al., 2015).

The amylase pattern observed in the present trial did not meet the constant activity decline in amylase from around mouth opening onwards described by Martínez et al. (1999), but is supported

by the increase in activity between 20 and 32 DAH described by Navarro-Guillén et al. (2015) for Senegalese sole. These differences between studies might be justified by the fact that Martínez et al. (1999) presented enzymatic ontogeny as specific activity (expressed per mg of protein), and this decline in specific enzyme activity may not be due to a diminution in enzyme synthesis but is the result of an increase in tissue proteins or due to differences in the analysis protocols used, as well as sample size. Moreover it is worthwhile mentioning that the amylase activity pattern is also variable depending if larvae are fed with live feed or microdiets (Ribeiro et al., 2002).

Oxidative stress happens when O_2 is metabolically transformed into ROS and as result of imbalances between the oxidation and reduction in the cells. Antioxidative defenses come in the form of glutathione, ascorbate, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase , glutathione-s-transferase and ascorbate peroxidase (Kelley et al., 2010; Valavanidis et al., 2006). In this study oxidative stress signals were detected, namely in the form of increased lipid peroxidation in post-larvae in MED treatment, which might suggest an imbalance derived from spermine supplementation. However, this higher level of lipid peroxidation did not compromise MED larvae growth performance. It is known that fish larvae display changes in oxidative stress status when subjected to toxic levels of compounds (Bauder et al., 2005; Cao et al., 2010; Jia et al., 2018) and that polyamines if in excess can generate toxicity effects (Pegg, 2013), nonetheless it would be expected, if this was the case, higher levels of oxidative stress indicators in HIGH larvae, however, since the underlying mechanisms in Senegalese sole are not fully known, further trials need to be conducted on the oxidative stress response to polyamine supplementation. By contrast, evaluating the values for glutathione, one of the major antioxidants, no differences were observed in the previously mentioned treatment, leading to suggest that other antioxidative defense might be acting against oxidative stress in this fish. In summary it can be stated that the analyzed oxidative stress indicators lead to inconclusive results. Thus, further studies with more samples and analysis need to be conducted in order to understand the relatively unexplored oxidative stress response in marine fish larvae.

Spermine is a polyamine with a crucial role in cell development (NCBI, 2020). Previous studies have described its possible function as promoter of early maturation of the digestive system and as enhancer of the antioxidative status, but with studies mainly conducted in organisms other than fish (i.e. Buts et al., 1993; Dufour et al., 1988; Elginaid Osman et al., 1998; Fang et al., 2016a,

2016b; Harada et al., 1994; Pères et al., 1997). The current work and the ones developed by Engrola et al. (2014), Lopes (2016) and Allon et al. (2016) are paving the way for *in ovo* modulation, a possible cornerstone in the field of early metabolic programing. Results support the need for further research in order to understand the link between gut maturation, oxidative stress and spermine supplementation.

6. Conclusion

Supplementation of spermine through the use of a sonophoresis protocol did not generate a negative effect in the hatching or survival rate, confirming the short- and long-term safety of this procedure. Despite spermine supplementation not revealing a clear advantage in growth compared to the control, supplemented larvae showed increased lipolytic capacity of *Solea senegalensis* at 14 DAH. Further research on early metabolic programming may not only contribute to the improvement *Solea senegalensis* culture, but also contribute to reduce the time and effort necessary for the weaning process, which might be also applied to other species. As world population will continuously grow, researchers focused on improving the aquaculture sector will slowly but surely trace the way to meet the increasing demand for fish and sea products.

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