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Escola Superior Agrária

Curso de Mestrado em Engenharia Alimentar

Development of a microencapsulated prototype to deliver low-molecular weight proteins at the onset of exogenous feeding of marine fish larvae

Bruno Filipe da Lança Guerreiro Nunes

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Development of a microencapsulated prototype to deliver low-molecular weight proteins at the onset of exogenous feeding of marine fish larvae

Relatório de dissertação de mestrado apresentado na Escola Superior Agrária do Instituto Politécnico de Beja

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Abstract

The goal of this Thesis was to develop a microencapsulated prototype, with low leaching, to deliver low-molecular weight proteins to marine fish larvae. A reduction of 75% of leaching was achieved with the microencapsulated prototype against a non-encapsulated Control. The microencapsulated prototype was then included in a commercial microdiet at two levels [8.5% (CAP8.5) and 30% (CAP30)] and tested in two aquaculture model species: gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*). Feed intake, growth performance and survival were evaluated in both species.

In gilthead seabream larvae a high replacement co-feeding strategy was used with experimental microdiets being introduced at first feeding. Gilthead seabream larvae showed a good acceptance of all microdiets since mouth opening, increasing their gut fullness during trial time and full weaning was possible at 23 DAH. Until 12 DAH, larvae presented a better growth performance with CAP30 microdiet. At the end of the trial (34 DAH), the better growth performance was presented by gilthead seabream larvae fed on CAP8.5 microdiet.

In Senegalese sole larvae, all microdiets were introduced at mouth opening with no live-prey being fed to the larvae. Results show that ingestion and gut fullness were, in general, low and variable during trial time, not showing a good acceptance of microdiet as observed for gilthead seabream larvae. The best growth performance was observed in larvae fed with CAP30 microdiet. Despite positive growth, larvae were unable to conclude metamorphosis. A positive survival was observed in all experimental treatments.

In conclusion, this Thesis describes the development of a low-leaching microencapsulated prototype with high biological potential was produced for inclusion in microdiets for fish larvae.

Keywords: Encapsulation, Microdiets, Fish larvae, First feeding, Nutrition, Weaning, Gilthead seabream, Senegalese sole, Aquaculture

Resumo

O objetivo desta tese foi o desenvolvimento de um protótipo microencapsulado de baixa lixiviação proteica, para fornecer proteínas de baixo peso molecular a larvas de peixe. Foi obtida uma redução de 75% de lixiviação proteica no protótipo microencapsulado em relação a um controlo não encapsulado. Procedeu-se à inclusão do protótipo microencapsulado numa microdieta comercial a dois níveis [8.5% (CAP8.5) e 30% (CAP30)] e testou-se em duas espécies modelo para a aquacultura: dourada (*Sparus aurata*) e linguado Senegalês (*Solea senegalensis*). A ingestão de alimento, o crescimento e a sobrevivência foram avaliados nas duas espécies.

Nas larvas de dourada foi utilizada uma estratégia de co-alimentação com alta substituição de alimento vivo pelas microdietas produzidas à abertura de boca. As larvas de dourada apresentaram uma boa aceitação das microdietas desde a abertura de boca, aumentando também a quantidade de alimento ingerido ao longo do ensaio, tornando possível o desmame aos 23 dias após eclosão. Até aos 12 dias após eclosão, as larvas apresentaram um melhor crescimento com a microdieta CAP30. No final do ensaio, aos 34 dias após eclosão, as larvas de dourada apresentaram um melhor crescimento com a microdieta *CAP8.5*.

Nas larvas de linguado Senegalês, as microdietas foram utilizadas à abertura de boca sem adição de alimento vivo. Os resultados mostram que a ingestão de alimento nas larvas de linguado Senegalês foi, no geral, baixa e variável durante o tempo do ensaio e não mostraram uma tão boa aceitação às microdietas como as larvas de dourada. O melhor crescimento foi obtido com a microdieta *CAP30*. Apesar de um bom crescimento, as larvas de linguado-do-Senegal foram incapazes de completar a metamorfose. Em todos os tratamentos experimentais, foram observados resultados positivos de sobrevivência.

Em conclusão, esta tese descreve o desenvolvimento de um protótipo microencapsulado com baixa lixiviação e alto potencial biológico, que poderá ser incluído em microdietas para larvas de peixe.

Palavras-chave: Encapsulação, Microdietas, Larvas de peixe, Primeira alimentação, Nutrição, Desmame, Dourada, Linguado-do-Senegal, Aquacultura

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Chapter 1: General Introduction

1.1. Aquaculture overview

Aquaculture can be defined as the rearing of aquatic organisms under controlled or semi-controlled conditions (Stickney, 2005) and its production can be divided in three main groups: food fish, aquatic plants and non-food products. In 2016, global fish production reached is maximum, about 171 million tonnes, in which aquaculture represented 47% of the total. Of the 171 million tonnes of total fish production in 2016, about 88% was utilized for direct human consumption and the major part of the remaining 12% used for non-food purpose was used for fishmeal and fish oil production (FAO, 2018).

Aquaculture products represented about 51% of total food fish consumption in 2015, and was estimated to be 53% in 2016, which means that world population is consuming more farmed than wild fish, which have remained relatively stable since the late 1980's. The group that contributes to a larger volume of production, 80 million tonnes in 2016, is the food fish, and it includes finfish, molluscs, crustaceans and other aquatic animals, such as turtles, sea cucumbers, sea urchins, frogs and edible jelly fish. Aquatic plant products are mainly seaweeds and a small part of microalgae and accounted for 30.1 million tonnes in 2016. Non-food products are ornamental shells and pearls, accounting for 37.9 tonnes in 2016 (FAO, 2018).

The world fish consumption has grown from 9.0 kg per capita in 1961 to 20.2 kg in 2015, growing at an average of about 1.5% per year. This means that at the same time that the world population expands, the request for food sources is increasing as well (FAO, 2018). In the same report by FAO (2018), it was estimated that in 2016 and 2017, per capita/year world fish consumption was 20.3 and 20.5 kg, respectively. In Europe, fish consumption was 24.3 kg per capita/year. In EUMOFA (2018) report regarding 2016, Portugal fish consumption was much higher than world and Europe average, 57 kg per capita/year, being the country within European Union countries consuming more fish.

The aquaculture sector has the potential for a more efficient supply of fish from the producer to the consumer and is generally able to address consumer concerns on sustainability and product origin more easily than wild fish sector (FAO, 2018). To improve the acceptance of aquaculture products by the consumers it is critical to produce fish juveniles that comply with the high-quality standards. The production of high-quality juveniles relies on a better understanding of larval nutritional requirements, and this production will increase the aquaculture industry efficiency. So, the advances on nutritional requirements of the farmed species, the actual knowledge on zootechnical conditions,

increased formulation quality, manufacturing and quality of microdiets may now allow to work on weaning of marine fish larvae at first feeding.

1.2. Ontogeny, physiology and nutrition of marine fish larvae

Newly hatched marine teleost fish larvae have a large yolk sac that provides nutrition to the developing organism until exogenous feeding starts after 2 to 5 days in most species. At this point, larval eyes are formed, but non-functional and at mouth opening, the eye becomes functional with the retina equipped with a monospecific layer of single cones already pigmented and the optic nerve is connected to the optic tectum. However, at early development, larvae have poor photopic perception and serious visual limitations (Yúfera et al., 2011). Marine teleost fish larvae hatch with a straight and undifferentiated digestive tract, a tubular segment, laying dorsally to the yolk sac with mouth and anus closed. At the onset of exogenous feeding, the digestive tract has histologically and functionally distinct regions, the bucco-pharynx, oesophagus, presumptive stomach, intestine and anus, also known as foregut, midgut and hindgut. The gut is coiled into a loop before exogenous feeding starts (Zambonino-Infante et al., 2008; Yúfera et al., 2011; Rønnestad et al., 2013) in most species.

The digestive functions follow a sequential chronology associated with morphological transformations (Figure 1) (Cahu and Zambonino Infante, 2001). The lack of functional stomach means that ingested protein do not suffer the denaturing conditions as on a gastric acid digestion (Hamre et al., 2013). When exogenous feeding starts, the gut of larval fish is functional and the enzymes responsible for digestion of proteins, lipids and carbohydrates are already present, turning possible to larvae absorb protein and lipids, but these enzymes have an optimal activity at a neutral or alkaline pH conditioning the range of proteins that fish larvae are able to digest (Yúfera et al., 2011; Hamre et al., 2013). The limited digestive capacity of fish larvae mentioned above and the higher nutritional requirements - larvae can grow 30% day^{-1} (Canada et al., 2017) suggest that the formulation of a first-feeding diet cannot be the same the formulation of a diet to fish juveniles.

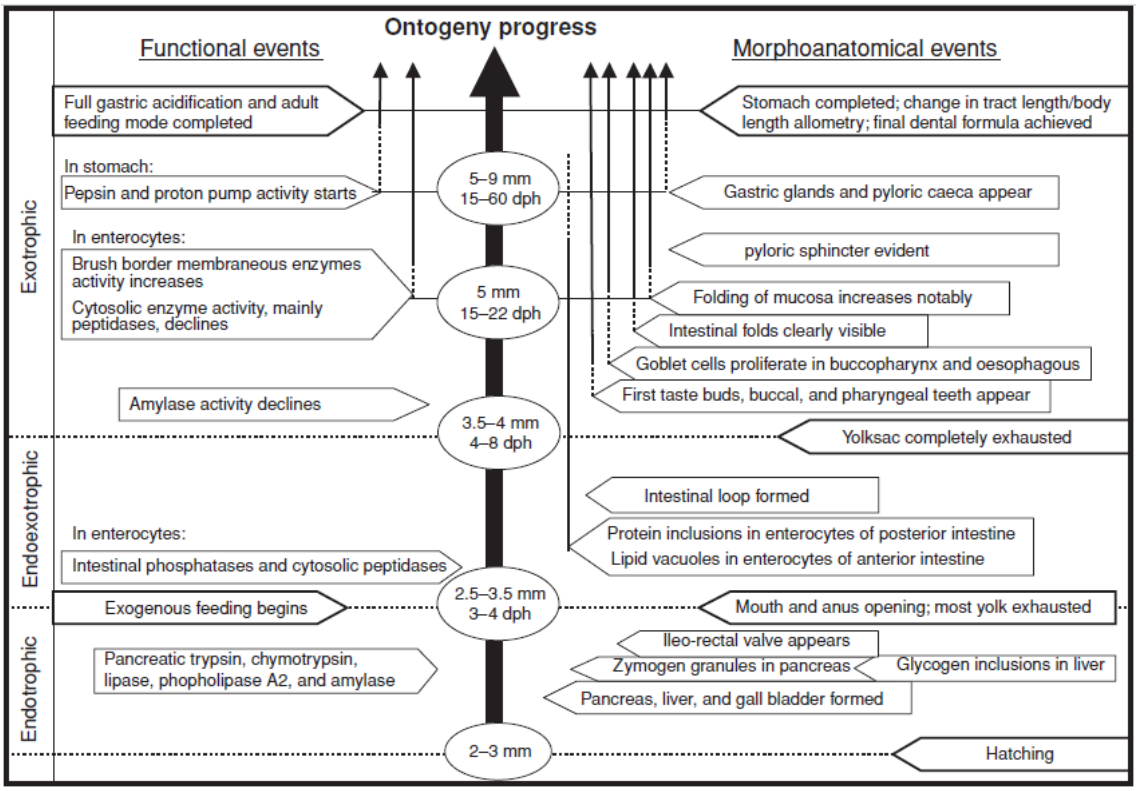

Figure 1 - General ontogeny progress of functional and morphoanatomical events of marine fish larvae. Adapted from Yúfera et al. (2011).

In commercial hatcheries, the production of fish larvae depends largely on supply of live-prey, such as rotifers and Artemia. Rotifers and Artemia are conveniently used due its adequate size to mouth of fish larvae [70-350 µm for rotifers (depending on strain and age) and 400-500 µm to Artemia nauplii]. Both are non-selective filtration feeders, which means that they can be enriched with selected nutrients or microalgae; both have fast growth rates of population and/or individually and good tolerance to culture conditions and handling. Rotifers and Artemia present a similar protein content (Aragão et al., 2004). The occurrence of collapses of the population, the effort required to maintain the whole plankton chain, the high bacterial load present in rotifers and Artemia during the culture and enrichment process may be detrimental to the larvae, being disadvantages on the use of these organisms as first feed for fish larvae. Artemia is the most convenient and least labour-intensive live food for aquaculture, due to its ability to form cysts that are very resistant to adverse environmental conditions and can be hydrated with water and hatch as a nauplii in less than 24 hours. However, it represents high costs due to its low and unreliable natural resources and increased demand. It also presents a low nutritional value, including low DHA and EPA content. A ratio of DHA:EPA above 2 is generally

recommended to marine fish larvae, since larvae require high amounts DHA to sustain larval growth and development of neural and visual systems, whose deficiency would led to major impacts on ability of larvae to capture their prey (Conceição et al., 2010). Artemia can be enriched in these fatty acids, but Artemia retroconverts DHA into EPA and tends to present a low ratio on DHA:EPA (Conceição et al., 2010).

The disadvantages mentioned above are reasons to demand the substitution and early weaning from live prey to a more nutritionally adequate diet. The nutritional inadequacy and variability of these organisms do not sustain growth at later stages (Cahu and Zambonino Infante, 2001; Conceição et al., 2003; Hamre et al., 2013). Optimal larval growth and development at first feeding is a first interest in the development of an inert microdiet. It would allow lower production costs and sustain the production of high and constant quality fish juveniles, contributing to increase knowledge of fish larvae nutrition and for progress of the aquaculture industry.

1.3. Technological processes for production of fish larval microdiets

In the last years, there has been a considerable progression in reducing the weaning age of fish larvae. This evolution is due to the increased quality of microdiets currently available, improved production technologies and knowledge on larval rearing and zootechnical conditions (Pinto et al., 2018). Yet, the utilisation of microdiets for first feeding larvae still faces several problems, such as: weak attractiveness when compared with live-prey, lower digestibility when complex proteins are present, and high losses of water-soluble molecules by leaching upon contact with rearing water, alongside with the lack of knowledge on fish larvae nutritional requirements (Yúfera et al., 2011).

The microdiets need to be able to admit any kind of compound, according to the formulation needs, and need to obey several structural and biochemical characteristics to be useful for feeding fish larvae: (1) stable enough to prevent particle disintegration after immersion in water and retain the maximum possible of hydrosoluble nutrients; (2) the particles should be accessible to fish larvae being available in the water column and having an appropriate diameter for ingestion; (3) particles have to be identified as a food item to be ingested, be digestible by the larval digestive system and has to meet properly the energetic and nutritional requirements for larval growth and development (Hamre et al., 2013). The attractiveness and digestibility can be promoted by including low-molecular weight proteins, such as protein hydrolysates where the increased availability of amino

acids and peptides helps the absorption in the gut (Hamre et al., 2013), while the high leaching can be reduced by entrapping the water-soluble molecules within a matrix by a process called microencapsulation.

Nowadays, most of the microdiets are produced by extrusion. Extrusion is the process where a determined mixture is provided a pellet shape depending on the cross section of the opening and cutting device by pushing out material through a narrow opening. When using low temperatures (cold-extrusion) the extruded material will not be majorly affected either physically and/or chemically by the extrusion process. The extruder is composed by a hollow cylindrical shell, called barrel, where a flighted screw drags the feed ingredients forwards. In the exit end of the extruder, called die, the extruded material is released and cut to a known size by a rotative blade (Berk, 2017). The current extrusion technology only allows a direct pellet size of 800 μm, at a relatively quick speed. Which means that if smaller sizes are needed, pellets are extruded to a larger size and then crumbled after drying. The crumbling process causes damage in the surface of the particles, increasing the leaching of water-soluble molecules.

Microencapsulation is a versatile technology that can ensure the protection of target molecules against environmental factors such as water, light, heat and oxygen, being currently applied in areas such as pharmaceutics, chemistry, food and agriculture. Some examples of microencapsulation technologies are spray drying, fluidized bed, emulsion and gelation (Sobel et al., 2014). Spray-drying is one of the most widely used microencapsulation technologies. Together with cold-extrusion it can help reducing leaching. Spray drying is a method where a liquid phase is converted to a dry powder by atomization into a flowing stream of hot air (Figure 2). The product to be transformed is initially sprayed into drops at the top of the chamber, travelling in the turbulent flow of heated air produced in the same region (Jacobs, 2014).

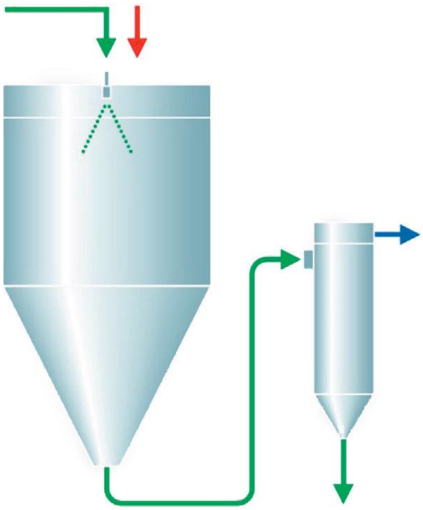

Figure 2 - Schematic configuration of a spray dryer. Adapted from Jacobs (2014).

1.4. Objectives

This Thesis aimed at developing a microencapsulated prototype able to reduce leaching of a protein hydrolysate commonly included in microdiets for marine fish larvae. This microencapsulated prototype was initially characterized for its effect on the reduction of protein leaching and, subsequently, included at two levels (8.5 and 30%) in complete microdiets. The inclusion of the microencapsulated prototype was evaluated for its effect on microdiet physical properties, elucidating the role of technological process for diet integrity in water after immersion. Furthermore, the newly-developed microdiets was teste in first-feeding gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*) larvae and its effect on larval growth performance, survival and microdiet acceptability were assessed in both species.

Chapter 2: Technological developments: encapsulation of low-molecular weight proteins

2.1. Materials and methods

2.1.1. Encapsulation of a fish protein hydrolysate by spray-drying technology

A spray dryer (GEA Niro A/S Production Mino, Søborg, Denmark) was used to encapsulate a mixture of a fish protein hydrolysate, containing 82-86% of proteins (PH, SOPROPÊCHE, France) and a protein concentrate (PC; Volac International Limited, United Kingdom), containing 80% of protein. Tap water was used to obtain the mixture of PC:PH, on a proportion 6:10, with a final concentration of 20% solids. The inlet temperature of the atomization chamber was 125ºC, the outlet temperature was 75ºC. The nozzle and exhaustion rotor speed were 25000 rpm and 2700 rpm, respectively. The resulting microparticles was collected to a stainless-steel container and stored sealed until the moment of analysis and incorporation on a microdiet.

2.1.2. Determining protein leaching in microencapsulated prototype particles

In order to determine how the encapsulation process affected the solubility of the microencapsulated prototype, 5 mg of powder was diluted with 5 mL of sea water in a RIA (Radio Immune Assay) tube. The tube was subsequently placed in an orbital shaker (PSU-20i Multi-functional Orbital Shaker, Biosan, Riga, Latvia) at a velocity of 40 rpm, simulating the water column of a tank. A 500µL aliquot was collected at 1, 5, 10, 15, 30, 60 and 120 minutes. Each time an aliquot was withdrawn, 500µL of sea water was added in order to prevent saturation of the water with protein. The former procedures we carried out in triplicate for each sampling point.

The protein content of the collected samples was quantified by Micro BCA Protein Assay Kit (Thermo Scientific, USA), using spray dried PC as Control. The Micro BCA methodology is based on the reduction of Cu^{2+} to Cu^{+} in presence of proteins in alkaline medium, using bicinchoninic acid (BCA) as the detection reagent. The purple-coloured reaction product formed by chelation of two BCA molecules with one cuprous ion (Cu^{+}) has a strong absorbance at 562nm. The macromolecular structure of protein, the number of peptide bonds and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) are responsible for colour formation with BCA.

The samples obtained were diluted ten times, as in previous studies, in MiliQ water (Millipore, Interface, Portugal). An aliquot of 100 µL of each sample was placed in triplicate, in a 96-wells microplate (Frilabo, Portugal) and 100 µL of BCA reagent were added.

As both proteins are soluble in water, non-encapsulated versions of Control and encapsulated prototype were diluted in MiliQ water and used as standard for spray-dried Control and encapsulated prototype. Sea water was used as blank for samples and MiliQ water as blank for standards. The microplate was then incubated at 37ºC during 2h and read in a microplate reader (Synergy HTX, Biotek, USA) at 562 nm.

2.1.3. Inclusion of microencapsulated particles in microdiets for first-feeding fish larvae

The novel microencapsulated prototypes were included in the formulation of a complete microdiet for fish larvae. A Sparos Lda (Olhão, Portugal) commercial microdiet (Commercial Control) was used as the base for two novel variants, where the microencapsulated prototype was included at 8.5% (CAP8.5) or 30% (CAP30). The microencapsulated prototype replaced protein sources included in the Commercial Control formulation, so that the three microdiets were formulated to be isonitrogenous.

According to target formulation, all three treatments were produced as follows: 1) dry powder ingredients were weighted and mixed together using a mixer (Felino, Portugal); 2) the mixture was ground on a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Germany); 3) the oil fraction was added and mixed, while adding enough water to connect the mixture. Cold extrusion equipment (Dominioni, Italy) was used to agglomerate the mixture and pelletize it. The resulting pellets were dried on a drying oven (HeatEvent 100/150, Vötsch Industrietechnik GmbH, Germany) for 3 hours at 70ºC, and after cooling, crumbled (Neuero Farm, Germany) and sieved to get the desired pellet size (100-200 µm, 200-400 µm and 400-600 µm).

2.1.4. Quality control in complete microdiets for fish larvae

In each diet mentioned above on sector 2.1.3, the anti-caking capacity, surface dispersion and buoyancy/sinking were evaluated. It was defined a scale of 1 to 5. The observation of the microdiets in its container was enough to evaluate the anti-caking capacity. About 2g of microdiet were drooped in water to evaluate surface dispersion and buoyancy/sinking.

2.1.5. Determining protein leaching in complete microdiets for fish larvae

As previously tested for the microencapsulated particles, the protein leaching after immersion of the microdiet in water was quantified. For this purpose, 2g of microdiet

were tossed in a 2000 mL capacity beaker containing around 1600 mL sea water, rotating at 40 rpm in an orbital shaker (PSU-20i Multi-functional Orbital Shaker, Biosan, Latvia). After desired time, at 2 and 30 minutes, the water was removed in the most laminar way possible to prevent any turbulence leading to protein leaching. The beaker was then placed in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) at 100ºC until complete evaporation of the remaining water, and the protein content was analysed in the remaining feed residue.

2.1.6. Data analysis

To calculate the amount of soluble protein released of the encapsulated prototype, a standard curve was used to obtain the result in the concentration used (µg mL^{-1}). The difference between the initial and final amount of protein in each time point was used to calculate the percentage of soluble protein released. To determine the protein leaching in the complete microdiet, the difference between the initial and final amount of total protein was performed and used to calculate the percentage of leached protein. Results expressed as percentage, an arcsine transformation (T) was performed prior to any statistical test: T = ASIN (SQRT (value / 100)), in order to comply with data normality. A Student's t-test was performed to calculate differences in each time point for each diet for protein leaching on the encapsulated prototype. One-way ANOVA, followed by a Tukey comparison test, was used to calculate differences in each time point for protein leaching in complete microdiet. The significance level considered was $p<0.05$ for all tests performed. All statistical analyses were performed with R Studio Version 1.2.1335.

2.2. Results

2.2.1. Protein leaching in microencapsulated particles

The results shown on Figure 3 represent the leaching profile of the encapsulated prototype. It was noticed a lower leaching for the encapsulated prototype when compared with Control. For both particles it was observed a quick protein leaching in the first 5 minutes. After 1 hour of immersion, Control kept increasing the protein leaching and reached values of around 60% while the encapsulated prototype only leached around 20% of protein, and after 2 hours of immersion Control kept increasing, peaking 90% of protein leaching, while the encapsulated prototype kept more stable, leaching only 24% of protein. Significant differences were found at 10, 15, 30, 60 and 120 minutes.

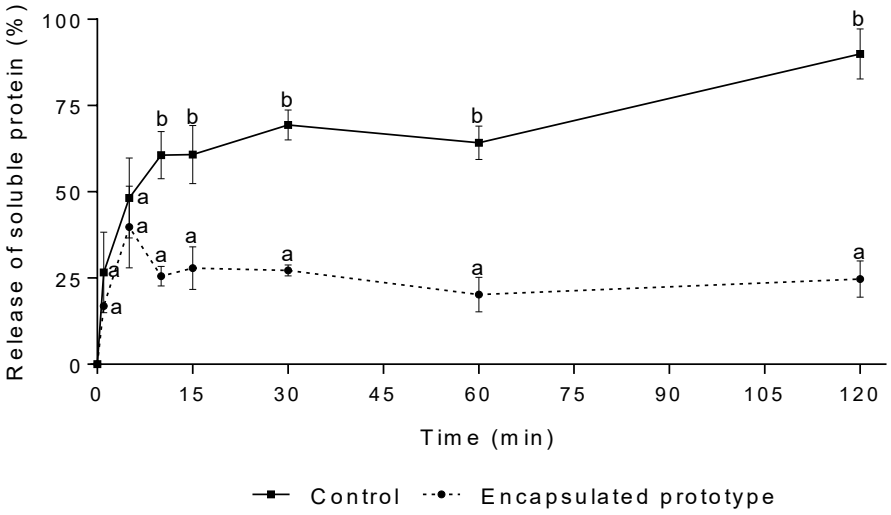

Figure 3 – Release of soluble protein in encapsulated microparticle used in manufacturing of microdiets used for gilthead seabream and Senegalese sole trials. Values presented as mean ± standard deviation (n=3). Different letters indicate statistical differences ($p<0.05$) between microparticles at the same time point.

2.2.2. Complete microdiets for fish larvae

The nutritional composition of the complete microdiets used in larval trials is presented on Table 1. It is possible to observe that no nutritional differences were found between each microdiet. Microdiets were composed by around 67% of crude protein, 20% lipids and 22 kJ g^{-1} energy.

Table 1 - Proximal nutritional composition of microdiets used in gilthead seabream and Senegalese sole trials.

	Commercial Control	CAP8.5	CAP30
Dry matter (%)	94.10	92.72	92.77
Energy (kJ g^{-1})	22.39	22.47	22.61
Crude protein (%DM)	66.99	67.15	67.55
Crude fat (%DM)	20.75	19.85	19.99
Ash (%DM)	13.21	12.90	12.20
Phosphorus (%)	1.92	1.89	1.78

In general terms, Figure 4 shows that protein leaching was higher in the smaller particle sizes of complete microdiets, with values achieved in the 100-200 µm particle size being higher than in the 200-400 and 400-600 µm sizes. In 100-200 µm size particles, there was a protein leaching of 52% for Commercial Control, 28% for CAP8.5 and 34%

for CAP30 after 2 minutes of immersion in water. A significantly lower leaching was found in CAP8.5 and CAP30 than in Commercial Control. After 30 minutes, Commercial Control protein leaching was 46% while CAP8.5 increased to 42% and CAP30 to 55%. After 30 minutes of immersion protein leaching did not follow the same trend as at 2 minutes, and a significant lower protein leaching was observed in CAP8.5 and Commercial Control than in CAP30.

In 200-400 μm size particles, after 2 minutes of immersion in water, Commercial Control leached 38% of protein while CAP8.5 and CAP30 leached about 30%. No significant differences were found at 2 minutes after immersion. After 30 minutes while Commercial Control kept stable, leaching only 37% of protein, CAP8.5 increased its leaching to 41% and CAP30 to 44%. A significant higher protein leaching was found in CAP8.5 and CAP30 than Commercial Control.

In 400-600 μm particles, Commercial Control leached 25% of protein, CAP8.5 leached 32% and CAP30 leached 36%, after 2 minutes of immersion in water. A significant higher leaching was observed in CAP8.5 and CAP30 than in Commercial Control. After 30 minutes of immersion, Commercial Control increased to 34% of protein leaching, CAP8.5 to 50% and CAP30 to 54%. The same trend was observed as after 2 minutes of immersion, and after 30 minutes, a significant higher leaching was observed in CAP8.5 and CAP30 than in Commercial Control.

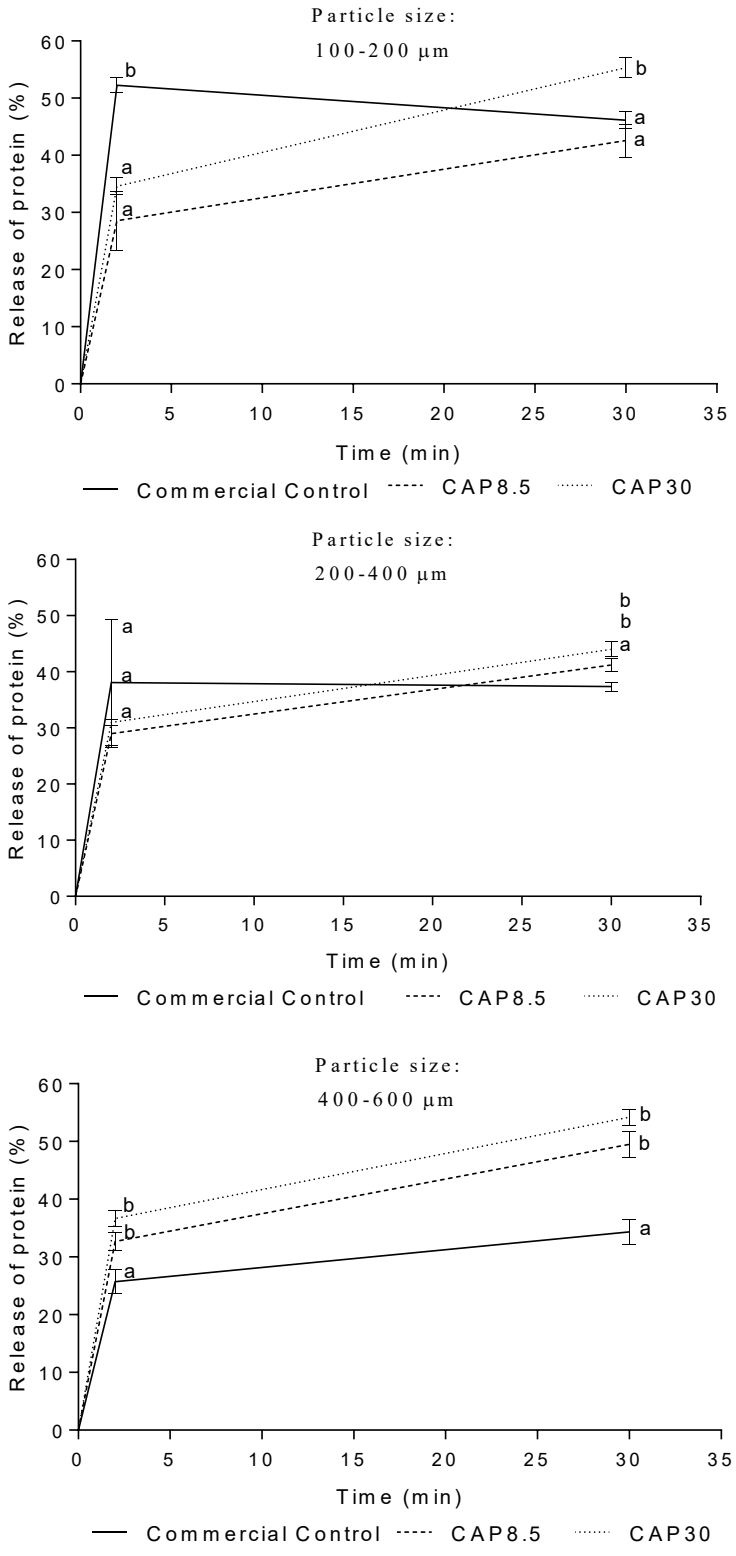

Figure 4 – Release of protein after immersion on microdiets used for the gilthead sea-bream and Senegalese sole trials. Values presented as mean ± standard deviation (n=2). Different letters indicate statistical differences (p<0.05) between microdiets at the same time.

2.2.3. Quality control in complete microdiets for fish larvae

In general, Table 2 data shows that smaller diet sizes are more susceptible to caking, but microparticles still presented a good surface dispersion when added to water. Particles size ranging between 100-200 µm presented a lower anti-caking capacity for all microdiets, and only the Commercial Control had fewer floating particles after addition to water. In the 200-400 and 400-600 µm sizes, all microdiets, presented an excellent behaviour after addition to water, having good surface dispersion and all had floating particles for a few minutes.

Table 2 - Quality control of physical parameters assessed on complete microdiets used for the gilthead seabream and Senegalese sole trial. Criteria were evaluated on a scale from 1 to 5, where 1 is the lowest quality value and 5 is ranked as highest.

Microdiet	Size (µm)	Anti-caking capacity	Surface dispersion	Buoyancy
Commercial Control	100-200	3	4	3
	200-400	5	5	4
	400-600	5	5	5
CAP8.5	100-200	4	5	4
	200-400	5	5	4
	400-600	5	5	5
CAP30	100-200	4	5	4
	200-400	5	5	4
	400-600	5	5	5

2.3. Discussion

Given the poor digestive capacity of larvae, it is critical to assure the production of a microdiet able to retain water-soluble nutrients to deliver to marine fish larvae, such as amino-acids, once absorption of these low-molecular weight protein is easier, providing attraction at the same time. Thus, microencapsulation emerged as a valuable tool to protect these water-soluble low-molecular weight proteins.

This chapter describes the successful production of a microencapsulated prototype of fish protein hydrolysate by spray-drying. The microencapsulated prototype was able to reduce the leaching of water-soluble protein in about 75% after 2 hours of immersion, while the PC alone did not retain almost any protein after the same time. Considering that both PH and PC are 100% soluble in water, the results are a significant achievement, because this prototype is able to provide low-molecular weight proteins to marine fish larvae. The spray-drying process, together with the formed interactions, decreased the

solubility of two soluble molecules probably due the proximity to its isoelectric point (pI), when immersed in seawater. At molecule's pI, the electrostatic forces between protein-protein interactions are at minimum, minimizing repulsive forces, meaning that less water interacts with the protein molecules (Pelegrine and Gasparetto, 2005). Based on soluble protein leaching results, it was decided to produce microdiets with two levels of inclusion of the microencapsulated prototype, at 8.5 (CAP8.5) and 30% (CAP30).

The microdiets were formulated to be isonitrogenous, being the only difference the amount of microencapsulated prototype. The leaching assessment showed that smaller particle size microdiets presented a higher leaching percentage, what is probably due to the higher surface-to-volume ratio and the short diffusion distance from the core to the surface (Hamre et al., 2013). Commercial Control showed a decrease on leaching content when the particles size increased, but this trend is not observed in the microdiets with the microencapsulated prototype. For these two microdiets, CAP8.5 and CAP30, the leaching kept stable when the microdiet size increased. These results suggest that the microencapsulated prototype played an important role on the physical and biochemical properties of the microdiets, being more effective in the smaller sizes. Looking closer to 400-600 μm particle size results (Figure 4c), Commercial Control microdiet present a lower leaching when compared to the microdiets containing the microencapsulated prototype, for the same size. These results are unexpected difficult to explain. The higher leaching of the CAP30 microdiet could be a concentration related phenomenon, as suggested by Önal and Langdon (2009) as per to the studies of Dubernet et al. (1990) and Jalil and Nixon (1990). In those studies, microcapsules containing higher core concentrations of ethylcellulose and polylactic acid exhibited a quick release of the core material compared to a sustained release of the microcapsules with lower core concentrations. The authors also suggested that the quick release might me due to the amount of the drugs in the surface of the capsules. Looking at the leaching of the microdiets containing the microencapsulated prototype, a higher inclusion of the prototype resulted in a microdiet with a higher leaching in the particle size 100-200 μm, what makes this explanation unlikely.

The quality control assessment was intended to analyse the impact of the addition of the microencapsulated prototype in the microdiets different particles size. The inclusion of the microencapsulated prototype, at the two levels, favoured microdiets small particle size (100-200 μm) in the three evaluated parameters (anti-caking capacity, surface dispersion and buoyancy). In the other particles size, Commercial Control, CAP8.5 and CAP30 presented similar characteristics, independently of the inclusion of the

microencapsulated prototype. Based on good overall characteristics, microdiets were used in larval trials.

In conclusion, the encapsulation of low-molecular weight proteins led to the successful production of a microencapsulated prototype with low-leaching. Its inclusion was promising, particularly in smaller diet sizes, which suggests its adequacy for first-feeding larvae.

Chapter 3: Biological testing of newly developed microencapsulated prototype: gilthead seabream larvae

3.1. Materials and methods

3.1.1. Larval rearing

Gilthead seabream eggs were obtained by natural spawning from the broodstock kept at Estação Piloto de Piscicultura de Olhão (EPPO) facilities (IPMA, Olhão), and transferred to Ramalhete facilities (CCMAR, Universidade do Algarve). After hatching, larvae were reared in 100 L cylindroconical fibre glass tanks inserted on a semi-open recirculation system, with a density of 120 larvae L^{-1}. The experimental system was equipped with a mechanical filter, a submerged biological filter, a protein skimmer (Deltec GmbH, Germany) and a UV sterilizer. Photoperiod was set at 12/12h (light/dark) from 3 DAH (days after hatching) onwards, provided by overhead fluorescent tubes to produce an intensity of 1000-1500 lx. Frozen *Nannochloropsis oculata* microalgae (Acuinuga SL, Spain) was thawed daily and added to the rearing tanks to provide green-water conditions. Temperature, oxygen and salinity averaged 18.9 ± 0.4 ºC, 92.8 ± 3.2 % and 35.1 ± 0.4 g L^{-1}. Daily purges were done to evaluate the need to increase or decrease the amount of provided food and to record the number of dead larvae. The trial ended at 34 DAH.

3.1.2. Experimental design

The microdiets mentioned in the previous section 2.1.3, Commercial Control, CAP8.5 and CAP30, were randomly distributed to 12 tanks, 4 replicates per treatment. Feed was manually provided to all tanks 3 times per day, at 9h, 12h and 17h. At 0h and 6h, feed was provided by an automatic feeder (FishMate F14, FishMate, UK). The amount of necessary feed was placed in 2 wells of the automatic feeder and set to provide feed at the specified times in cycles of 2h of feeding followed by 1h break. A co-feeding regime was adopted with a reduction of the amount of the live-prey usually recommended. Rotifers from 3 DAH until 13 DAH. Artemia from 12 DAH until 23 DAH. The amount of live prey provided decreased along time during the trial. Larvae were fed with the microdiets since mouth opening, at 3 DAH, until the end of the trial, at 34 DAH. From 23 DAH onwards, larvae were only fed microdiet. On Table 3 the feeding plan used is described.

Table 3 – Feeding protocol used in gilthead seabream larvae trial. During co-feeding period, the initial amount of live-prey provided decreased. DD = Daily dose based on recommended feeding protocol.

DAH	Rot mL^{-1} (DD (%))	AF mL^{-1} (DD (%))	M24 mL^{-1} (DD (%))	Microdiet (mg)	Feed size (µm)
3	12 (80%)			150	100-200
4	16 (80%)			150	100-200
5	16 (80%)			150	100-200
6	16 (80%)			150	100-200
7	12 (60%)			150	100-200
8	13 (60%)			200	100-200
9	14 (60%)			200	100-200
10	14 (60%)			200	100-200
11	14 (60%)			500	100-200
12	7 (30%)	0.3 (60%)		500	100-200/ 200-400 (50/50)
13	4 (30%)	0.3 (60%)		500	100-200/ 200-400 (50/50)
14		0.3 (60%)		750	100-200/ 200-400 (50/50)
15		0.3 (60%)		1000	100-200/ 200-400 (50/50)
16		0.3 (60%)		1000	100-200/ 200-400 (50/50)
17		0.3 (60%)		1000	100-200/ 200-400 (50/50)
18		0.3 (40%)		1200	100-200/ 200-400 (50/50)
19		0.3 (40%)		1200	100-200/ 200-400 (50/50)
20		0.3 (40%)		1200	100-200/ 200-400 (50/50)
21		0.3 (30%)		1200	100-200/ 200-400 (50/50)
22		0.3 (30%)		1200	100-200/ 200-400 (50/50)
23		0.3 (30%)	0.2 (40%)	1500	100-200/ 200-400 (50/50)
24				1500	100-200/ 200-400 (50/50)
25				1600	200-400
26				1600	200-400
27				1900	200-400
28				2000	200-400
29				2100	200-400
30				2200	200-400
31				2300	200-400
32				2400	200-400
33				2500	200-400

Rot: Rotifers; AF: Artemia nauplii AF Strain; M24: 24h Artemia metanauplii AF Strain and Feed: Commercial Control, CAP8.5 and CAP30. Rotifers are expressed as number of rotifers/mL, Artemia are expressed as number of Artemia/mL and feed daily ration are expressed as g/tank/day.

3.1.3. Feed intake, growth performance and survival

During trial, ingestion pattern was evaluated. The evaluation of the gut content and filling were performed daily in a sample of 5 to 8 larvae per tank. Each larvae was observed to determine if it was ingesting microdiet, as well as the percentage of gut fullness. A binocular microscopes (Leica EZ4 HD, Leica microsystems, Germany) was used for this purpose.

Growth performance was evaluated as dry weight (DW) and total length (TL) assessment at 12, 20, 27 and 34 DAH. At 3 DAH a pool of 30 larvae was collected form each tank to assess DW. At 12, 20 and 27 DAH, 15 individual larvae were collected from each tank, and at 34 DAH, 20 individual larvae were collected from each tank. All sampled larvae were washed with distilled water to remove any residual feed and seawater. To determine TL, each larvae was orderly distributed in a Petri dish to photograph and placed on an Eppendorf tube. Snap freeze with liquid nitrogen to kill larvae and freeze drying thereafter. DW was assessed on freeze fried larvae. Non sampled larvae from each tank were counted at the end of the trial to determine survival (S, %).

3.1.4. Data analysis

Relative growth rate (RGR, % day^{-1}) was calculated as: $RGR = (e^{g} - 1) \times 100$, where $g = (\ln Wt - \ln W0) \times t^{-1}$. *Wt* and *W0* correspond to the final and initial dry weights, respectively, at a chosen period t. Survival (S, %) was calculated as: $S = (Lf / Li) \times 100$, where L*i* and L*f* correspond to the initial and final number of larvae in the tanks, respectively. Sampled larvae were considered alive for survival purposes. An arcsine transformation (T) was performed as it follows: T = ASIN (SQRT (value / 100)), for results expressed as percentage. One-way ANOVA, followed by a Tukey´s multiple comparison test, was used to calculate differences in feed intake, growth performance and survival between each treatment. The significance level considered was $p<0.05$ for all tests performed. All statistical analyses were performed with R Studio Version 1.2.1335.

3.2. Results

3.2.1. Feed intake, growth performance and survival

The results presented in Figure 5 show that, in general, larvae from all experimental treatments were ingesting feed from the beginning of the trial. A microdiet feed intake increasing was observed during trial. At 11 DAH, a significantly lower microdiet

ingestion was observed in larvae from CAP30 than in larvae from Commercial Control and CAP8.5. 50% of larvae from CAP30 had ingested microdiet while 65% of larvae from Commercial Control and 75% of larvae from CAP8.5 had ingested microdiet. At 23 DAH, there was no significant different percentage of larvae ingesting microdiet between treatments. 90% of larvae from Commercial Control and CAP30 and 95% of larvae from CAP8.5 had ingested microdiet. At 33 DAH, a significantly higher ingestion was observed in larvae from CAP8.5 than in larvae from CAP30. 100% of larvae from CAP8.5 had ingested microdiet while 97% of larvae from Commercial Control and 94% of larvae from CAP30 had ingested microdiet.

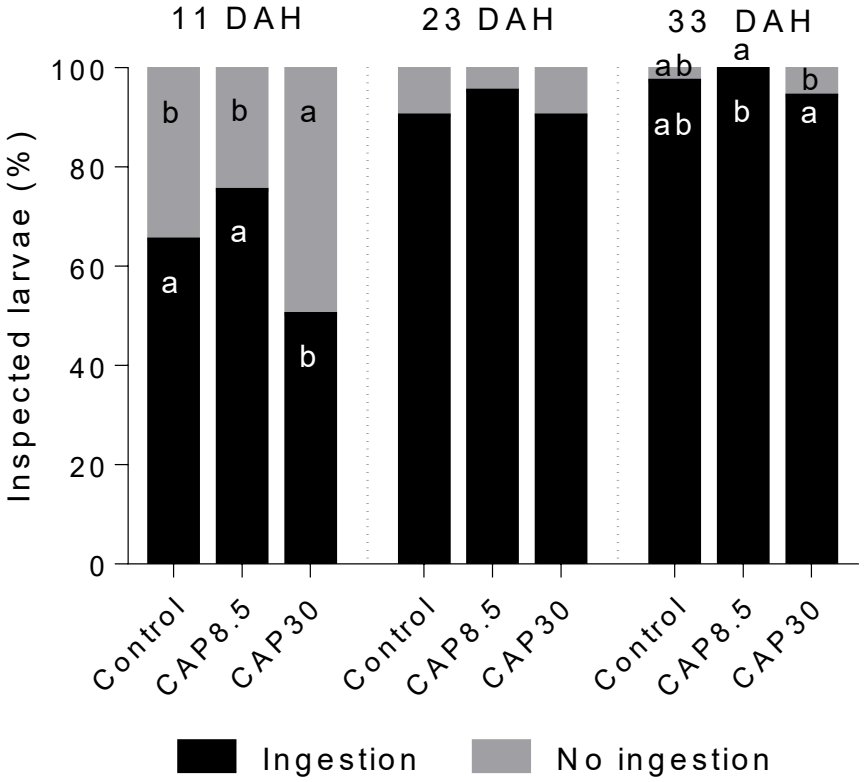

Figure 5 - Ingestion pattern in gilthead seabream larvae fed with different experimental microdiets at 11, 23 and 33 DAH. Results expressed as treatment mean (11 and 23 DAH n=20; 33 DAH n=32). Different letters indicate statistical differences (p<0.05) between treatments at the same age.

Table 4 – Gut fullness pattern evaluated in Gilthead seabream larvae fed on experimental microdiets at 11, 23 and 33 DAH. Results expressed as treatment mean ± standard deviation (11 and 23 DAH n=20; 33 DAH n=32). Different letters indicate statistical differences (p<0.05) between treatments and gut fullness at the same age.

11 DAH				
Gut fullness (%)	**Commercial Control**	**CAP8.5**	**CAP30**	***p* value**
0	35.0±34.2	25.0±25.2	50.0±11.5	0.275
25	20.0±16.3	30.0±20.0	50.0±11.5	0.113
50	20.0±16.3^{b}	30.0±11.5^{b}	0.0±0.0^{a}	1.66E-3
75	20.0±0.0^{b}	10.0±11.5^{ab}	0.0±0.0^{a}	3.06E-8
100	5.0±10.0	5.0±10.0	0.0±0.0	0.494
23 DAH				
Gut fullness (%)	**Commercial Control**	**CAP8.5**	**CAP30**	***p* value**
0	10.0±11.5	5.0±10.0	10.0±20.0	0.830
25	35.0±19.2	30.0±25.8	35.0±25.2	0.836
50	20.0±16.3	45.0±30.0	30.0±20.0	0.419
75	35.0±19.2	20.0±16.3	25.0±25.2	0.474
100	0.0±0.0	0.0±0.0	0.0±0.0	1.000
33 DAH				
Gut fullness (%)	**Commercial Control**	**CAP8.5**	**CAP30**	***p* value**
0	3.1±6.3	0.0±0.0	6.3±7.2	0.290
25	34.4±18.8	18.7±12.5	37.5±25.0	0.462
50	28.1±15.7	34.4±18.7	53.1±23.7	0.255
75	21.9±6.3^{b}	40.6±15.7^{b}	3.1±6.3^{a}	1.17E-2
100	12.5±17.7	6.3±7.2	0.0±0.0	0.206

In Figure 6 and Table 4 is possible to observe an increasing in gut fullness through the duration of the trial. At 11 DAH, no significant difference was found in the number of larvae with 0, 25 and 100% of the gut full between treatments. A significantly higher number of larvae from Commercial Control and CAP8.5 than larvae from CAP30 were found with 50% of the gut full and a significantly higher number of larvae from Commercial Control than larvae from CAP30 were found with 75% of the gut full. At 23 DAH, no significant differences were found in larvae from all treatments with the same gut fullness. At 33 DAH, a significantly higher number of larvae from Commercial Control and CAP8.5 were found with 75% of the gut full when compared with larvae from CAP30. No significant differences were found in the remaining percentage of gut fullness between each treatment.

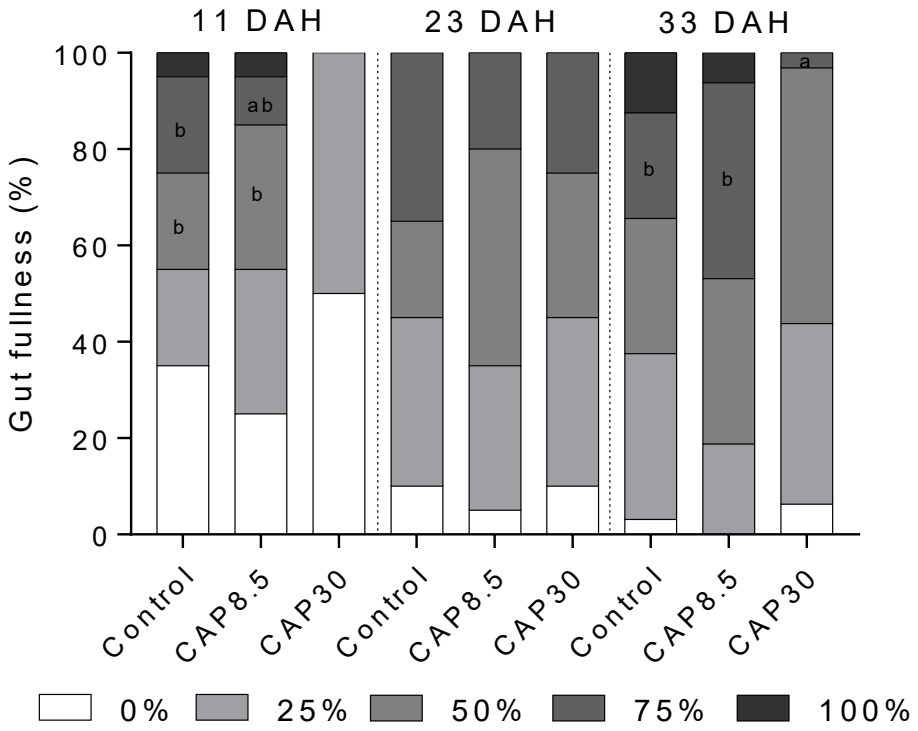

Figure 6 - Gut fullness evaluated in Gilthead seabream larvae fed on experimental microdiets at 11, 23 and 33 DAH. Results expressed as treatment mean ± standard deviation (11 and 23 DAH n=20; 33 DAH n=32). Different letters indicate statistical differences ($p<0.05$) between treatments and gut fullness at the same age.

The results shown in Figure 7 make possible to observe at 12 DAH, larvae from Commercial Control and CAP8.5 had a TL of around 4 mm while larvae from CAP30 had a TL of around 4.5. A significantly higher TL was observed in larvae from CAP30 than larvae from Commercial Control and CAP8.5. At 20 DAH this trend was not maintained. Larvae from Commercial Control and CAP8.5 had a TL of around 6 mm while larvae from CAP30 had a TL of around 5.5 mm. A significantly higher TL was found in larvae from Commercial Control than in larvae from CAP30. At 27 DAH, larvae from the three treatments had a TL of approximately of 6.6 mm and no significant differences were found between them. In the final of the trial, at 34 DAH, larvae from Commercial Control and CAP8.5 had a TL of around 7.5 and larvae from CAP30 a TL around 7 mm. A significantly higher TL was found in larvae from Commercial Control and CAP8.5 than in larvae from CAP30.

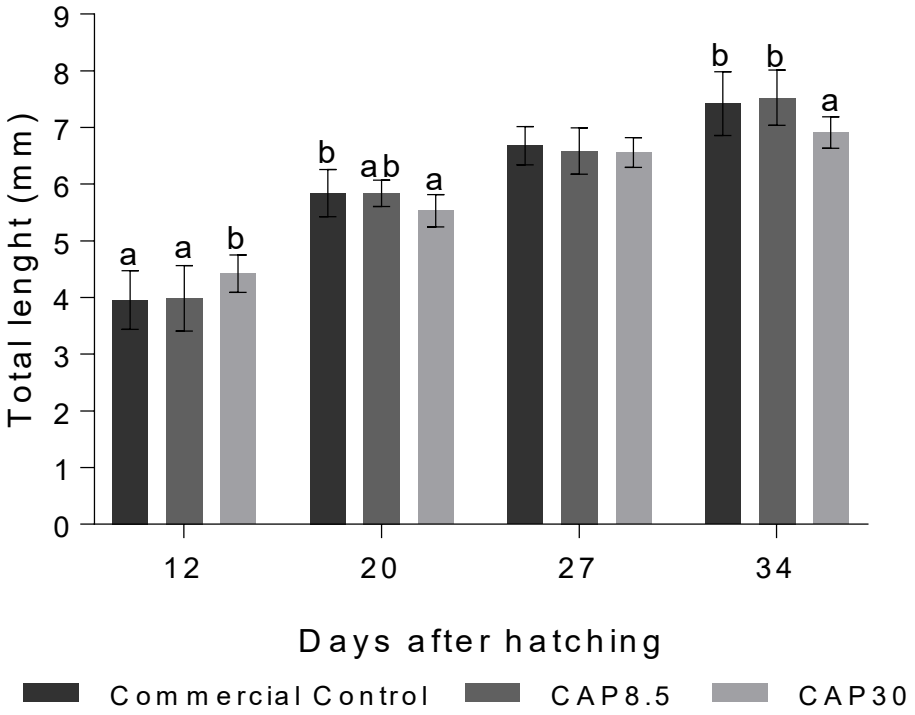

Figure 7 - Total length assessed in Gilthead seabream larvae reared under different dietary treatments throughout the trial. Results expressed as treatment mean ± standard deviation. At 12, 20 and 27 DAH n=60 larvae, and at 34 DAH n=80 larvae. Different letters indicate statistical differences (p<0.05) between treatments at the same age.

The results presented in Figure 8 show a variable, but positive, DW increase in all three treatments. At 12 DAH, larvae from Commercial Control had a DW around 0.03 mg, larvae from CAP8.5 and CAP30 had a DW of around 0.04 mg. A significantly higher DW was found in larvae from CAP8.5 and CAP30. At 20 DAH, the trend changed and larvae from Commercial Control and CAP8.5 had an increasing of DW to around 0.18 mg and larvae from CAP30 to around 0.14 mg. A significantly higher DW was found in larvae from Commercial Control when compared with larvae from the other experimental treatments. At 27 DAH, larvae from all treatments had DW around 0.23 mg and no significant differences were found between the three experimental treatments. At 34 DAH, end of the trial, larvae from Commercial Control presented a DW of around 0.38 mg, larvae from CAP8.5 presented a DW of around 0.44 mg and larvae from CAP30 presented a DW of around 0.35 mg. A significantly higher DW was found in larvae from CAP8.5 when compared with larvae from CAP30.

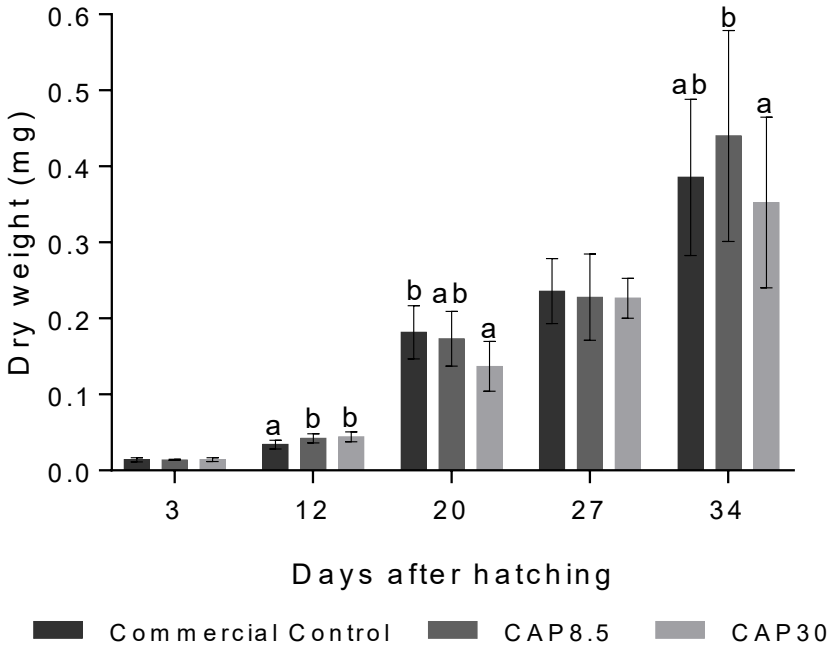

Figure 8 - Dry weight assessed in Gilthead seabream larvae reared under different dietary treatments throughout the trial. Results expressed as treatment mean ± standard deviation. At 3 DAH n= 3 pool of 30 larvae, at 12, 20 and 27 DAH n=60 larvae, and at 34 DAH n=80 larvae. Different letters indicate statistical differences (p<0.05) between treatments at the same age.

In Figure 9, it can be observed that between 3 and 12 DAH, larvae from Commercial Control presented an RGR of around 11%, larvae from CAP8.5 presented an RGR of around 13% and larvae from CAP30 presented an RGR of around 13.5%. A significantly higher RGR was found in larvae from CAP8.5 and CAP30 than in larvae from Commercial Control. Between 12 and 20 DAH, larvae from Commercial Control presented a significantly higher RGR than larvae from CAP8.5 and CAP30, reaching RGR values of around 23, 19 and 15% respectively. Between 20 and 27, a decreasing in RGR values was observed, when compared with the last time period, and larvae from Commercial Control and CAP8.5 had an RGR of around 4% while larvae from CAP30 had an RGR of around 8%. No significant differences were found between treatments. Between 27 and 34 DAH, larvae from CAP8.5 had the RGR increased to around 10%, Commercial Control RGR increased to around 7% and larvae from CAP30 RGR decreased to around 6%, but no significant differences were found between larvae RGR from the three dietary treatments. Between the beginning and the end of the trial (3 and 34 DAH), no significant differences

were found between larvae RGR from the three experimental treatments with an average RGR of around 11% DW day^{-1}.

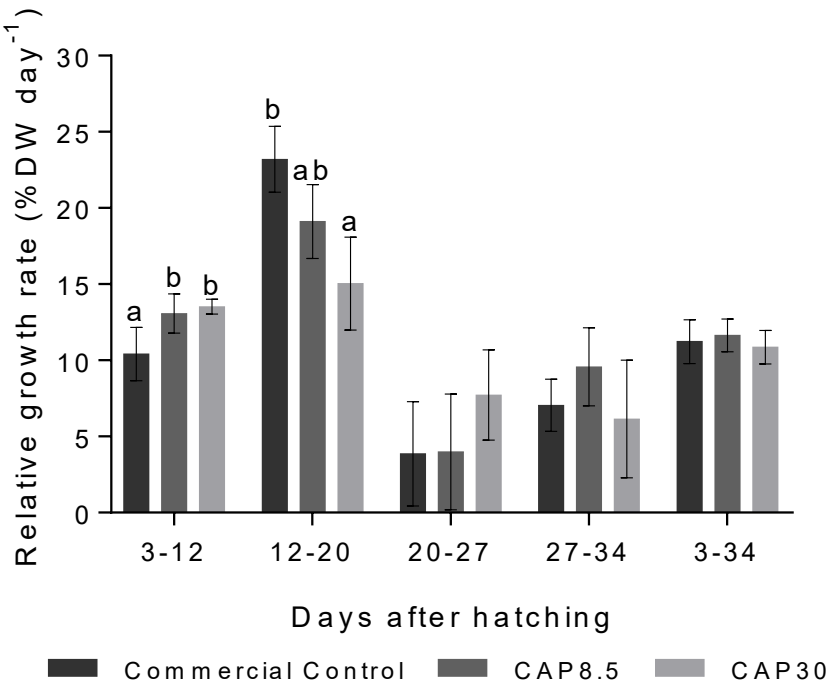

Figure 9 - Relative growth rate (%DW day^{-1}) determined in Gilthead seabream larvae reared under different dietary treatments throughout the trial. Results expressed as treatment mean ± standard deviation between Different letters indicate statistical differences (p<0.05) between treatments at the same age period.

In Figure 10 the survival results are expressed. The calculated survival ranged from 8% in larvae from Commercial Control to 5% in larvae from CAP8.5 and 6.5% in larvae from CAP30. No significant differences were found between the three experimental treatments.

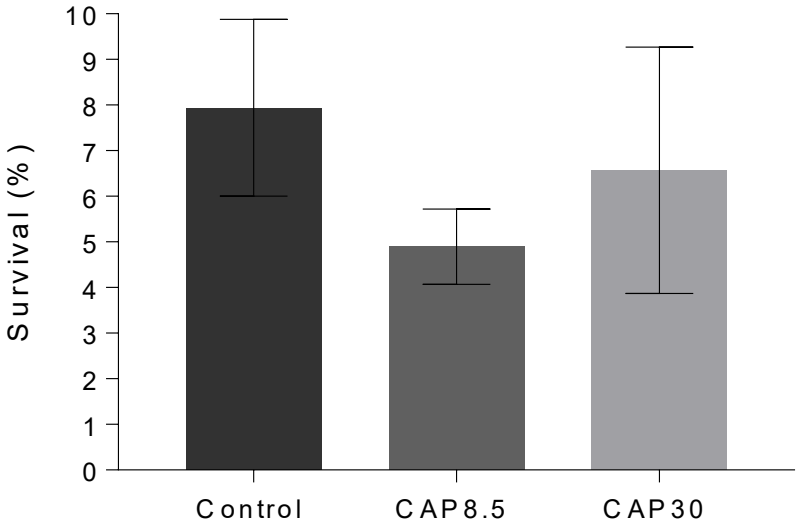

Figure 10 - Gilthead seabream larvae survival (%) at the end of trial (34 DAH). Results expressed as mean ±standard deviation (n=4). Absence of letters indicate no statistical differences ($p>0.05$) between treatment.

3.3. Discussion

The acceptance of microdiets by fish larvae and the digestion and absorption of nutrients are the major concern at first-feeding, since larvae does not possess a fully complete digestive capacity. Larvae lack a functional stomach, meaning a weak digestive capacity as in gastric acid conditions, but have some digestive enzymes that have an optimal activity at neutral or alkaline pH, as pancreatic enzymes, conditioning the range of proteins and lipids that larvae are able to digest (Yúfera et al., 2011; Rønnestad et al., 2013; Canada et al., 2017). Although the nutritional requirements for fish larvae are not clear (Hamre et al., 2013), it is known that larvae digestion is easier and growth is promoted if there is a balanced and appropriate dietary amino acids/ indispensable amino acids ratio (Aragão et al., 2004; Canada et al., 2016a). So, the retention of these water-soluble nutrients when producing a digestible microdiet for fish larvae needs to be assured. With this in mind, the novel encapsulated prototype was tested in gilthead seabream using two inclusion levels, 8.5 and 30%.

The approach taken was to observe the gut of the larvae to determine if the microdiets were being ingested and how much full the gut was. This allowed to adjust the feeding protocol, as for example, increase the provided microdiet. Ingestion of the microdiets was observed since mouth opening, indicating a good acceptance by gilthead

seabream larvae. Ingestion of microdiet increased during trial time. At 11 DAH, a lower percentage of larvae from CAP30 were ingesting microdiet, even though this experimental microdiet presented a higher leaching after 30 minutes of immersion, which suggests that gilthead seabream larvae may have more visual than olfactory predatory behaviour (Yúfera et al., 2011). As CAP30 microdiet presented lighter colour (data not shown), it may explain the lower ingestion once larvae present limited visual capacity at early stages. Lower palatability and loss of nutritional value can also be an explanation, once after 30 minutes, CAP30 had leached more than 50% of its total protein and probably vitamins and minerals. In the remaining sampling days, a constant increase was observed, which is translated in the larvae gut fullness. The gut fullness increasing means a growing adaptation of larvae to the microdiets. This increasing ingestion and gut fullness was also a translation of the increased amount of microdiet. Ortiz-Monís et al. (2018) also observed that gut filling tends to increase with larvae age.

The use of an early co-feeding strategy with high replacement of live-prey allowed a full weaning at 23 DAH. The weaning to microdiet was performed based on visual inspection of microdiet ingestion and at 23 DAH bream larvae presented a higher intake of microdiet than live-prey which led to the decision to stop live-prey feeding. The weaning age is later than in the Yúfera et al. (1999), Yúfera et al. (2000) and Yúfera et al. (2005) studies, where a sudden weaning was performed from live-prey to an inert diet at 8 DAH. Saleh et al. (2014) completely weaned seabream larvae at 16 DAH. It is worth to mention that throughout the experiments, larvae were only receiving a small fraction of live-prey in comparison with what normally used in commercial hatcheries, revealing success in the implementation of microdiets and respective feeding protocol. In most seabream hatcheries, weaning occurs when larvae reach around 30 to 35 DAH (Wilson Pinto, personal communication).

The survival results obtained are low when compared to other studies, which is a response to zootechnical conditions and a more agressive feeding protocol. Survival of 7.94±1.94, 4.89±0.82 and 6.57±2.70 % were obtained with Commercial Control, CAP8.5 and CAP30 microdiets, respectively. For example Yúfera et al. (1999) obtained 70% survival in gilthead seabream larvae fed microcapsules from 8 DAH until 15 DAH and Sandel et al. (2010) obtained 73% survival in larvae co-fed from 20 DAH until 33 DAH. Izquierdo et al. (2019) obtained a lower survival when compared to these authors. Survival of 19% was obtained in gilthead seabream larvae fed with rotifers from mouth opening until 16 DAH, and microdiet from 16 DAH onwards. Even though survival was low,

the results are acceptable to the low rearing tank volume used in the experiment. No events of high mortality were recorded during trial time and the non-statistical difference between each treatment suggests that the microdiets type had no negative effect on survival.

The best growth performance was obtained with CAP8.5 microdiet, where gilthead seabream larvae achieved, at 34 DAH, 0.44±0.14 mg DW, 7.52±0.49 mm TL and an average RGR of 11.6±1.07 % DW day^{-1}, followed by Commercial Control microdiet where larvae achieved 0.39±0.10 mg DW, 7.42±0.56 mm TL and an average RGR of 11.23±1.44 % DW day^{-1}, and CAP30 microdiet where larvae achieved 0.35±0.11 mg DW, 6.90±0.28 mm TL and an average RGR of 10.85±1.10 % DW day^{-1}. Similar DW and TL were found in gilthead seabream larvae in several studies: exclusively fed on live-prey in Mata-Sotres et al. (2016); Izquierdo et al. (2019) obtained TL values of 7.20 mm at 31 DAH with gilthead seabream larvae fed with rotifers from mouth opening until 16 DAH and microdiet from 16 DAH onwards. Better growth performances were obtained by Morais et al. (2006), who obtained at 33 DAH, gilthead seabream larvae with 0.9 mg DW at 34 DAH in a co-feeding strategy since 23 DAH and 1.8 mg DW in gilthead seabream larvae fed exclusively on live prey; Sandel et al. (2010) obtained 0.9 mg DW at 33 DAH in gilthead seabream larvae in a co-feeding and larvae exclusively fed with live-prey achieved 2 mg. Taken together, the present results are comparable with some studies in the bibliography, meaning that although a high replacement strategy was adopted, the results were positive and the experiment was successfully conducted.

In the first two weeks of development, larvae from CAP8.5 and CAP30 presented a better growth than larvae from Commercial Control, which indicates a more adequate nutritional profile than the Commercial Control diet, thus improving growth. After this time, a growth performance increasing was observed in larvae from Commercial Control, presenting a higher growth performance, which was not maintained until the end of the trial. The growth rate increasing of larvae fed on this experimental microdiet was probably due to the significant increasing amount of microdiet provided in the tanks at 12 DAH onwards together with increasing in the digestive capacity of complex protein. After this first two weeks, a decreased growth performance was also observed in CAP30 larvae. It has been suggested that including PH in microdiets for culture of fish larvae can improve performance and quality because the dietary nitrogen source thus provided is more readily available than intact proteins to fish larvae (de Vareilles et al., 2012). But the higher amount of encapsulated prototype in the CAP30 microdiet may have caused an

overloading of amino acids, that being absorbed at different rates and different efficiencies, leading to transitory amino acids imbalances and subsequent reduced protein deposition and growth (Canada et al., 2017).

In conclusion, the high live prey replacement in the co-feeding protocol adopted in gilthead seabream larvae led to an early weaning with growth performance identical to studies were larvae were exclusively fed on live-prey until weaning. In order to get the maximum possible results, gilthead seabream larvae can be fed with CAP30 microdiet in the first two weeks of development and after that with CAP8.5 microdiet. This microdiet composition sequence seems to be better adapted to larval digestive and absorption capacity during the first weeks of development.

Chapter 4: Biological testing of newly developed microencapsulated prototype: Senegalese sole larvae

4.1. Materials and methods

4.1.1. Larval rearing

Senegalese sole eggs were obtained by natural spawning from the broodstock kept at EPPO facilities and transferred to Ramalhete facilities. After hatching, larvae were reared in 100 L cylindro-conical fibre glass tanks, with a stock density of 86 larvae L^{-1}, in semi-open recirculation system. The experimental system was equipped with a mechanical filter, a submerged biological filter, a protein skimmer and a UV sterilizer. Photoperiod was set 12h light/ 12h dark from 3 DAH onwards, provided by overhead fluorescent tubes to produce an intensity of 1000-1500 lx. Thaw *Nannochloropsis oculata* microalgae was added on a daily basis to the water to provide green-water conditions. Temperature, oxygen and salinity averaged 19.2 ± 0.3 ºC, 91.5 ± 4.7 % and 35.1 ± 0.3 g L^{-1} (mean ± SD). Daily purges were done to evaluate the need to increase or decrease the amount of given food and to record the number of dead larvae. The trial ended at 20 DAH

Table 5 - Feeding protocol used in Senegalese sole larvae trial.

DAH	Feed (mg)	Feed size (µm)
3	250	100-200/ 200-400 (50/50)
4	1350	100-200/ 200-400 (50/50)
5	2500	100-200/ 200-400 (50/50)
6	2500	100-200/ 200-400 (50/50)
7	2500	100-200/ 200-400 (50/50)
8	2500	100-200/ 200-400 (50/50)
9	2500	100-200/ 200-400 (50/50)
10	2500	100-200/ 200-400 (50/50)
11	2500	200-400
12	2500	200-400
13	2500	200-400
14	2500	200-400
15	2500	200-400
16	3000	200-400
17	3000	200-400
18	3000	200-400
19	3000	200-400
20	3000	200-400

4.1.2. Experimental design

The microdiets mentioned in the previous section 2.1.3, Commercial Control, CAP8.5 and CAP30, were randomly distributed to 9 tanks, 3 replicates per treatment. During the trial time, only experimental microdiets were provided to the tanks. The daily

amount of microdiet was placed in 8 wells of the automatic feeder and set to provide microdiet at every 3 hours in cycles of 2h of feeding followed by 1h break. Automatic feeders were programmed to release microdiet at 0h, 3h, 6h, 9h, 12h, 15h, 18h and 21h. After being release, the microdiet would fall in a tube, where it was hydrated, and then released to the rearing water. The feeding plan can be seen on Table 5.

4.1.3. Feed intake, growth performance and survival

The gut content and filling evaluation was assessed as previously described on section 3.1.3. The only difference was that sampling only occurred two times per week, at 4, 6, 10, 13 and 17 DAH, and 8 larvae were sampled each time.

DW and TL were assessed at 9 and 20 DAH. At 2 DAH, a pool of 30 larvae was also collected from each tank to assess DW. At 9 and 20 DAH, 15 and 20 individual larvae were collected from each tank, respectively. The remaining procedure was the same of the section 3.1.3.

4.1.4. Data analysis

Data analysis was performed as previously described on section 3.1.4. Relative growth rate (RGR, % day^{-1}), Survival (S, %) were calculated. Sampled larvae were considered alive for survival purposes and an arcsine transformation (T) was conducted for results expressed as percentage. One-way ANOVA, followed by a Tukey´s multiple comparison test, was used to calculate differences in feed intake, growth performance and survival between each treatment. The significance level considered was $p<0.05$ for all tests performed. All statistical analyses were performed with R Studio Version 1.2.1335.

4.2. Results

4.2.1. Feed intake, growth performance and survival

The results presented in Figure 11 show that, in general, larvae from all experimental treatments were not ingesting microdiet at the beginning of the trial (4 DAH), but after 6 DAH some feed was observed in the gut and ingestion kept stable during trial duration. At 6 DAH, 45% of larvae from Commercial Control were found ingesting microdiet, while 30 and 25% of larvae from CAP8.5 and CAP30 were ingesting microdiet, respectively. A significantly higher ingestion was found in larvae from Commercial Control than in larvae from CAP30. At 10 DAH, around 50% of larvae from CAP8.5 and

CAP30 and around 40% of larvae from Commercial Control were ingesting microdiet, but no significant differences were found between ingestion in all three treatments. At 13 DAH, around 40, 30 and 25% of larvae from Commercial Control, CAP8.5 and CAP30 were found ingesting microdiet, respectively. A significantly higher ingestion was found in larvae from Commercial Control than in larvae from CAP30. At 17 DAH, the same trend was observed, and larvae from Commercial Control presented a significant higher ingestion than larvae from CAP8.5 and CAP30.

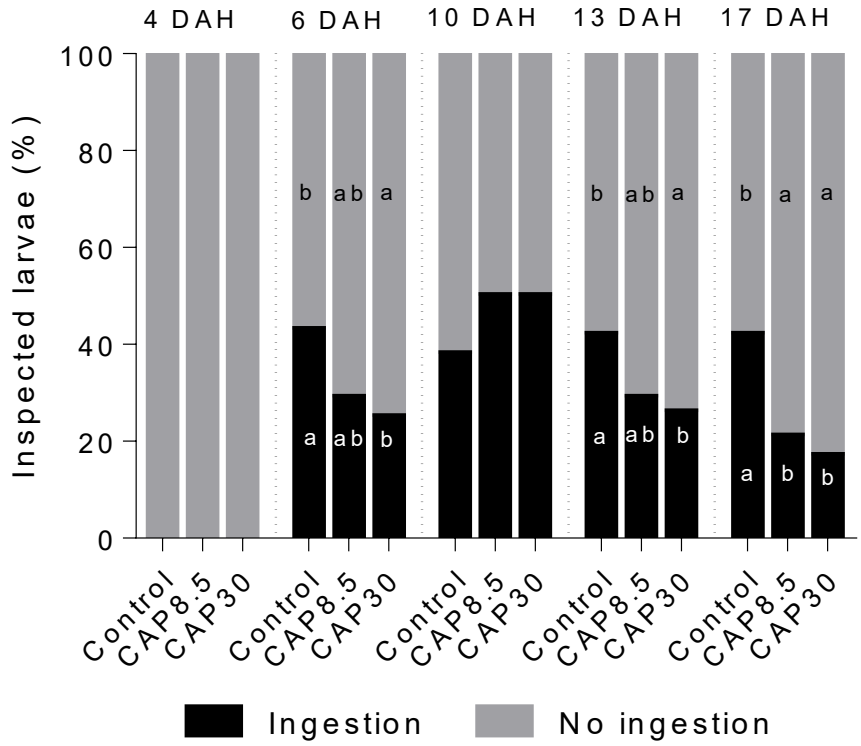

Figure 11 – Senegalese sole larvae ingestion pattern of different experimental microdiets at 4, 6, 10, 13 and 17 DAH. Results expressed as treatment mean (n=24). Different letters indicate statistical differences (p<0.05) between treatments at the same age.

Table 6 - Gut fullness evaluated in Senegalese sole larvae fed on experimental microdiets at 4, 6, 10, 13 and 17 DAH. Results expressed as treatment mean ± standard deviation (n=24). Different letters indicate statistical differences (p<0.05) between treatments and gut fullness at the same age. NA=Not applicable.

4 DAH				
Gut fullness (%)	**Commercial Control**	**CAP8.5**	**CAP30**	***p* value**
0	100.0±0.0	100.0±0.0	100.0±0.0	1.000
6 DAH				
Gut fullness (%)	**Commercial Control**	**CAP8.5**	**CAP30**	***p* value**
0	56.5±6.3	70.8±14.4	75.0±25.0	0.3382
25	39.3±3.1^{b}	25.0±12.5^{ab}	8.3±14.4^{a}	0.1410
50	0.0±0.0	4.2±7.2	8.3±14.4	0.5348
75	4.2±7.2	0.0±0.0	4.2±7.2	0.5348
100	0.0±0.0	0.0±0.0	4.2±7.2	0.6857
10 DAH				
Gut fullness (%)	**Commercial Control**	**CAP8.5**	**CAP30**	***p* value**
0	62.5±12.5	50.0±21.7	50.0±33.1	0.6484
25	33.3±14.4	29.2±19.1	12.5±12.5	0.3785
50	4.2±7.2	12.5±12.5	12.5±12.5	0.6522
75	0.0±0.0	4.2±7.2	16.7±14.4	0.2949
100	0.0±0.0	4.2±7.2	8.3±7.2	0.2950
13 DAH				
Gut fullness (%)	**Commercial Control**	**CAP8.5**	**CAP30**	***p* value**
0	58.3±19.1	70.8±26.0	73.2±15.3	0.6544
25	25.0±0.0	20.8±26.0	8.3±7.2	0.2708
50	12.5±21.7	8.3±14.4	13.7±14.3	0.8701
75	4.2±7.2	0.0±0.0	4.8±8.2	0.5348
100	0.0±0.0	0.0±0.0	0.0±0.0	1.0000
17 DAH				
Gut fullness (%)	**Commercial Control**	**CAP8.5**	**CAP30**	***p* value**
0	58.3±19.1	79.2±19.1	83.3±14.4	0.3611
25	25.0±12.5	20.8±19.1	12.5±12.5	0.5585
50	12.5±12.5	0.0±0.0	4.2±7.2	0.3110
75	4.2±7.2	0.0±0.0	0.0±0.0	0.6857
100	0.0±0.0	0.0±0.0	0.0±0.0	1.0000

In Figure 12 and Table 6 the results concerning the larval gut fullness throughout the trial are expressed, and it is possible to observe that at the beginning of the trial (4 DAH) larvae from all treatments were not ingesting microdiet, but an increasing ingestion was observed in the next days. At 6 DAH, a significantly higher number of larvae from Commercial Control was found with 25% of the gut full than larvae from CAP30. No significant differences were found between the remaining gut fullness sampling points.

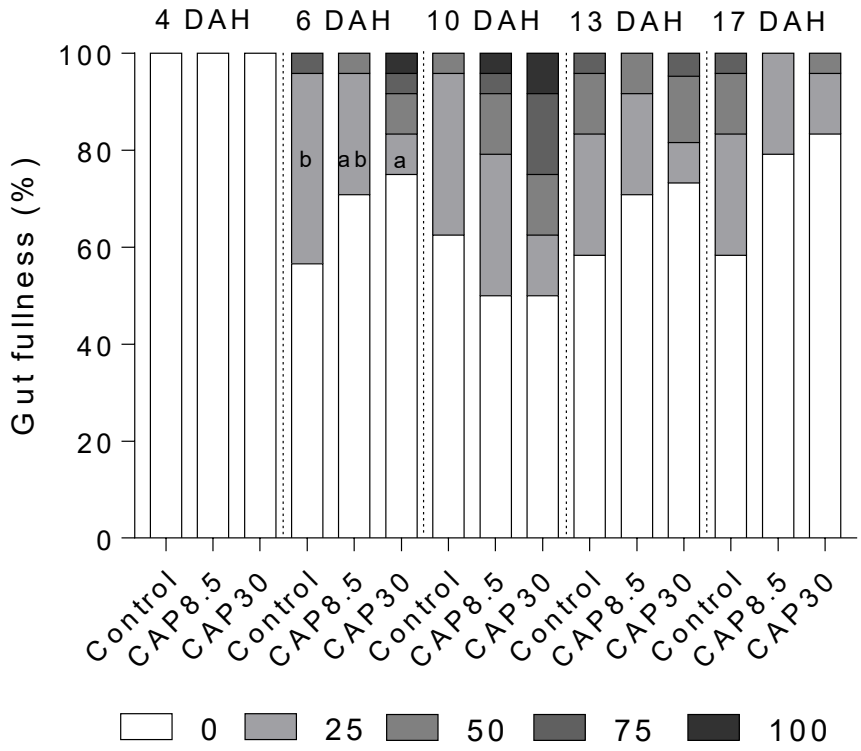

Figure 12 - Gut fullness evaluated in Senegalese sole larvae fed on experimental microdiets at 4, 6, 10, 13 and 17 DAH. Results expressed as treatment mean ± standard deviation (n=24). Different letters indicate statistical differences (p<0.05) between treatments and gut fullness at the same age.

Results presented in Figure 13, concerning TL, show that at 9 DAH larvae had a TL of around 4 mm and no significant differences were found between treatments. At 20 DAH, larvae TL varied from around 5.20 mm in Commercial Control to 5.00 mm in CAP8.5 and 5.10 mm in CAP30, but no significant differences were found between the three experimental treatments.

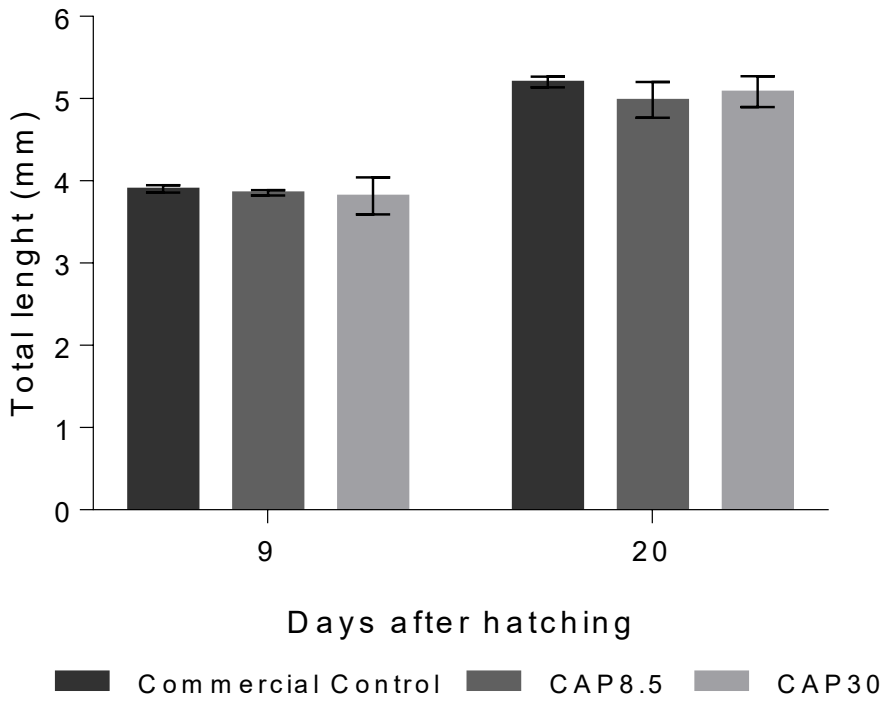

Figure 13 – Total length assessed in Senegalese sole larvae reared under different dietary treatments throughout duration of the trial. Results expressed as treatment mean ± standard deviation. At 9 DAH n=45 larvae, and at 20 DAH n=60 larvae. Absence of letters indicate no statistical differences (p>0.05).

Senegalese sole DW results at 9 DAH (shown on Figure 14), had approximately 0.05 mg DW in the three experimental treatments and no significant DW were found in any experimental treatment. At 20 DAH, larvae from Commercial Control had around 0.15 mg DW, from CAP8.5 0.13 mg and from CAP30 0.19 mg. A significantly higher DW was found in larvae from CAP30 in comparison with larvae from CAP8.5.

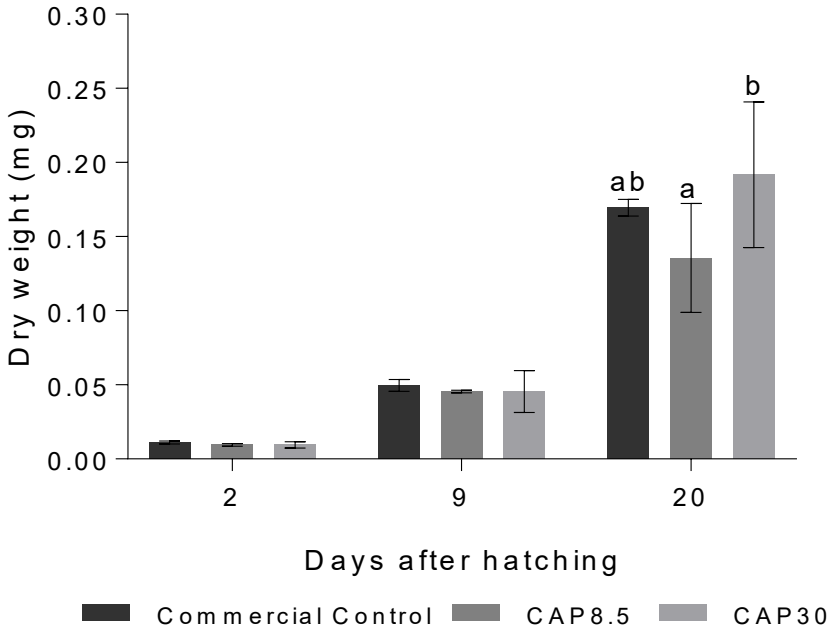

Figure 14 – Dry weight assessed in Senegalese sole larvae reared under different dietary treatments throughout duration of the trial. Results expressed as treatment mean ± standard deviation. At 2 DAH n= 3 pool of 30 larvae, at 9 DAH n=45 larvae and at 20 DAH n=60 larvae. Different letters in indicate statistical differences for a given age (p<0.05).

Senegalese sole RGR presented in Figure 12 shows between 2 and 9 DAH, larvae from all three experimental treatments presented similar RGR of around 24% and no significant RGR was found. Between 9 and 20 DAH, larvae from Commercial Control had an RGR of around 12%, larvae from CAP8.5 had an RGR of around 10% and larvae from CAP30 had an RGR of around 14%. No significant RGR was found between the three experimental treatments in this period. During trial, between 2 and 20 DAH, no significant differences in larvae RGR were found, growing at an average RGR of 16%DW day^{-1}.

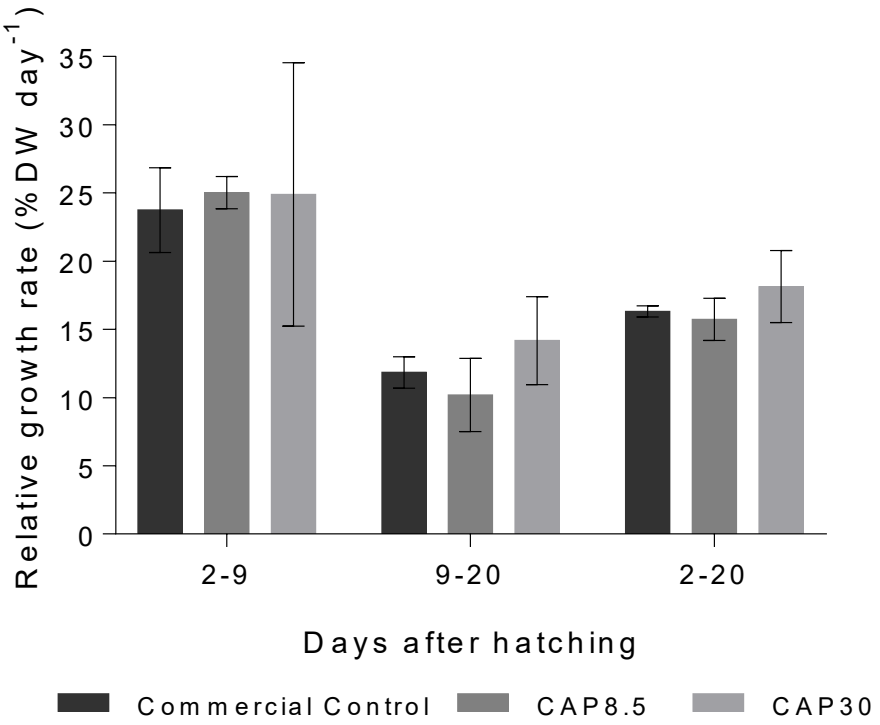

Figure 15 – Senegalese sole larvae relative growth rate (%DW day^{-1}) determined throughout duration of the trial. Results expressed as treatment mean ± standard deviation between a given time. Different letters indicate statistical differences (p<0.05) between treatments at the same age period.

The shown survival results on Figure 16 show that no significant differences were found in the experimental treatments. Survival of 70% was found in Commercial Control and 55% in CAP30.

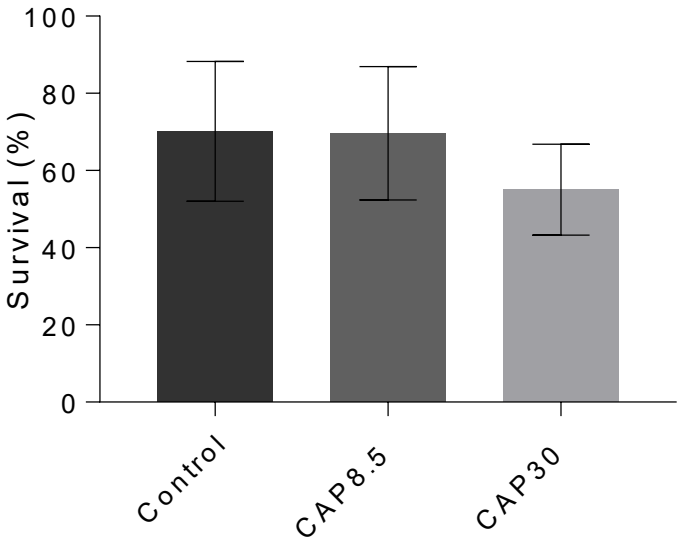

Figure 16 - Senegalese sole larvae survival at the end of the trial (20 DAH). Results expressed as mean ± standard deviation (n=3). Absence of letters indicate no statistical differences (p>0.05) between treatment.

4.3. Discussion

Senegalese sole is a high value species for aquaculture industry in the Southern Europe. The newly hatched larvae are pelagic and usually undergo an accentuated metamorphosis between 11 and 19 DAH (Dinis et al., 1999). In general, Senegalese sole larvae are more robust than gilthead seabream larvae, so a more risky protocol was adopted.

No ingestion was observed in the first days after mouth opening, suggesting a weak acceptance of all experimental microdiets by larvae at the beginning of the trial. After 6 DAH onwards some ingestion was observed in all three experimental treatments, but always less than 50% of the inspected larvae were ingesting microdiet. A lower ingestion observed in larvae from CAP30 at 6 and 13 DAH in comparison with larvae from Commercial Control, and at 17 DAH, CAP8.5 and CAP30 larvae presented lower ingestion than larvae from Commercial Control. So, in general, larvae ingested Commercial Control microdiet better than microdiets containing the microencapsulated prototype. In comparison with gilthead seabream larvae, Senegalese sole larvae presented a lower ingestion, but the ingestion pattern of CAP30 microdiet was the same in both trials. Both gilthead seabream and Senegalese sole larvae presented lower ingestion of CAP30 microdiet in relation to Commercial Control microdiet.

The lower ingestion and gut fullness observed in Senegalese sole larvae, compared to gilthead seabream larvae, could be due to species behaviour, difference in feeding protocol, or even microdiet hydration. It is possible that, after hydration, particles sink faster than if there was no hydration and thus may stay less time available in water column. Almost double of the microdiet amount was being administrated in Senegalese sole larvae when compared to gilthead seabream feeding plan. With the observation of these conditions, it is possible that a large amount of the provided microdiet was not available to larvae to fed. Further testing is needed to verify these conditions, since only first observations were made, in order to validate the correct microdiet administration form.

The feeding regime adopted allowed, at the end of the trial (20 DAH), Senegalese sole larvae fed on Commercial Control microdiet reach 5.20±0.06 mm TL, 0.17±0.01 mg DW and RGR of 16.32±0.40 % DW day^{-1}; larvae fed on CAP8.5 microdiet reached 4.98±0.22 mm TL, 0.14±0.04 mg DW and RGR of 15.73±1.54 % DW day^{-1}; larvae fed on CAP30 microdiet reached 5.08±0.19 mm TL, 0.19±0.05 mg DW and RGR of 18.14±2.65 % DW day^{-1}. Senegalese sole larvae TL was not affected by any of the three experimental treatments, but at the end of the trial larvae from CAP30 had a significantly higher DW than larvae from CAP8.5. Even though no significant differences were found in RGR values, larvae from CAP30 grew more in DW than larvae from CAP8.5. Comparing Senegalese sole larvae with gilthead seabream larvae, it seems that Senegalese sole larvae have a similar capacity to digest and absorb high amounts of PH at initial stages (Canada et al., 2017). These results are an advance comparing to the results obtained by Pinto et al. (2010) in a co-feeding regime since mouth opening, with DW values of around 0.22 mg and TL around 6 mm at 20 DAH, but a lower RGR, around 9.1%. Better results were obtained by Engrola et al. (2009) with 1 mg DW at 20 DAH in a co-feeding regime with live-prey replacement; Engrola et al. (2010) achieved 0.74 mg DW and RGR of 18.78% at 20 DAH in a co-feeding regime with high replacement of live-prey; Lobo et al. (2014) achieved 1 mg DW and around 8 mm TL at 20 DAH in a live-prey feeding regime; Canada et al. (2016b) with DW of around 1 mg and RGR around 25% at 19 DAH in a co-feeding regime; Canada et al. (2017) with DW of around 0.5 mg and RGR of around 26% at 16 DAH. Although the DW results obtained in this trial are similar to the ones of Pinto et al. (2010) with lower RGR and Engrola et al. (2010) obtained higher DW with similar RGR, one need to take into account that the initial Senegalese sole DW was very low (0.01±0.001 mg DW), which may suggest a lower quality of egg/larval hatch. Senegalese sole larvae DW at mouth opening is recorded to be around 0.04 mg (Dinis et

al., 1999; Engrola et al., 2010). The obtained growth performance was a response to the adopted feeding protocol.

The microdiets were able to sustain high and no significant different survival, indicating a positive influence. Survival of 70.13 ± 18.12 %, was found in larvae from Commercial Control, 69.69 ± 17.26 % in larvae from CAP8.5 and 55.06 ± 11.75 % in larvae from CAP30. The obtained survival results are higher than the 28% obtained by Pinto et al. (2010) and 30% obtained by Engrola et al. (2010). So, in terms of survival, the experiment was very positive. Unfortunately, none of the experimental treatments allowed metamorphosis to be completed until the end of the trial.

In conclusion, at early stages Senegalese sole larvae should be fed a microdiet containing the protein hydrolysate at relatively high levels of inclusion (CAP30). It can be said that the growth performance, and particularly survival, achieved by Senegalese sole larvae were promising considering the risky feeding protocol adopted. However, it would be interesting to extend the duration of the trial to verify if larvae would eventually be able to conclude metamorphosis, reaching the juvenile stage with a feeding protocol based on microdiets only. Yet, considering the low growth observed, the microdiet formulation probably needs further improvements, in order to reach good growth performance.

Chapter 5: General conclusions

This Thesis supports the following conclusions:

- A low-leaching microencapsulated prototype including a protein hydrolysate was successfully produced and included in a complete microdiet, reducing leaching (up to 75 % in two hours following immersion) and improving microdiet physical properties at the first-feeding sizes.

- The use of a high replacement of live-prey in co-feeding strategy resulted in a good growth performance and promoted weaning at 23 DAH in gilthead seabream larvae, with reasonable survival. The daily observation of microdiet in the digestive tract helped to adjust the feeding plan to get the full performance of the larvae.

- Gilthead seabream larvae may be fed with CAP30 microdiet in the first two weeks of development. After this time, larvae may be fed with CAP8.5 microdiet.

- In with Senegalese sole larvae, a risk feeding regime was adopted, with larvae feeding on microdiet alone during the experiment. The microdiets were able to sustain high survival but not a good growth performance.

- The best results were obtained with CAP30 microdiet, indicating a need for high levels of dietary protein during the first developmental weeks.

- Further research should explore the benefits of using the microdiets produced during this Thesis, or further improved prototypes, on the downstream quality of gilthead seabream and Senegalese sole juveniles.

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