**UNIVERSIDADE DO ALGARVE**

**Universidade do Algarve**

**Faculdade de Ciências e Tecnologias**

# Effects of a fast growth microdiet on larval development of gilthead seabream (*Sparus aurata*, Linnaeus, 1758) and meagre (*Argyrosomus regius*, Asso, 1801)

**Sofia Isabel Martins Viegas**

Dissertation for the degree of Master of

Aquaculture and Fisheries

Work performed on the orientation of:

Pedro Pousão-Ferreira

Sofia Engrola

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**Declaração de autoria de trabalho**


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


_____

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**Para os meus Pais e Irmão**

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# Resumo

A produção de aquacultura Mundial continua em crescimento. O aumento da população humana, provoca um aumento na procura de produtos de origem animal e seus derivados sendo, por isso, importante continuar a sua expanção, melhorando técnicas de cultivo e aumentando o número de espécies cultivadas, uma vez que se prevê que a população atinja as 9 biliões de pessoas em 2050, e onde a aquacultura representa grande parte do fornecimento de alimento no Mundo.

A dourada, *Sparus aurata* é uma espécie produzida em aquacultura com elevado valor comercial, existindo práticas de cultivo bem estabelecidas e tecnicamente dominadas. Todavia esta espécie apresenta períodos críticos no seu desenvolvimento, nomeadamente a fase larvar.

A corvina, *Argyrosomus regius*, foi recentemente introduzida na aquacultura por apresentar características necessárias a uma produção sustentável: requisitos biológicos facilmente atingíveis (crescimento, fecundidade e rápida adaptação na criação em cativeiro) e requisitos de mercado (alto valor comercial e disponibilidade anual), que em conjunto com o custo de produção reduzido, podem competir com as espécies já cultivadas. É um peixe carnívoro, que, com a alimenação apropriada, consegue atingir 1kg em menos de um ano (cultivo em jaulas), pelo que um rácio de proteína:lipido apropriado será a resposta para esse elevado crescimento.

O aumento da produção em aquacultura e a possibilidade de cultivar novas espécies de alto valor comercial vai depender no sucesso de produção de larvas e juvenis em cativeiro de alta qualidade. Para isso, a nutrição é considerada crítica para promover o melhor desenvolvimento e qualidade das larvas, que deve ser analisada em diferentes espécies. Hoje em dia, a maior parte dos protocolos de cultivo das corvinas são baseados nos protocolos já existentes para douradas e outras espécies comerciais.

Para uma dieta apropriada, as rações necessitam de um valor ideal de proteina, lipidos, aminoácidos e ácidos gordos essenciais que permitem criar balançoes energéticos que irão sustentar o crescimento. Os ácidos gordos são inseridos nas rações através de óleos de peixe, que contêm ácidos gordos saturados, monoinsaturados e polinsaturados, sendo estes últimos aqueles que possuem efeitos benéficos para o ser humano – ómega-3.

Os ácidos eicosapentaenóico (EPA) e docosahexaenóico (DHA) são os principais ácidos gordos fornecidos pelos óleos de peixe, e essenciais a todos os peixes. Os hidratos de carbono são utilizados na formulação das rações, servindo como uma fonte de uso limitada para a produção de energia, bem como para dar forma e estabilidade às rações. Os minerais e vitaminas também são introduzidos nas rações, para suplementar deficiências a esse nível.

Se a nutrição não for bem implementada desde os estados larvares, não só a qualidade dos peixes diminui como podem ocorrer malformações a nível osseo, levando a despesas desnecessárias na produção. Além disso, peixes com malformações na boca, barbatanas, ou coluna demonstram qualidades natatórias e de caça inferiores, tendo consequentemente baixas taxas de alimentação, crescimento e maior susceptibilidade ao stess e a agentes patogénicos que outros indivíduos não deformados.

Tendo em mente os problemas mencionados acima, foram realizados dois ensaios: larvas de douradas durante 35 dias (desde os 23 ao 58 dias após a eclosão); e larvas de corvinas durante 26 dias (desde os 20 ao 46 dias após a eclosão). Os objectivos deste estudo são avaliar o impacto de duas novas microdietas em comparação com uma comercial (controlo), no crescimento, sobrevivência e qualidade na cultura de larvas de douradas e corvinas.

Para a avaliação do crescimento foi analisado o crescimento total, o peso seco e a taxa específica de crescimento em algumas amostragens. No final dos ensaios para avaliação da qualidade foi feita uma análise de malformações e a sobrevivência foi comparada entre os tratamentos.

Ambas as espécies (*Sparus aurata* e *Argyrosomus regius*) foram alimentadas com alimento vivo até ao inicio dos ensaios (rotíferos e artémia). Em ambos os ensaios foram testadas duas dietas de crescimento rápido, com variações nos níveis de poteina (61 e 64%) e lípidos (16 e 22%), sendo denominadas de FAST61/22 (dieta para crescimento larvar rápido com altos níveis de proteina/altos níveis de lipidos) e FAST64/16 (dieta para crescimento larvar rápido com altos níveis de proteina/baixos níveis de lipidos), em comparação com uma dieta comercial (COOM), normalmente utilizada em larvas de douradas e robalos.

No geral dos parâmetros analisados (comprimento total, peso seco e taxa específica de crescimento), pode-se concluir que a dieta FAST61/22 promoveu um melhor desenvolvimento e sobrevivência nas larvas de ambas as espécies. A análise de malformações não deu resultados estaticamente diferentes.

Em futuros estudos é recomendada a utilização da ração FAST61/22, uma vez que neste estudo fornece um melhor crescimento e desenvolvimento larvar. As corvinas podem necessitar de um conteúdo proteico e lipidico mais elevado, devido ao seu crescimento rápido, enquanto que para as douradas um conteúdo lipidico mais elevado pode ser suficiente para garantir o seu melhor desenvolvimento.

Palavras-chave: Dourada; corvina; microdietas; malformações; crescimento larvar.

# Abstract

Word aquaculture production continues to grow. Nowadays, due to the huge increase in human population, demand for animal products and its derivates is increasing drastically, where aquaculture represents one of the most forms of supplying the world with food.

Gilthead seabream, *Sparus aurata* plays an important commercial role in fisheries and aquaculture in the Mediterranean Sea and north-eastern Atlantic Ocean. Meagre (*Argyrosomus regius*) have a high growth and food conversion rates, high marketable value, the quality of the fillet and resistance to diseases is considerably higher than those of seabream and seabass.

Fish feeding is one of the most important factors in intensive fish farming. Nutritional requirements to sustain survival and growth in marine fish larvae are slightly different from those of juveniles. In the larval rearing, the quality of food is essential for the larvae obtain necessary nutrients for normal development.

The aims of this study were to evaluate the impact of two new formulated microdiets, in comparison with a commercial diet (control), on the growth, survival and quality of gilthead seabream (*Sparus aurata*) and meagre (*Argyrosomus regius*) larvae.

To evaluate growth were performed (1) total length; (2) dry weight; and (3) specific growth rate analysis. At the end of the experiments, to evaluate quality were performed malformations analysis and the survival was compared between the different treatments.

For future studies is recommended the used of microdiet FAST61/22, since in these experiments gives to the larvae the best growth performance and larval development. While meagre seem to require higher dietary protein and lipid, due to their much fast growth, for gilthead seabream a higher dietary lipid may be sufficient to guarantee maximum performance.

Keywords: Gilthead seabream; meagre; microdiets; malformations; growth.

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# Abreviations, Siglas and Simbols

**μg** – Microgram;

**AA** – Amino acids;

**a.m**. – "Ante *Meridiem*", meaning before noon.

**B.C** – Before Christ;

**Cº** - Degree Celsius;

**COMM** – Commercial microdiet;

**DAH** – Days After Hatch;

**DHA** – Docosahexaenoic acid;

**DW** – Dry weight;

**E.g.** – for example;

**EPA** – Eicosapentaenoic acid;

**EPPO –** Estação Piloto de Piscicultura de Olhão;

**FA** – Fatty acids;

**FAO** – Food and Agriculture Organization;

**FAST61/22** – Prototype feed for fast growing larvae with high protein/high lipid;

**FAST64/16** - Prototype feed for fast growing larvae with high protein/low lipid;

**FBW** – Final Body Weight;

**HCl** – Hydrochloride acid;

**HUFA** – Highly unsaturated fatty acids;

**IBW** – Initial Body Weight;

**IPMA** – Instituto Português do Mar e da Atmosfera;

**KOH** – Potassium hydroxide;

**L** – Liter;

**mg** – Milligram;

**PBS** – Phosphate-buffered saline solution;

**PUFA** – Polyunsaturated fatty acids;

**S** – Survival;

**SGR** – Specific Growth Rate;

**Spp**. – Species;

**SPSS** – Statistical Package for the Social Sciences;

**T** – Temperature;

**TL** – Total length;

**UALG** – Universidade do Algarve;

**UK** – United Kingdom.

# 1. Introduction

## 1.1. Aquaculture – present situation and trends

World aquaculture production continues to grow (Figure 1). According to the latest available statistics collected globally by Food and Agriculture Organization (FAO), world aquaculture production attained 73.8 million tonnes, in 2014, of a total of 167.2 million tonnes of world fish capture by fisheries and aquaculture production (FAO, 2016). The fish for human consumption achieve 146.3 million tonnes (Figure 2), where fish remains among the most traded food commodities worldwide (FAO, 2016). Besides, aquaculture is one of the most modern types of farming practiced in the world. It started around 2000-1000 B.C., with the Chinese being the first ones to put the knowledge into practice (Rabanal, 1988).
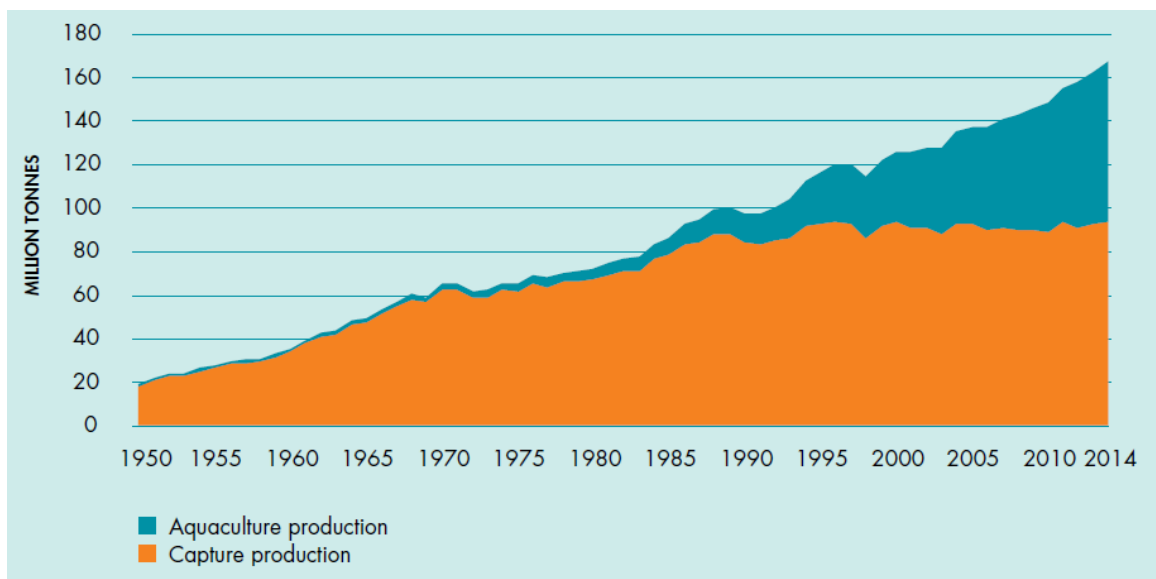


Figure 1 – World capture by fisheries and aquaculture production by FAO, (2016).

Nowadays, due to the huge increase in human population, demand for animal products and its derivates is increasing drastically, where aquaculture represents one of the most forms of supplying the world with food (Brugère and Ridler, 2004). In Europe, the main aquaculture producers are Norway, Spain, France, United Kingdom (UK) and Italy (FAO, 2014).

The rapid growth in inland aquaculture of finfish reflects the fact that it is a relatively easy-to-achieve type of aquaculture in developing countries when compared with mariculture (FAO, 2014), continuing a positive trend that has resulted in a 37 percent increase in the last decade. Sixteen countries have annual inland water catches exceeding 200 000 tonnes, and together they represent 80 percent of the world total (FAO, 2016).
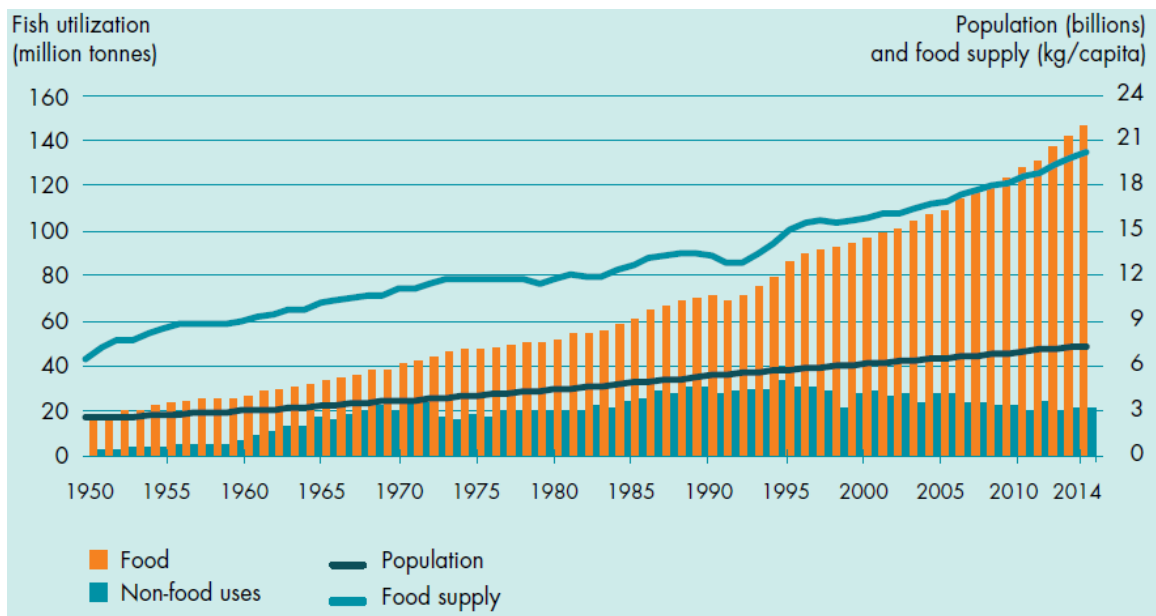


Figure 2 – World fish utilization and supply, by FAO, (2016).

The extensive studied biology and optimized feeding protocols, allows the production of marine and diadromous fishes such as Atlantic salmon (*Salmo spp.*), rainbow trout (*Oncorhynchus mykiss*), gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*), besides this species also Atlantic cod and tilapia are between the most studied species (Karakatsouli, 2012). However, most of the species cultivated are produced in small quantities, the major production is of relatively small number of species, mostly because of economical issues, where fish prices are influenced by demand and supply factors, including the costs of production and transportation, but also of alternative commodities (e.g. meat and feeds) (FAO, 2014). In contrast, overall demand for fish meals continued to grow, pushing prices to historic highs until January 2013, with an increase of 206 percent between January 2005 and January 2013, however between January 2013 and January 2014, prices declined by 20 percent (FAO, 2014). Most likely due to increased consumption, since in 2012, aquaculture contributed about

49 percent of the fishery output for human consumption, impressive growth compared with its 5 percent in 1962, 37 percent in 2002 and 45.7% in 2008 (FAO, 2012; FAO, 2014).

The increase in aquaculture production and the possibility of cultivating new species of high economic value (Imsland *et al*., 2003) are dependent on the success in producing larvae and juveniles in captivity, healthy and high-quality (Piccinetti *et al*., 2014; Conceição *et al*., 2010b). Proper nutrition is considered critical to the promotion of a normal and healthy growth in fish.

Fish larvae often go through very complex processes of morphogenesis and differentiation during growth. Development of organs and changes in morphoanatomical characters occur in a stepwise fashion. Therefore, differential relative growth (allometry) of body parts is a common feature of fish development (Loy *et al*., 2001). Body structures develop according to their importance for primary living functions (Stoner and Livingston, 1984 *In* Russo *et al*., 2007; Sagnes *et al*., 1997). Owing to its small size and behavior, marine fish larvae, have specific nutritional needs, requiring for it to have available prey with movements compatible with their capture mechanisms and predation, and size appropriate to their size mouths, allowing its easy capture and ingestion (Pousão-Ferreira, 2009). Fish generally use one or more sensory systems for acquiring feed, such as visual detection, sound, water turbulence and chemical stimuli released by food (Pillay, 1990). In the larval stage is still necessary to provide not only small but also live prey in quantity, to facilitate their date and capture (Pousão-Ferreira, 2009).

Rural diets in many countries may not be particularly diverse and, thus, it is vital to have good food sources that can provide all essential nutrients in people's diets. Micronutrient deficiencies affect hundreds of million people, particularly women and children in the developing world. More than 250 million children worldwide are at risk of vitamin A deficiency, 200 million people have goitre (with 20 million have learning difficulties as a result of iodine deficiency), 2 billion people (more than 30 percent of the world's population) are iron deficient, and 800 000 child deaths per year are attributable to zinc deficiency (FAO, 2014). Fallowing this problem and according with Karakatsouli, (2012), nutritional treatments exceed by far all other experimental factors investigated accounting for 78.14% of the studies. Next in rank come the investigation of rearing conditions (e.g., density, temperature, stress etc.) and comparisons between farmed

species and their wild conspecifics. The majority of selected papers use juvenile fish, followed by larvae and fish of commercial size (Karakatsouli, 2012). So it is of most importance to carry on the study of nutrition and improve the quality of the fish food since the larval stage and in consequence the quality of the fish for human consumption.

## 1.2. *Sparus aurata* **specie**

Gilthead seabream, *Sparus aurata* (Linnaeus, 1758), (Figure 3) is one of the most important species in Mediterranean aquaculture (Monroig *et al*., 2006). The gilthead seabream is a perciform fish, belonging to the family Sparidae, common in the Mediterranean Sea, and present along the Eastern Atlantic coasts from Great Britain to Senegal (Moretti *et al*., 1999). Is a protandrous hermaphrodite with a breeding season ranging from October to December, being a functional male in the first two years and at over 30 cm in length becomes female. The planktonic larval stage lasts about 50 days at 17-18ºC.



Figure 3 Gilthead seabream fish, by FAO.

Recent data from European Aquaculture Production Report (FEAP, 2015), shows that, between 2005 and 2014, gilthead seabream production increase from 108.795 tonnes, in 2005, to 146.467 tonnes, in 2014. Also seabream juveniles production increase from 442.115 thousands, in 2005, to 599.972 thousands, in 2014 (total values from Turkey; Greece; France; Spain; Italy; Cyprus; Croatia; Portugal).

The gilthead seabream is an euryhaline and eurythermal species, being characterized by high trophic flexibility (Kraljević, M. and Dulčić, J. 1997; Parra and Yúfera, 2000; Mariani *et al*., 2002; Tancioni *et al*., 2003) and by remarkable anatomical changes (e.g. in dentition and in the gut) throughout its life history. Furthermore, this species is known to undergo ontogenetic shifts in feeding habits (Mariani *et al*., 2002; Tancioni *et al*., 2003).

It seems that for the practical range of application for gilthead seabream (first two years of life and sub-optimal (<25∘C) temperatures), growth is exponentially dependent on body size and linearly dependent on both temperature and feed ration. Apparently the availability of food is the limiting factor under these conditions (Seginer, 2016). According with Monroig *et al*., (2006), standard protocols have been established for gilthead seabream larval production and its availability makes this species an appropriate candidate to test new diets for larviculture.

For these reasons we attempt to increase the knowledge about microdiets and we choose gilthead seabream because is an important demersal commercial species, highly appreciated as food fish for its flesh (Crosetti *et al.*, 2014).


## 1.3. *Argyrosomus regius* specie


Meagre (*Argyrosomus regius*) (Figure 4) is a teleost fish species that belongs to the Sciaenidae family, and has a wide distribution, being present in the Mediterranean and Black seas, Atlantic coast of Europe and west coast of Africa, living in inshore or coastal waters, close to the bottom or near the surface (range depths from 15 to 200m) (Cabral and Ohmert, 2001; Poli *et al*., 2003; El-Shebly *et al*., 2007). Is also a coastal species with high potential, as a candidate for marine finfish diversification on commercial aquaculture in Mediterranean and Eastern Atlantic coasts mainly related to their production performance (high growth and food conversion rates), high marketable value, the flesh quality and high nutritional value (*regius* for royal quality of flesh), and resistance to diseases is considerably higher than those of seabream and seabass (Poli *et al*., 2003; Piccolo *et al*., 2008; Monfort, 2010; Roo *et al*., 2010; Chatzifotiset *et al*., 2011; Mylonas *et al*., 2013; Vallés and Estévez, 2013). As an euryhaline species, meagre has a

high tolerance to salinity and therefore can be reared in different environments such as cages in the sea or earthen ponds in estuaries (Ribeiro *et al*., 2013). Is found in waters of the Mediterranean including Portugal, Spain, France, Italy, Greece and Turkey and it has become a commercially valuable marine fisheries product (Ermre *et al*., 2016).
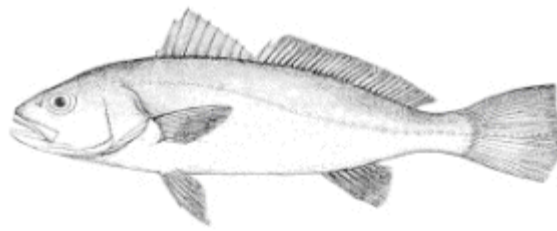


Figure 4 Meagre fish, by FAO.

Adding to the characteristics already described, it's a specie that tolerates wide ranges of salinity, temperature and can be reared in brackish water ponds (El-Shebly *et al*., 2007), being ideal for the aquaculture industry.

This species culture techniques are based on rearing of gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*), but the elevated growth rate of meagre let's reach 1 kg in ten months of culture (Jiménez *et al*., 2005; Roo *et al*., 2010). Farmed meagre market size usually ranges between 1 and 2 kg (FAO, 2013), depending on the production and management systems (Ribeiro *et al*., 2013), but recently fish of smaller size (from 600g) have started being commercialised as well (Monfort, 2010; FAO, 2014), with a total production just over 14 000 tonnes, in 2011, produced in several Southern European countries. Also, recent data from European Aquaculture Production Report (FEAP, 2015), shows that meagre production increase from 907 tonnes, in 2005, to 5 021 tonnes, in 2014 [total values from Croatia; Cyprus; France; Italy; Portugal; Spain; and Turkey (only data in 2014)].

In recent years the research effort has been focused on identifying the best conditions for larval rearing, such as larval density, live prey feeding sequence (Roo *et al*. 2010), to standardize the culture protocols and then undertake studies on the nutritional requirements of the larva. Given the characteristics of meagre larva (high voracity, visual feeding in conditions of relatively high luminosity, and cannibalism) their nutritional requirements, in the form of protein or fat, will be very different from those of other marine fish species in culture (Vallés and Estévez, 2015). According with this information, we choose *Argyrosomus regius* specie to increase the knowledge about the larval development and quality rearing with different microdiets, formulated with different content of lipids and fatty acids, to increase growth performance, since optimization of growth is closely related to the knowledge of protein metabolism aiming for a supply of good quality protein in quantities fulfilling larval requirements (Conceição *et al*., 2003), and, at the moment, is the species with the highest potential for intensive farming in this region.

## 1.4. Nutritional requirements

Most information regarding nutritional requirements for fish are performed with seabream and other commercial species (Monroig *et al*., 2006; Guerreiro, *et al*., 2010; Piccinetti *et al*., 2014; Fernández-Díaz and Yúfera, 1997; Moyano *et al*., 1996; Peres *et al*., 1996; García-Meilán, et al., 2013; Russo *et al*., 2007; Yúfera *et al*., 2004; Zeytin *et al*., 2016). Fish feeding is one of the most important factors in intensive fish farming (Mílan *et al.*, 2014), the nutritional requirements to sustain survival and growth in marine fish larvae are slightly different from those of juveniles (Cahu *et al*., 2003).

Since 50% of fish larval composition (in dry matter) is protein, growth optimisation is closely linked to the supply of dietary protein of appropriate quality and quantity (Conceição *et al.*, 2003). Growth and food conversion efficiencies can be maximised by manipulating the composition of the dietary amino acids (AA). AA imbalances in the diet cause increased AA oxidation and lead to decreased food conversion efficiencies (Fauconneau et al., 1992 *in* Conceição, et al., 2003). AA losses have particular importance in fish larvae, which have a growth potential ranging from 10 to 50%/day (Houde, 1989). The requirement for dietary protein has two components: (1)

a need for indispensable AA that he fish cannot synthesize either at all or at a rate commensurate with its need for protein deposition or commensurate with the synthesis of a variety of other compounds with metabolic functions, and (2) a supply of either dispensable AA or sufficient amino nitrogen to enable the fish to synthesize them. Insofar as synthesis of dispensable AA requires expenditure of energy, feeding dietary proteins that most nearly meet the needs of fish for both indispensable and dispensable AA, will result in the most efficient growth by the fish (Thoman *et al.*, 1999; Chatzifotis *et al.*, 2011). Dietary protein constitutes one of the primary nutrient costs of the feed and is the initial source of nitrogen waste products entering a culture system.

The lipids present in teleost fish species may be divided into two major groups: the phospholipids and the triglycerides. Phospholipids make up the integral structure of the unit membranes in the cells, thus, they are often called structural lipids. The triglycerides are lipids used for storage of energy in fat depots, typically located in the subcutaneous tissue in the belly flap muscle and in the muscles moving the fins and tail (Huss, 1995). To best fulfill the needs in lipids for optimal development and growth, oils derived from fish that contains materials with valuable dietary and pharmaceutical properties as well as having physical properties, now used as a source of long-chain (n-3) fatty acids (FA) (Chatzifotis et al., 2010).

The main purpose of using processed feeds is to ensure that the animals under culture receive a balanced diet that meets their nutritional requirements (Pillay, 1990).

In the larval rearing, the quality of food is essential for the larvae obtain necessary nutrients for normal development. As the marine fish larvae have a high growth potential, also have specific nutritional needs (Ajiboye *et al.* 2011; Rønnestad *et al.* 1999; Van der Meeren *et al.* 2008), requiring the appropriate nutritional value of the food to each cultivated specie. Normally, it is necessary to use bioencapsulation techniques or nutritional enrichment allowing the production of prey with high nutritional requirements, especially in fatty acids of the n-3 highly unsaturated fatty acids (HUFA) and amino acids, essential for marine fish (Pousão-Ferreira, 2009; Monroig *et al.*, 2006). During the last decade there is a gradual and steady increase of research papers concerning farmed fish fatty acids (FA), papers published in 4 years, that is, 2007–2010, representing 53.04% of selected studies. In about 50% of the 394 papers assessed, research took place in Europe, followed by Asia and North America (Karakatsouli, 2012). Fatty acid profile

may also play a role on flesh texture as a higher content of polyunsaturated fatty acids may decrease hardness due to higher fluidity of biological membranes (Palmeri *et al*., 2008; Saavedra *et al*., 2015b).

The step leading to DHA is usually the net result of two elongations, along with a few saturates and oleic acid, the (n-6) and (n-3) polyenes make up the fatty acids found in most plants, animals, and commodity oils and fats. The (n-3) long-chain, polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), are important nutritionally and are mainly obtained from oily fish and fish oils where they are present at levels from 5 to 20% (Das, 2006). Maintaining high levels of (n-3) PUFA, as well as low levels of (n-6) FA, in farmed fish, is considered desirable to provide high value product for human consumption (Das, 2006; Martins *et al*., 2007).

The search for a food capable of supporting growth and larval development has increased its importance, with the main objective of maintaining the quality of fish for consumption and minimizing the need for living organisms, such as microalgae , rotifers and Artemia (Piccinetti *et al*., 2014; Guerreiro, *et al*., 2010; Yúfera *et al*., 2005). The formulation of appropriate diets has undergone several changes, driven by the increasing demands of farming and fish nutritional needs, and the suitability of an inert diet for larval fish depends on the characteristics of both diet and larvae, as well as the rearing system used (Fernández-Díaz and Yúfera, 1997). When balanced, these diets provided the nutrients required for proper physiological functioning and can positively influence the health of fish that feed upon it.

Studies on enzymatic development in larvae have contributed useful information concerning the proper time to introduce prepared feeds and whether the addition of exogenous enzymes is required. There is a relationship between larval age and digestive enzyme activity in marine fish (Moyano *et al*., 1996; Peres *et al*., 1996) and, consequently, the ability to digest and assimilate an inert diet (Fernández-Díaz and Yúfera, 1997). Furthermore, according to Russo *et al*., (2007) there is also a correspondence between shape changes and feeding shifts.

Digestion and absorption are key processes in the optimal use of a diet (García-Meilán *et al*., 2013). Digestion processes begin in the stomach, where hydrochloride acid (HCl) denatures protein and converts pepsinogen to active pepsin (Yúfera *et al*., 2004).

Absorption processes occur by diffusion, facilitated transport or active transport (Mailliard *et al*., 1995) and take place throughout the entire intestine, which differentiates fish from mammals (García-Meilán *et al*., 2013). When dietary composition changes, intestinal enzymatic activities and nutrient absorptive capacity may be modulated in fish. Voluntary feed intake may also change to improve feed use and assure growth performance (Karakatsouli, 2012).

The minerals, for instance, are required by all animals either in their elemental form or incorporated into specific compounds, for various biological functions such as the formation of skeletal tissue, respiration, digestion and osmoregulation (Pillay, 1990). The minerals are required in extremely small quantity in the diet, with excess supplementation through a continuous process can cause severe toxicity. In deficient condition, growth is retarded with abnormal metabolism in particular (Chanda *et al*., 2015). Fish can absorb part of the required minerals directly from the water through gills or even through their entire body surface, but this absorption from the water do not meet the total requirement and a certain supplementation through the diet is required whether in natural food or supplementary feed (Pillay, 1990; Chanda *et al.,* 2015), so it is very important to achieve the right amounts of each mineral, proteins, amino acids, fatty acids, etc., to have a complete and balance diet for fish. The knowledge of feeding requirements allows better management of early larval rearing and also the introduction of new feeding methods (Papandroulakis *et al*., 2000).

Although considerable improvements in the quality of formulated diets were achieved in the last decades (Hamre *et al*., 2013), for larval fish (e.g., Fernandez-Diaz and Yufera, 1997; Cahu and Infante, 2001; Koven *et al*., 2001), current feeding protocols of marine fish larvae still depend on live feeds, especially in the first-feeding stages and early larval stages (Conceição *et al*., 2010a). Several studies have been evaluating the feeding behavior and digestive physiology of seabream larvae with regard to microdiet ingestion (Fernández-Díaz *et al*., 1994; Kolkovski *et al*., 1997; Parra and Yúfera, 2000). Nevertheless, there is little and inconclusive data available to determine the optimum feeding time and frequency for the different developmental stages of larvae under a feeding regime applying to microdiet (Zeytin *et al*., 2016).

Compound diets are now being formulated, usable from first feeding onwards in some marine fish larvae (Cahu *et al.*, 2003). The major aim of the studies conducted now in larval nutrition is to improve larvae quality.

## 1.5.Malformations considerations

The presence of skeletal anomalies in farmed teleosts is a constant world-wide problem in aquaculture and it entails economic, biological and animal welfare issues (Boglione *et al*, 2013; Darias *et al*., 2011). The fact that an accurate study of skeletogenesis during larval development is of utmost importance for the recognition and identification of abnormalities in skeletal structures was suggested by the scientific community long ago, as can be seen in the statement of McMurrich (1883).

However, the problem persists and many hypotheses for the causes of skeletal anomalies are still being discussed today, because different causative factors can have a common symptomatology and frequently act synergistically. The present difficulties in separating the causes of the many genetic and non-genetic factors that interact in aquatic organisms remain an open problem (Boglione *et al*, 2013).

It is generally considered that four classes of mineralized tissues can be identified in vertebrates: bone, cartilage, dentine and enamel/enameloid. Teleost fish display a large range of intermediate skeletal tissues as part of their mature – non-pathological, non-regenerating – skeleton (Benjamin, 1990, *In*: Boglione *et al*, 2013).

Skeletal anomalies in reared fish can affect all skeletal tissues, but from a production related viewpoint, alterations of the notochord, cartilage and bone abnormalities are the most important. Anomalies of dermal skeletal elements, such as teeth, scales (and fin rays), are possibly indicative for the skeletal heath status of the animal (Persson *et al*. 1997, 2000); however, anomalies affecting teeth and scales are rarely studied (Boglione *et al*, 2013). The presence of deformed fish concerns also ethical issues: fish with a deformed mouth, fins or vertebral axis show impaired feeding and swimming performances, with consequent lower feeding rates, slower growth rates and a higher susceptibility to stress and pathogens than healthy nondeformed individuals. These

deformed fish cannot be considered to be in a proper welfare condition (Boglione *et al,* 2013), therefore, it is considered malformation analysis in gilthead seabream and meagre, at the end of the trials.

The vertebral column of gilthead seabream and meagre has 24 vertebrae distributed in four regions in anterior–posterior direction: cranial, pre-haemal, haemal and caudal (Boglione *et al*., 2001; Gisbert *et al*. 2012).

According to Boglione *et al.,* (2013), pugheadness, cross-bite and lower jaw reduction or elongation are the main types of jaw abnormalities that can affect Mediterranean aquaculture reared finfish, but data from both experimental and reared fish demonstrate that pugheadness is the most frequent jaw abnormality in gilthead seabream. And according with Georgakopoulou *et al.*, (2010) the involvement of the gill-cover in respiration function, could affect the survival of deformed larvae, directly and/or indirectly through a decrease of the growth rate and a subsequent increased sensitivity to cannibalism by the larger individuals of the population. The common response of gill-cover and caudal-fin deformities to water temperature could be explained by the fact that the elements of both structures develop during the early larval stage (Koumoundouros *et al*., 1997). Moreover, temperature might affect bone development indirectly, via modifications of the species nutritional optima, or via changes of the nutritional status of the planktonic organisms used for the larval feeding (Georgakopoulou *et al*., 2010). Also according with Cahu *et al*., (2003) nutrients also affect development and particularly skeletal formation.

Koumoundouros *et al*., (1997) affirms that the abnormality of the gilthead seabream yolk-sac larvae may not be lethal and the specimens continued developing, but seriously affecting the quality of the following larvae and juveniles, being very important to detect malformations at early live stages, to reduce costs of production.

## 1.6. Objectives

The aims of this study were to evaluate the impact of two new formulated microdiets, in comparison with a commercial diet (control), on the growth, survival and quality of gilthead seabream (*Sparus aurata*) and  meagre (*Argyrosomus regius*) larvae.

To evaluate growth were performed (1) total length; (2) dry weight; and (3) specific growth rate analysis. At the end of the experiments, to evaluate quality were performed malformations analysis and the survival was compared between the different treatments.

# 2. Materials and Methods

The experiments take place at the Aquaculture Research Station (EPPO) of IPMA (Portuguese Institute for the Ocean and Atmosphere) in Olhão (Portugal). In these experiments two similar dry microdiets formulated by SPAROS, Lda. (Olhão, Portugal), were tested, with different lipid/ protein ratio, in comparison with a commercial diet (control), to observe the larval development with each diet. The same diets were tested in gilthead seabream (*Sparus aurata*) and meagre (*Argyrosomus regius*) larvae.

## 2.1. Experimental fish

Gilthead seabream (*Sparus aurata*) and meagre (*Argyrosomus regius*) larvae's were obtain throw natural breeding of reproducers kept in captivity in IPMA's - EPPO facilities. The eggs were incubated in 200L cylindroconical white tanks, with filtered salt water at 18ºC, for 2 days until hatch. After hatch was calculated the survival and hatching rate and reares in a 1500L tank until the beginning of the trial. At this age (23, and 20 DAH, respectively), the larvae were counted and distributed equally to the tanks of the trials.

## 2.2. Experimental trials

### 2.2.1. *Sparus aurata* trial

The trial was conducted at the Aquaculture Research Station of IPMA in Olhão (Portugal), from January 29th to March 4th (35 days), with gilthead seabream larvae bred in captivity at the station. The fish larvae were counted into nine cylindroconical white tanks of 200L (Figure 5), each treatment in triplicate, at an initial density of 5 larvae/L (1000 larvae/tank).

Figure 5 Cylindroconical white tank of 200L. The letters correspond: a) automatic feeder; b) aeration tube; c) water renewal tube; d) filter; and e) purge site.

These tanks are individually set with in a semi-closed system (partial recirculation), with continuous aeration, and physicochemical parameters were registered daily, until the end of the trial (Table I).

**Table I Average salinity (ppt), temperature (ºC), oxygen saturation (%) and photoperiod (light:dark) in gilthead seabream rearing.**

| Salinity (ppt) | Temperature (ºC) | Oxygen saturation (%) | Photoperiod (light:dark) |
|---|---|---|---|
| $37 \pm 1$ | $21.6 \pm 0.7$ | $> 80$ | 18:6 |

Temperature of the air was controlled by the use of an air conditioner ($20 \pm 0.5°C$), and the temperature of the water by a thermostat ($21 \pm 1.0°C$). From 0 to 22 DAH (days after hatch) the fish were cultured with a mixture of natural and halogen light lamps were used (halogen lights with an intensity of 1000-1200 lux) above the water surface, with a 16:8h light:dark photoperiod. The light intensity was measure using a Delta OHM Luxmeter HD 8366, on the first day of the trial, and all the tanks had similar values of light intensity. From 23 to 58 DAH were used only halogen light lamps with a 18:6 light:dark photoperiod, starting at 8 a.m.. The water renewal of the system was controlled

every day of the trial and adjusted to larvae behavior, progressively increased (Table II). Surface skimmers set at 3 DAH until 22 DAH and manual skimming if needed.

**Table II Water renewal, in percentage, according with the age of the larvae (DAH) in gilthead seabream rearing.**

| Age (DAH) | Water renewal (%) |
|-----------|-------------------|
| 22 – 30   | 20                |
| 30 – 37   | 30                |
| 37 – 38   | 35                |
| 38 – 39   | 40                |
| 39 – 42   | 45                |
| 42 – 50   | 55                |
| 50 – 58   | 60                |

Each morning the tanks were aspirated and purged twice a day, once in the morning and another in the afternoon, with the larval development and the increasing of meal administered was increased the number of purges for a greater water renewal. Every day, dead larvae were removed from the tanks and counted after cleaning the tanks and purge. The filters in the tanks were washed with fresh water twice or more times per day, as needed. From the start of the trial until 24 DAH were kept the 250μm filter during the day and the 350μm filter overnight to better water renewal. From this day until the end of the trial, with the increased size of the artemia and the amount of meal, was used only 500μm filters, both day and night.

Feeding until 22 DAH was entirely of rotifers and *Artemia sp.*.Rotifers were grown on ω3Yeast 60 (Bernaqua, Belgium) and enriched with Red Pepper (Bernaqua, Belgium) according to manufacturer's specifications. Artemia strain used was AF (INVE, Belgium) until 22 DAH and from 22 DAH until 34 DAH EG (INVE, Belgium), enriched with Red Pepper (Bernaqua, Belgium). Live preys were divided into 4-5 meals/day, with amount depending on number of preys in the tanks and water renovation. A mature

culture of *Isochrysis* aff. *galbana* and *Nannochloropsis oculata* was added 2-3 times/day (green water technique).

From 23 to 34 DAH seabream larvae were co-fed with enriched Artemia and dry microdiets using automatic feeders manually programmed to feed in a certain amount following the larval development (equal amount in all treatments). From 34 DAH larvae were feed dry microdiets alone until 58 DAH (Figure 8). Initial feeding time was set at 8 a.m. and lasted until 2 a.m. of the next day. Three microdiet were used: a commercial microdiet (COMM) widely used in seabream hatcheries; a prototype for fast growing larvae with high protein/high lipid (FAST61/22); and a prototype for fast growing larvae with high protein/low lipid (FAST64/16), and three different sizes were used following larval development: 100-200µm; 200-300/400µm; and 300/400-500/600µm.

At the end of the experiment all fish were counted for the calculation of the survival (S):

$$S = \frac{Final\ number\ of\ fish}{Inicial\ number\ of\ fish} \times 100$$

### 2.2.2.*Argyrosomus regius* trial

The trial was conducted at the Aquaculture Research Station of IPMA in Olhão (Portugal), from April 22nd to May 18th (26 days), with meagre larvae bred in captivity at the station. The fish larvae were counted into nine tanks of 300L, each treatment in triplicate (Figure 6), at an initial density of 15 larvae/L (4500 larvae/tank).
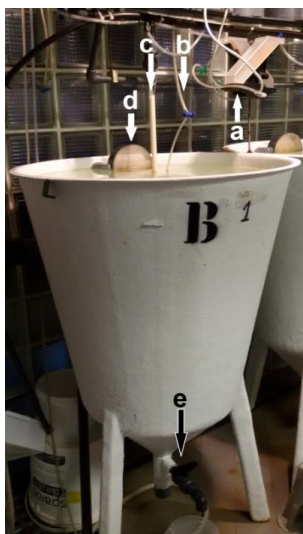
Figure 6 Blue tank of 300L. The letters correspond: a) automatic feeder; b) aeration tube; c) water renewal tube; and d) purge site.

These tanks are individually set with in a semi-closed system (partial recirculation), with continuous aeration, and physicochemical parameters were registered daily, until the end of the trial (Table III). Temperature of the air was controlled by the use of an air conditioner ($20 \pm 0.5°C$), and the temperature of the water by a thermostat ($21 \pm 1.0°C$).

**Table III Average salinity (ppt), temperature (°C), oxygen saturation (%) and photoperiod (light:dark) in meagre rearing.**

| Salinity (ppt) | Temperature (°C) | Oxygen saturation (%) | Photoperiod (light:dark) |
|---|---|---|---|
| $37 \pm 1$ | $21 \pm 1$ | $> 80$ | 18:6 |

From 0 to 20 DAH (days after hatch) the fish were cultured in a 1500L tank with a mixture of natural and halogen light lamps were used (halogen lights with an intensity of 1000-1200 lux) above the water surface and with a 16:8h light:dark photoperiod. The light intensity was measure using a Delta OHM Luxmeter HD 8366, on the first day of the trial, and all the tanks had similar values of light intensity. From 20 to 46 DAH were used only halogen light lamps with a 18:6h light:dark photoperiod, starting at 8.am.. The water temperature was controlled at an average of 18°C from 0 to 20 DAH, and ate an

average of 21±1ºC from 20 to 46 DAH as mentioned previously. The water renewal of the system was controlled every day of the trial and adjusted to larvae behavior, progressively increased (Table IV). Surface skimmers set at 6 DAH until 20 DAH and manual skimming if needed.

**Table IV Water renewal, in percentage, according with the age of the larvae (DAH) in meagre rearing.**

| Age (DAH) | Water renewal (%) |
|-----------|-------------------|
| 20 – 25   | 20                |
| 25 – 28   | 30                |
| 28 – 31   | 40                |
| 31 – 35   | 50                |
| 35 – 46   | 60                |

Each morning the tanks were aspirated and purged twice a day, once the morning and another in the afternoon, with the larval development and the increasing of meal administered was increased the number of purges for a greater water renewal. The dead larvae were counted daily after cleaning the tanks and purge. The filters in the tanks were washed with fresh water twice or more times per day, as needed. From the start of the trial until 25 DAH were kept the 250μm filter during the day and the 350μm filter overnight to better water renewal. From this day until the end of the trial, with the increased size of the artemia and the amount of meal, was used only 500μm filters, both day and night.

Feeding until 20 DAH was entirely of rotifers and *Artemia sp.*.Rotifers were grown on ω3Yeast 60 (Bernaqua, Belgium) and enriched with Red Pepper (Bernaqua, Belgium) according to manufacturer's specifications. Artemia strain used was AF (INVE, Belgium) until 19 DAH and from 20 DAH until 30 DAH EG (INVE, Belgium), enriched with Red Pepper (Bernaqua, Belgium). Live preys were divided into 4-5 meals/day, near 9h, 12h30, 14h30 and 18h, with amount depending on number of preys in the tanks and water renovation. A mature culture of *Isochrysis* aff. *galbana* and *Nannochloropsis oculata* was added 2-3 times/day (green water technique).

From 20 to 30 DAH meagre larvae were co-fed with enriched Artemia and dry microdiets using automatic feeders manually programmed to feed in a certain amount following the larval development (equal amount in all treatments). From 30 DAH larvae were feed dry microdiets alone until 46 DAH (Figure 9). Initial feeding time was set at 8 a.m. and lasted until 2a.m.. Three microdiet were used: a commercial microdiet (COMM) widely used in seabream hatcheries; a prototype for fast growing larvae with high protein/high lipid (FAST61/22); and a prototype for fast growing larvae with high protein/low lipid (FAST64/16), and three different sizes were used following larval development: 100-200µm; 200-300/400µm; and 300/400-500/600µm.

At the end of the trial all fish were counted to calculate the survival (S):

$$S = \frac{Final\ number\ of\ fish}{Inicial\ number\ of\ fish} \times 100$$

### 2.2.3.Live feed

Strains *Brachionus* spp. are selected from natural populations, whose life cycle is fully controlled in laboratory. In this assay *Brachionus* spp. were produced by the upscaling technique normally applied to this cultivation in the EPPO according Pousão-Ferreira (2009).

Artemia cysts are not produced in the EPPO and comes from commercial manufacturers. The strains used in this assay, strain A.F. (480 INVE Aquaculture, Ghent, Belgium) and strain EG (Artemia Systems SA, Ghent, Belgium) are decapsulated according to a known protocol and then incubated according to the needs and protocols currently used in the EPPO as described in Pousão-Ferreira (2009).

Both live foods are fortified with the assistance of commercial emulsions, such as Red Pepper (Bernaqua) rich in fatty acids and important nutrients often lacking in diets based on yeast or lipid emulsions. Its use was followed according to the manufacturer's instructions for both cultures.

The microalgaes used in this assay are, as previously mentioned, *Isochrysis galbana* and *Nannochloropsis oculata*, produced according to standard protocols. Large cultures were grown from pure microalgae stocks using the upscaling method and practices described in Pousão-Ferreira (2009).



Figure 7 *Isochrysis galbana* and *Nannochloropsis oculata* culture in different stages, at IPMA facilities.

## 2.3. Feeding regimes

The feeding regime used for gilthead seabream larvae before and during the trial, from 23 to 58 DAH, is represented in Figure 8, and the feeding regime used for meagre larvae before and during the trial, from 20 to 46 DAH, is represented in Figure 9.
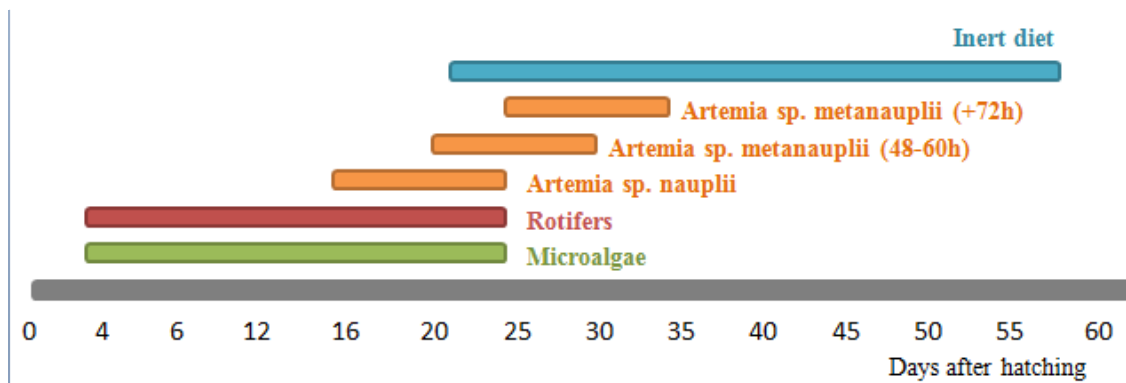


Figure 8 Feeding regime of gilthead seabream (*Sparus aurata*), at ±20ºC.
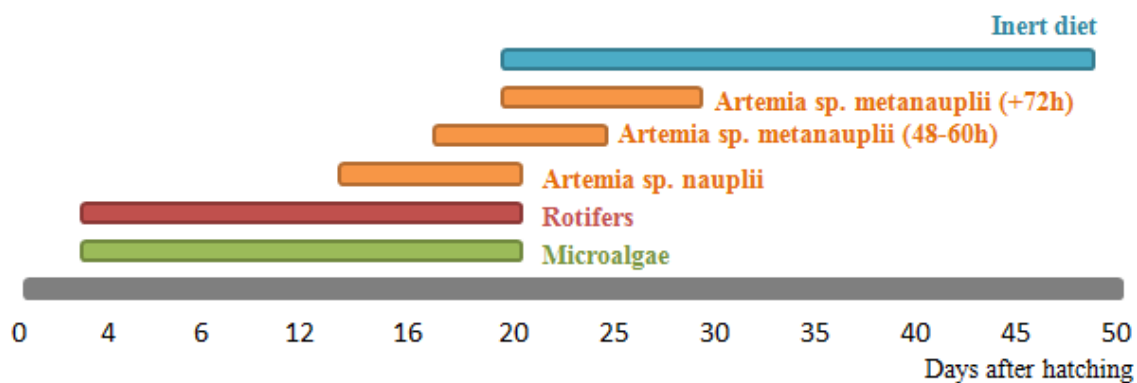
Figure 9 Feeding regime of meagre (*Argyrosomus regius*), at ±20ºC.

The size of the inert food was increase conforming the larvae behaviour and growth.

## 2.4. Experimental Diets

A novel microdiet (FAST) with two different dietary lipid levels was formulated by SPAROS, Lda. (Olhão, Portugal), commercial feed company, using microencapsulation technologies to protect some nutrients, was compared with a current premium microdiet (COMM) for marine fish larvae, in a growth performance trial with seabream (*Sparus aurata*) and meagre larvae (*Argyrosomus regius*).

The commercial microdiet (COMM) widely used in seabream/seabass hatcheries, with 62% crude protein and 17% crude lipid, and where the main ingredients are fish, krill, fish roe, soybean lecithin, brewer's yeast, microalgae, fish gelatine, squid meal, vegetable fat; a prototype for fast growing larvae with high protein/high lipid (FAST61/22), with 61% crude protein and 22% crude lipid; and a prototype for fast growing larvae with high protein/low lipid (FAST64/16), with 64% crude protein and 16% crude lipid (Table V). The main ingredients used in both prototypes were fishmeal, squid meal, shrimp meal, wheat gluten, fish solubles, fish oil and soy lecithin. The daily ration provided ad libitum, but was always equal for all 3 treatments.

**Table V Main ingredients, protein and lipid percentage of the three dietary treatments (COOM; FAST64/16; FAST61/22), used in the trials of gilthead seabream and meagre.**

| Dietary Treatments | Protein | Lipids | Main ingredients |
|---|---|---|---|
| COOM | 62 | 17 | Fish krill, fish roe, lecithin, brewer's yeast, microalgae, gelatin, squid meal and vegetable fat |
| FAST64/16 | 64 | 16 | Fishmeal, squid meal, shrimp meal, wheat gluten, fish soluble, fish oil and lecithin |
| FAST61/22 | 61 | 22 | |

## 2.5. Sampling methods

At the beginning of the seabream trial (23 DAH) were collected 20 larvae (initial pool) to measure initial total length and dry weight. At 35 DAH, when larvae stops eating live food, were collected 20 larvae from each tank (60 larvae/treatment) to measure total length and dry weight. At the end trial (58 DAH) were collected 40 larvae (120 larvae/treatment), to measure the final total length and dry weight, and 20 larvae from each tank for malformations analysis (60 larvae/treatment). To water quality analysis were collected water samples from the different treatments (COMM, FAST64/16 and FAST61/22) at 36, 43 and 46 DAH

At the beginning of the meagre trial (20 DAH) were collected 30 larvae (initial pool) to measure initial total length and dry weight. At 31 DAH, when larvae stops eating live food, were collected 40 larvae from each tank (120 larvae/treatment) to measure total length and dry weight. At 40 DAH, to observe the growth of the larvae in 9 days feeding just the microdiet, were collected 30 larvae (90 larvae/treatment), to measure total length and dry weight. At the end trial (46 DAH) were collected 30 larvae (90 larvae/treatment), to measure total length and dry weight, and 20 larvae from each tank for malformations analysis (60 larvae/treatment). To water quality analysis were collected, each week, water samples from the different treatments (COMM, FAST64/16 and FAST61/22) at 27, 34, 39 and 46 DAH.

## 2.6. Analytical methods

### 2.6.1. Biometry

In each sampling was measured the total length of each fish by micrometric magnifier glass Zeiss® Stemi 2000-C. With representative photographs of each sample record with the camera Cannon PowerShot® GS 5,0 MP, incorporated into the magnifying glass. The same larvae were then washed rapidly with distilled water to remove the salt, placed in 1.5 ml microtubes and frozen immediately in liquid nitrogen, where they remained stored until determination of dry weight. After lyophilization (to remove all the water from the fish), dry weight of larvae was determined by the balance Sartorius® Pro 11 with a precision of 1 µg.



Figure 10 Example of a dry meagre larva.

In both species was determined specific growth rate (SGR) using the formula:

$$SGR = \frac{[\ln(FBW) - \ln(IBW)]}{DAH} \times 100$$

In this formula, FBW is the final mean body weight (mg); IBW, the initial mean body weight (mg); and DAH, the number of days after hatch. The SGR allows the comparisons of growths rates between treatments made with similar IBW.

### 2.6.2. Water quality analysis

The water quality analysis was performed by the laboratory of Oceanography and Chemistry of IPMA. The water samples were filtered with Nuclepore membranes (0.45 µM) and preserved at -4 ºC, in a maximum period of 2 months, in polypropylene vials. The nutrients: ammonia ($NH_4+$), nitrate ($NO_3-$), nitrite ($NO_2-$), phosphates ($HPO_42-$) and silicates ($Si(OH)4$), were analyzed with an autocatalizator "Skalar" with four simultaneous channels, with the Technicon Industrial Technology (Grasshoff, 1983).

### 2.6.3. Malformations

From gilthead seabream and meagre rearing were taken, at the end of the trials, 60 larvae/treatment for sampling, meaning that a total of 180 larvae were analyzed from each trial.

For malformation analysis, since the coloration was performed in different species at different ages (different size and bone formation), was used similar stain protocols. Some differences between them are the time to stain or to wash the larvae. Both protocols were performed according to Gavaia *et al*. (2000). Alcian blue and alizarin red staining solutions were use to stain cartilage and bones, respectively (Gavaia *et al*., 1999).

### 2.6.3.1.    *Sparus aurata* malformations protocol

The fish were fixed with 10% buffered formaldehyde for 24h in a ratio of 1:10. After this time, the larvae were subjected to 3 successive washings with PBS (in intervals

of 15 minutes) and tap water, and kept in 70% alcohol until the time of stain. During coloring, the first step consisted of 20 minute baths of alcohol at 50% and at 25%. Then the larvae were immersed for one hour in Alcian Blue staining solution, after this time was removed by proceeding to a wash with 96% ethanol splashes. It was added a potassium hydroxide (KOH) solution 1% for about 1h45min, in order to neutralize the excess acidity caused by the dye. Then they took place new 20 minute washes of 80% ethanol, 70%, 50%, 25% and finally distilled water. Thereafter, it proceeded to staining with Alizarin Red solution for 3h at a ratio of 1.5: 50 ml. Ending staining, the larvae were soaked in a solution of KOH 1% for 48h, proceeding to point substitutions, where necessary (generally when the solution started to become yellowish). Larvae were then subjected to glycerol bath at increasing percentages: 50% of glycerol 25% + 50% of KOH1%; 50% of glycerol 50% + 50% of distilled water; 50% of glycerol 75% + 50% of distilled water; and 100% glycerol for better preservation until analysis. This protocol was performed according to the described by Gavaia *et al.* (2000).

The malformations analysis was done by Leica® M26 magnifying glass and a camera Canon G12 PowerShot® with 10 MP and 5x optical zoom, examining in each fish the whole body, and more in detail each body area: cephalic; haemal; and caudal, recording and taking photographs of each malformation.

The data were compared for each treatment according to the number of malformed fish, the malformations severity, the effect of the malformation in the phenotype of the larvae, malformations per fish and, finally, the malformations on the anatomical areas.

The severity levels used in this analysis were used according to Gavaia (Com Pess.), which considers 5 levels of severity according with Table VI.

### 2.6.3.2. *Argyrosomus regius* malformations protocol

The fish were fixed with 10% buffered formaldehyde for 24h in a ratio of 1:10. After this time, the larvae were subjected to 3 successive washings with PBS (in intervals of 15 minutes) and tap water, and kept in 70% alcohol until the time of stain. During

coloring, the first step consisted of 20 minute baths of alcohol at 50% and at 25%. Then the larvae were immersed for one hour in Alcian Blue staining solution, after this time was removed by proceeding to a wash with 96% ethanol and KOH 2% in a ratio of 100:0.1. It was added a potassium hydroxide (KOH) solution 2% for about 1h30min, in order to neutralize the excess acidity caused by the dye. Then they took place new 20 minute washes of 80% ethanol, 70%, 50%, 25% and finally distilled water. Thereafter, it proceeded to staining with Alizarin Red solution overnight at a ratio of 1.5: 50 ml. Ending staining, the larvae were soaked in a solution of 2% KOH for 48h, proceeding to point substitutions, where necessary (generally when the solution started to become yellowish). Larvae were then subjected to glycerol bath at increasing percentages: 50% of glycerol 25% + 50% of KOH 1%; 50% of glycerol 50% + 50% of distilled water; 50% of glycerol 75% + 50% of distilled water; and 100% glycerol for better preservation until analysis. This protocol was performed according to the described by Gavaia *et al*. (2000).

The malformations analysis was done by Leica® M26 magnifying glass and a camera Canon G12 PowerShot® with 10 MP and 5x optical zoom, examining in each fish the whole body, and more in detail each body area: cephalic; haemal; and caudal, recording and taking photographs of each malformation.

The data were compared for each treatment according to the number of malformed fish, the malformations severity, the effect of the malformation in the phenotype of the larvae, malformations per fish and, finally, the malformations on the anatomical areas.

The severity levels used in this analysis were according to P. Gavaia (Com. Pess.), which considers 5 levels of severity according with Table VI.

**Table VI Severity levels for malformation analysis according and adapt from P. Gavaia (Com. Pess.)**

| Severity level | Description |
|:---:|:---|
| 0 | Normal - do not show any malformations |
| 1 | Small malformations in fins or archs that do not affect the external phenotype |
| 2 | Fusions or malformations that do not affect the phenotype |
| 3 | Fusions or malformations that affect the phenotype |
| 4 | Fusions or malformations that affect the phenotype with severity |

## 2.7.Statisitical analysis

The results obtained in this study were expressed as mean arithmetic followed by respective variation of the standard deviation for all parameters analyzed, except the incidence, severity level, influence on the phenotype and malformations distribution by anatomical area, which were treated by counting. All means and standard deviations, as well as charts and tables were made with the help of the Office Excel® for Windows®.

As the main objective was to compare diets for all tests, in both species, the null hypothesis was used (H0): there are no significant differences between the study diet, against the alternative hypothesis (H1): there are significant differences between diets studied. The significance level used (α) was always 0.05, considering there are significant differences in values of $p < 0.05$, rejecting the null hypothesis.

All data concerning malformations were tested using the chi-square test. In the remaining data sets were tested the assumptions of normality, the Kolmogorov-Smirnov, Liliefors and Shapiro-Wilk. If the data agree with the assumptions, a parametric statistical analysis was used. If the opposite were to happen, even after processing the data by the neperian logarithm, was applied a non-parametric statistics.

The results were tested by analysis of variance (ANOVA), when the conditions were met, or the nonparametric equivalent, Kruskal-Wallis test to compare means, when the conditions were absent. Respectively, where checked differences between diets, these were analyzed by the Tukey - HSD, or the analysis of multiple comparisons of the p-value.

All statistical analysis was performed with the *software* IBM SPSS Statistics 23.

# 3. Results

## 3.1. *Sparus aurata* rearing results

Gilthead seabream larvae pictures at 23, 35 and 58 DAH, are showed bellow, as an example of the larvae at that age.



**Figure 2 - Gilthead seabream (*Sparus aurata*) larvae with 23 DAH.**
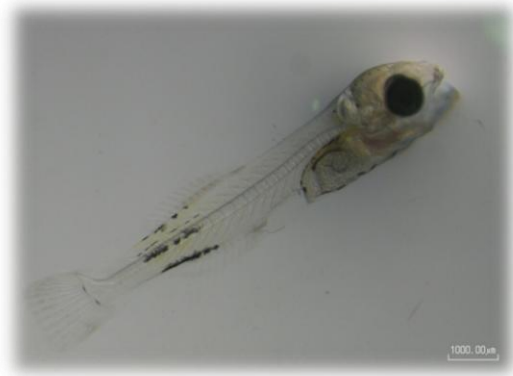


**Figure 12 - Gilthead seabream (*Sparus aurata*) larvae with 35 DAH.**



**Figure 13 - Gilthead seabream (*Sparus aurata*) larvae with 58 DAH.**

### 3.1.1. Growth performance

The different performances in gilthead seabream larvae with the three dietary treatments are represented in Figure 14. SGR (Specific Growth Rate) between treatments range from 8 % to 11%. The best growth performance was observed in the larvae of treatment FAST61/22 with a SGR of $11.03 \pm 0.58\%$, while treatment COMM had the lowest SGR ($8.28 \pm 0.41\%$), and treatment FAST64/16 had a SGR of $10.35 \pm 0.34\%$.
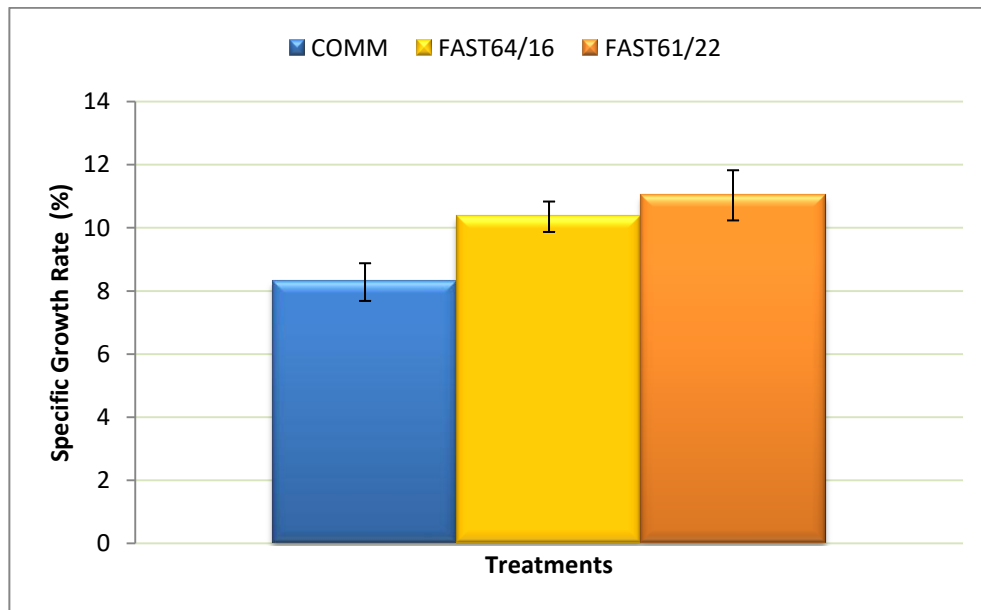


Figure 14 - Specific Growth Rate (in percentage) of gilthead seabream (*Sparus aurata*) larvae fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), since 23 DAH until 58 DAH. Standard deviations are represented by vertical bars.

The mean values of TL (Total length) at the different ages, in the three treatments, shows a exponential tendency (Figure 15). At the beginning of the trial (23DAH), the mean TL of the larvae, in all treatments was similar. Passing 12 days, at 35 DAH, the difference between treatments was very small, since the larvae were still fed with live food and microdiets. The treatments, at this age (35 DAH), had a TL of $8.2 \pm 0.2$mm, $8.4 \pm 0.1$mm and $8.5 \pm 0.2$mm (COMM, FAST64/16 and FAST61/22, respectively).

Figure 35 - Total Length mean values, in millimeters, of gilthead seabream (*Sparus aurata*) larvae fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), at 23, 35, and 58 DAH. Letters mean that differ significantly (p<0.05), and standard deviations are represented by vertical bars.

Between 35DAH and 58DAH the larvae only resource of food was the dietary treatments, and is observed in the graph that from this age (35DAH), the TL mean values between treatments diferenciam-se. At 58DAH, TL is significantly different (p<0.05) between treatments, larvae from the treatment FAST61/22 had the highest TL mean values (18.1 ± 1mm), while treatment COMM had the lowest TL mean values (14.2±0.5mm), and treatment FAST64/16 had a TL mean values of 16.9±0.7mm.

### 3.1.2. Dry weight

The mean values of DW (Dry weight) (Figure 16) at the beginning of the trial (23DAH) were similar in all treatments (0.25 ± 0.04mg). Passing 12 days, at 35 DAH, the difference between treatments was very small, since the larvae were still fed with live food and microdiets, as previously mentioned. The treatments, at this age, had a DW of 0.53 ± 0.023mg, 0.66 ± 0.05mg and 0.67 ± 0.02mg (COMM, FAST64/16 and FAST61/22, respectively).
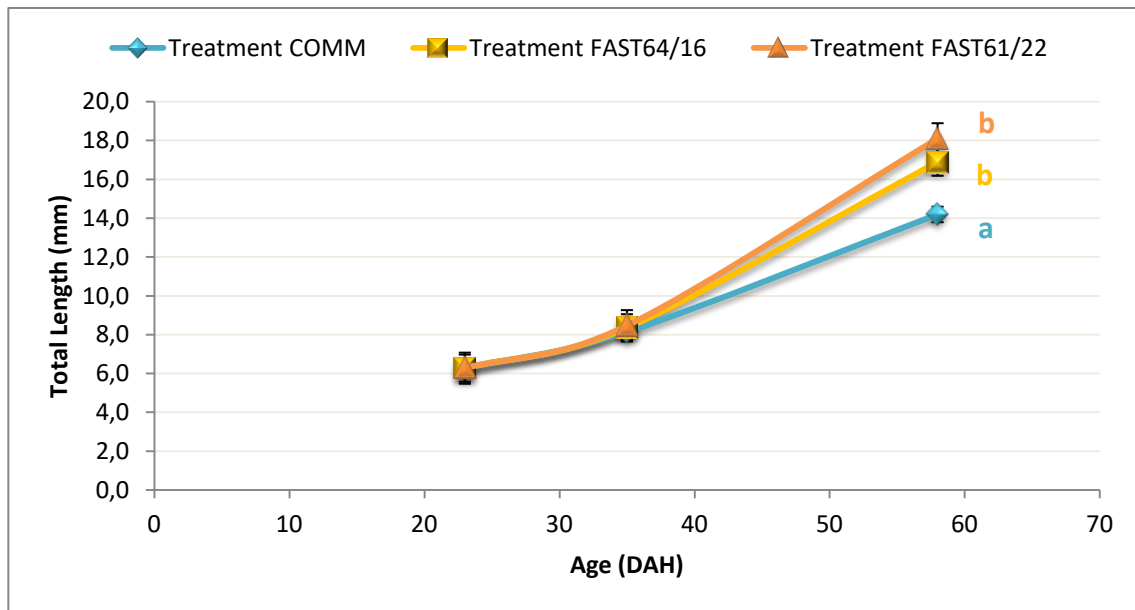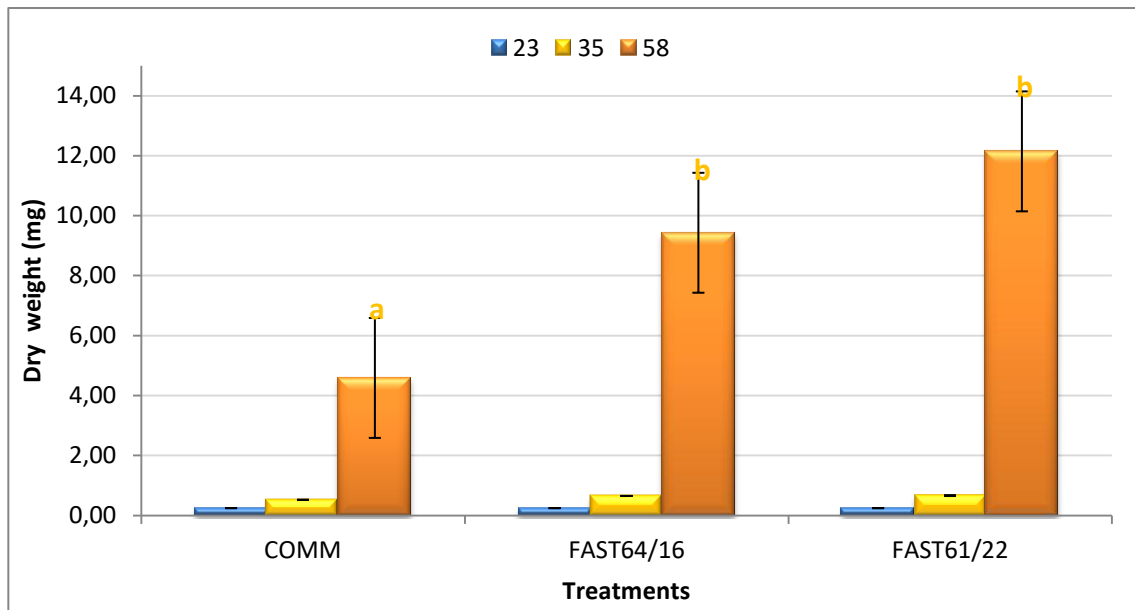
Figure 164 - Dry weight mean values, in milligrams, of gilthead seabream (*Sparus aurata*) larvae fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), at 23, 35, and 58 DAH. Letters mean that differ significantly (p<0.05), and standard deviations are represented by vertical bars

At 58 DAH, DW is significantly different (p<0.05) between treatments, larvae from the treatment FAST61/22 had the highest DW mean values (12.1 ± 2.3mg), while treatment COMM had the lowest TL mean values (4.6 ± 0.6mg), and treatment FAST64/16 had a TL mean values of 9.4±1.2mg.

### 3.1.3.Survival

In the rearing of gilthead seabream larvae, until 42 DAH the mortality remain constant (0 or 1%), from 42 DAH until 50 DAH mortality increase in all tanks of the experiment. Increase to 2, 3%, and 4 ± 1% in COMM, FAST64/16, and FAST61/22 treatment, respectively (data not show).
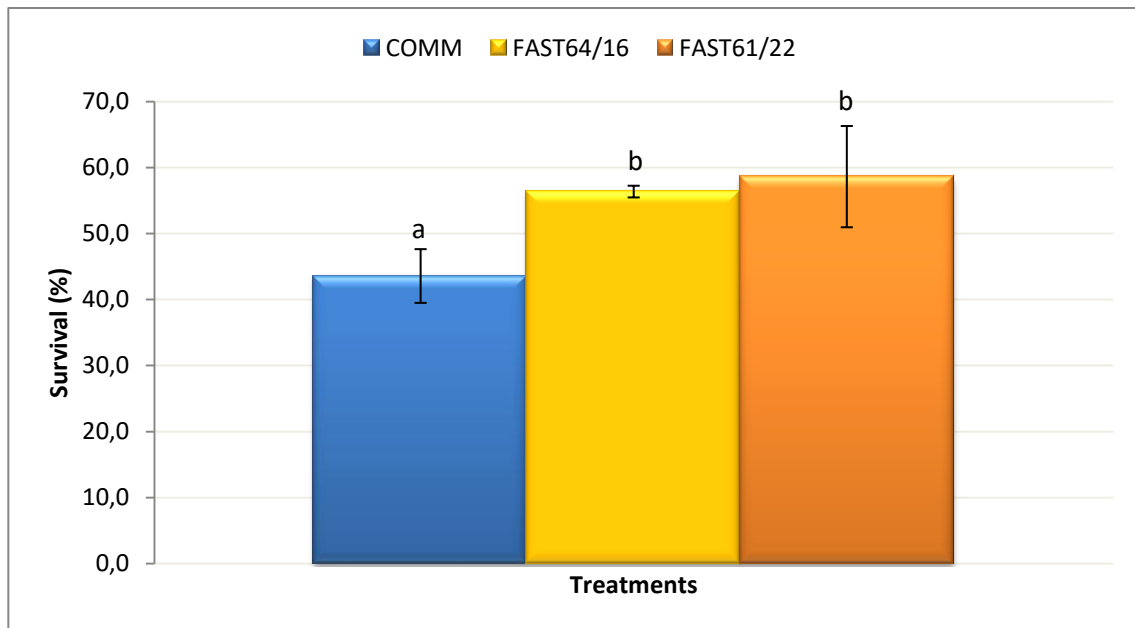
Figure 17- Survival mean values, in percentage, of gilthead seabream (*Sparus aurata*) larvae fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), since 23 DAH until 58 DAH. Letters mean that differ significantly (p<0.05), and standard deviations are represented by vertical bars.

The treatment FAST61/22 had, at the end of the trial the best survival on this experiment, with a survival of 58.7 ± 5.6% (Figure 17). The treatment COMM had the lowest survival (43.6±2.9%), and the treatment FAST64/16 had a survival of 56.4±0.7%. Between treatment the survival is significantly different (p<0.05).

### 3.1.4.Feeding behavior

Seabream adapts very easily to captivity, during the rearing seams that the larvae eats regularly, learning the times that the food is given. Nevertheless, some measures had to be taken since they react when people pass close to the tanks, the system rearing was in a close room with little movement to prevent stress. This larva goes for the food since the moment the feed gets on the top of the water, and when satisfied go to the middle or the bottom of the tank. Few hyperventilated larvae that maintain in the top of the water during the rearing, eat swimming in circles.

### 3.1.5. Water quality analysis

In seabream trial water samples were collected at 36 DAH, when larvae stop eating live feed, at 43 DAH, because of an increase of the mortality, and 3 days later for control.

Ammonia concentrations in all treatments increase from 36 to 43 DAH, especially on COOM treatment, and decrease at 46 DAH. These values varies from 0.0013 until 0.1513 mg/L, being bellow the total values recommended for ammonia (between 0.4 and 1 mg/L) for juvenile and adult fishes (Figure 18).
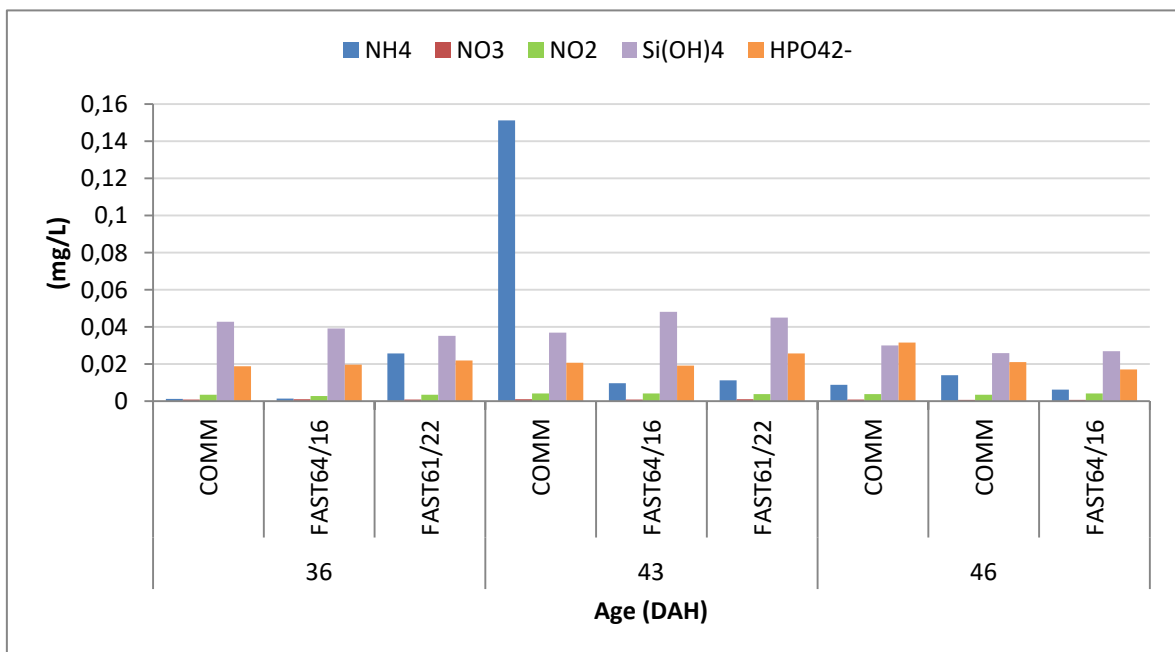


Figure 18 Variation of $NH_4^+$ (mg/L), $NO_3^-$ (mg/L), $NO_2^-$ (mg/L), $Si(OH)_4$ (mg/L) and $HPO_4^{-2}$(mg/L) concentrations, at 27, 34, 39 and 46 DAH, during seabream (*Sparus Aurata*) larvae rearing fed with three different dietary treatments (COMM; FAST64/16; FAST61/22).

Nitrite and nitrate concentrations were constants during the sampling, having a small increasing from 36 to 43 DAH. The nitrite values range between 0.0007 and 0.0011 mg/L, and nitrate values range between 0.0028 and 0.0043 mg/L, but keeping bellow the limit values recommended (between 0.1 and 0.3 mg/L for nitrite and between 1 and 3 mg/L for nitrate).

Silicate concentrations were slightly superior at 43 DAH and slightly lower at 46 DAH. The values range from 0.0260 and 0.0482 mg/L, as shown in Annex A.

Phosphates concentrations were constant during the sampling and range between 0.0171 and 0.0316 mg/L.

**3.1.6.Malformations**

In gilthead seabream rearing the most common malformation observed was fusions in the caudal area, an example of a caudal fusion is shown in Figure 21. Same larvae showed different malformations, like a lordosis as shown in Figure 20.



Figure 19 Gilthead seabream larva without

malformations.
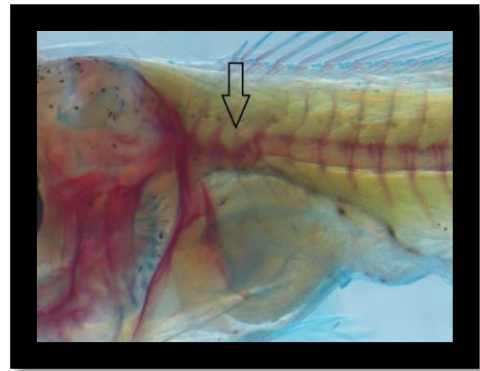


Figure 20 Example of a lordosis pre-haemal,

in a gilthead seabream larva.
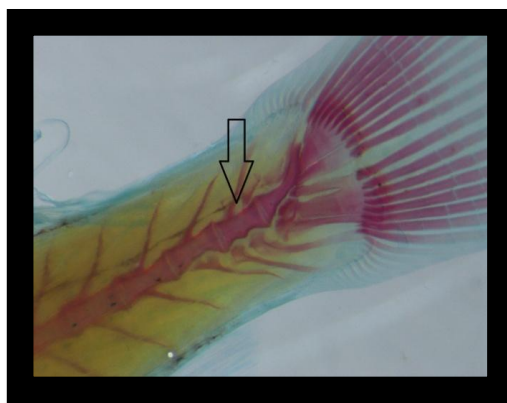


Figure 21 Example of a caudal fusion, in a gilthead seabream larva.

To evaluate the malformations of the gilthead seabream larvae, were analyzed the incidence of malformations, the severity level, the effect of the malformations on body appearance, the number of malformations per fish and the malformations distribution for anatomic area.

All statistical analysis performed between the treatments COMM, FAST64/16, and FAST61/22 show no significant differences ($p > 0.05$).

### 3.1.6.1. *Incidence of malformations*

In 60 gilthead seabream larvae taken for malformations analysis from each treatment, the highest percentages of malformed fish was observed in treatment FAST61/22, with 44 ± 10%, right fallowed by treatment COMM, with 41 ± 16%, and treatment FAST64/16 had the lowest percentages of malformed fish (32 ± 19%), as demonstrated in Figure 22.
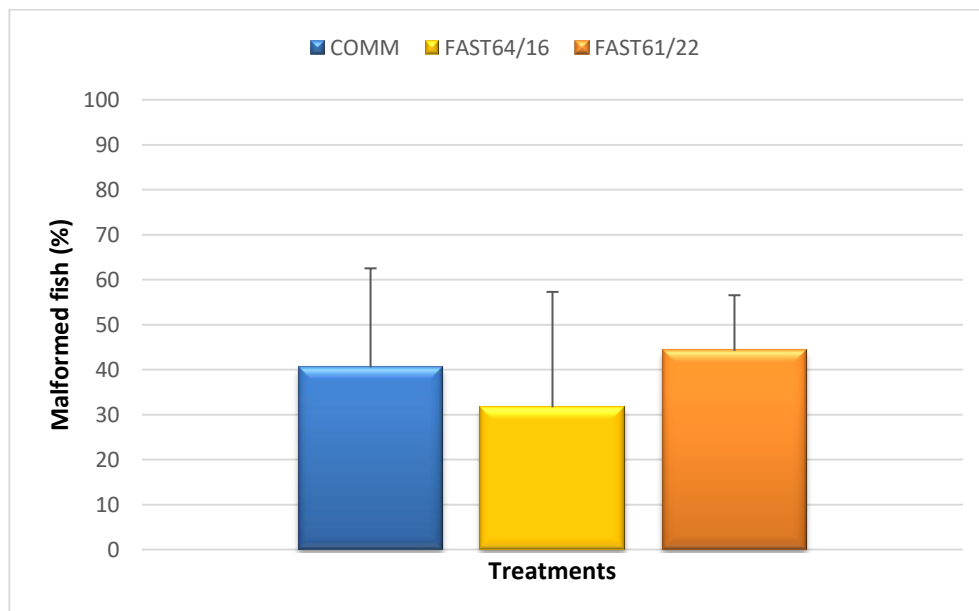


Figure 22 - Malformed fish mean values, in percentage, of gilthead seabream (*Sparus aurata*) larvae fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), since 23 DAH until 58 DAH. Standard deviations are represented by vertical bars.

### 3.1.6.2. *Severity level*

The severity level analysis was accordind the levels described in the Table VI (Gavaia (Com Pess.)).

The percentage of malformations with severity level 1 in the treatments COMM and FAST64/16, was only observed in one of the three tanks of the treatment. The mean percentage value for malformations with severity level 1 was higher in treatment FAST61/22 ($16 \pm 3.6\%$), the treatment COMM had the lowest percentage value ($4 \pm 5\%$), and the treatment FAST64/16 had a percentage value for malformations with severity level 1 of $6 \pm 7.6\%$ (Figure 23).

The percentage of malformations with severity level 2 in the treatments FAST64/16, was only observed in two of the three tanks of the treatment. The mean percentage value for malformations with severity level 2 was higher in treatment FAST61/22 ($50 \pm 30\%$), the treatment COMM had the lowest percentage value ($55 \pm 14.2\%$), and the treatment FAST64/16 had a percentage value for malformations with severity level 2 of $17 \pm 22.3\%$.

The mean percentage value for malformations with severity level 3 was higher in treatment COMM ($39 \pm 18.6\%$), followed by treatment FAST64/16 that had a percentage value of $38 \pm 28.2\%$, and the treatment FAST61/22 had the lowest percentage value for malformations with severity level 3 ($29 \pm 20.77\%$).
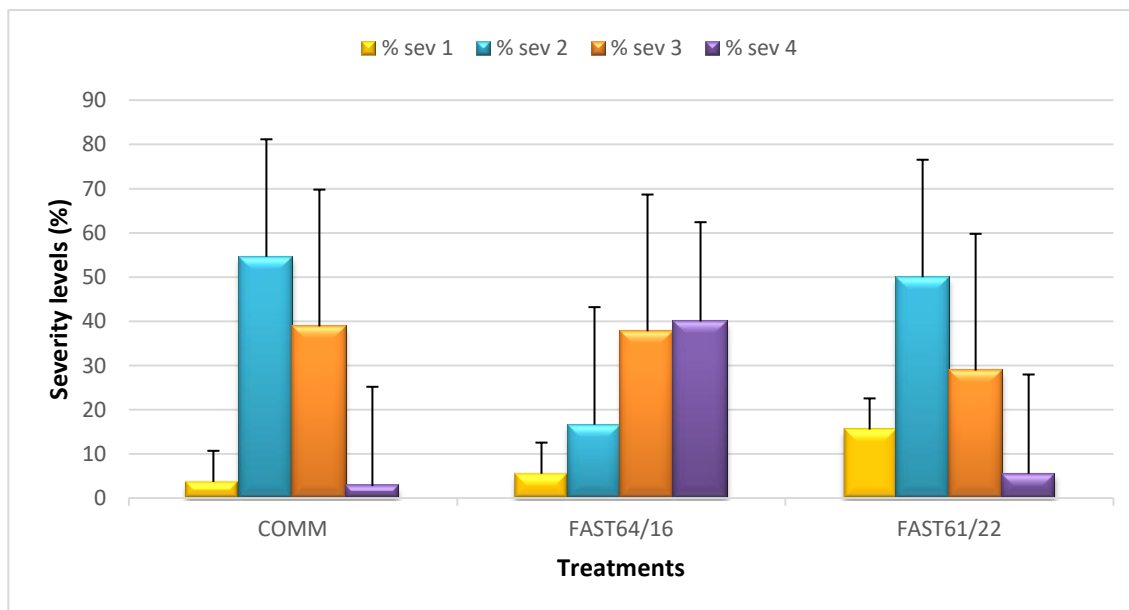
Figure 23 - Severity levels, in percentage, of the malformations in gilthead seabream (*Sparus aurata*) larvae fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), since 23 DAH until 58 DAH. Severity levels (Table VI), in this analysis according to Gavaia (Com Pess.), and standard deviations are represented by vertical bars.

The percentage of malformations with severity level 4 in the treatments COMM and FAST61/22 was only observed in one of the three tanks of the treatment, and in treatment FAST64/16 was only observed in two of the three tanks of the treatment. The mean percentage value for malformations with severity level 4 was higher in treatment FAST64/16 ($23 \pm 17.7\%$), the treatment COMM had the lowest percentage value ($4 \pm 3.8\%$), and the treatment FAST64/16 had a percentage value for severity level 4 of $6 \pm 7.6\%$.

### 3.1.6.3. *Effect of the malformations on body appearance*

The treatment FAST61/22 had the highest mean percentage of fish with malformations were the phenotype was not affected ($66 \pm 16.9\%$), followed by treatment COMM with a mean percentage of $58 \pm 16.6\%$. The treatment FAST64/16 had the lowest percentage of $22 \pm 27.6\%$ (two of the three tanks of the treatment present 0% of fish with malformations were the phenotype was not affected.
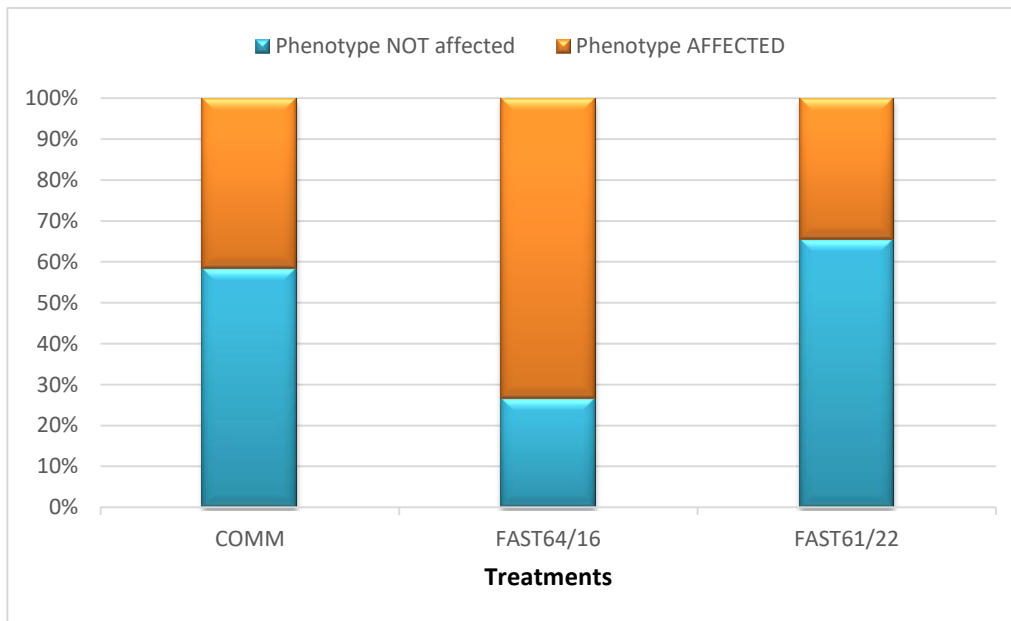
Figure 24 - Mean percentage of malformed gilthead seabream (*Sparus aurata*) fish depending if affects the phenotype or not, fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), since 23 DAH until 58 DAH.

The mean percentage of fish with malformations were the phenotype was affected, were higher in treatment FAST64/16 (61 ± 35.2%) where two of the three tanks of the treatment present 100% of fish with malformations were the phenotype was affected, but in one of this tanks only two larvae of the 20 had malformations. The treatment COMM had a mean percentage of 42 ± 16.8%, followed by treatment FAST61/22 that had the lowest percentage of 34 ± 16.9%. The Figure 24, represent the mean percentage of fish with malformations were the phenotype affect, or not, the phenotype of the fish.

### 3.1.6.4.    *Malformations per fish*

The mean values of malformations per fish were very similar between treatments. The treatment COMM had the highest mean value of 1.4 ± 0.13 malformation/fish, followed by treatment FAST61/22 with a mean value of 1.3 ± 0.1 malformation/fish, and treatment FAST64/16 had a mean value of 1.2 ± 0.1 malformation/fish.
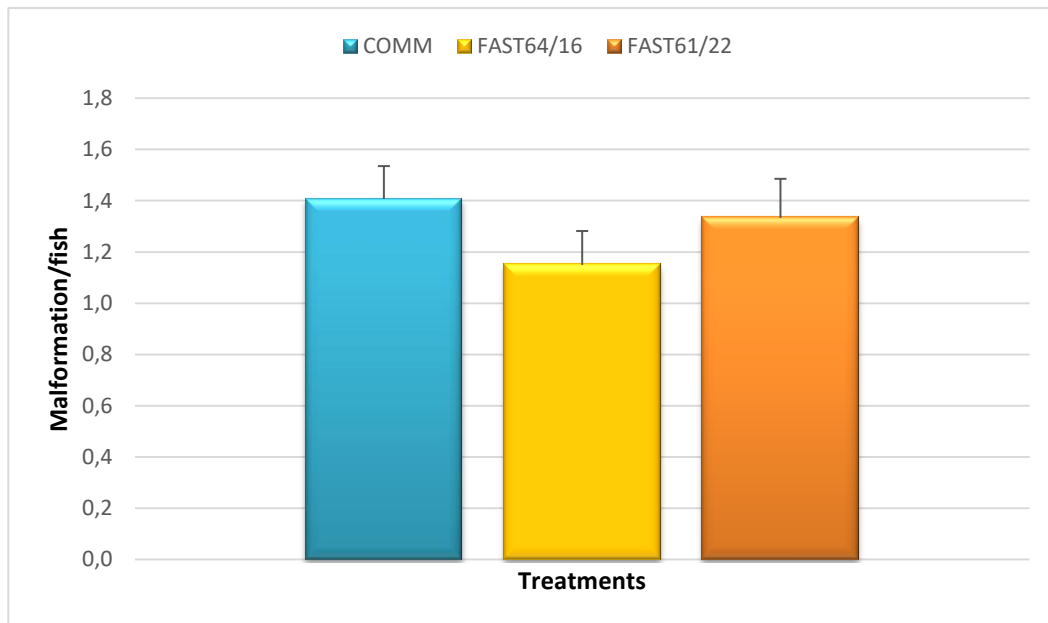
Figure 25 - Mean values of malformations per fish, in percentage, of gilthead seabream (*Sparus aurata*) larvae fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), since 23 DAH until 58 DAH. Standard deviations are represented by vertical bars.

Meaning that the majority of the analyzed fish only have one or two malformations (Figure 25).

### 3.1.6.5. *Malformations distributions in each anatomic area*

The mean percentage of malformations per anatomic area of the fish is represented in Figure 26. The mean percentage of malformations in the cephalic area in the treatments COMM and FAST64/16, was only observed in one of the three tanks of the treatment. The mean percentage value for malformations in the cephalic area of the fish was higher in treatment FAST64/16 (6 ± 8%), the treatment FAST61/22 had the lowest percentage value (0%), and the treatment COMM had a percentage value of malformations in the cephalic area of 2 ± 2.8%.

The mean percentage of malformations in the pre-haemal area of the fish in the treatments COMM and FAST64/16, was only observed in two of the three tanks of the treatment. The mean percentage value for malformations in pre-haemal area was higher

in treatment FAST64/16 (40 ±40%), followed by treatment COMM with a percentage value for malformations in the pre-haemal area of 38 ± 25.1%, and the treatment FAST61/22 had the lowest percentage value of 31 ± 9.2%.

The mean percentage value for malformations in the haemal area was higher in treatment FAST61/22 (36 ± 5.4%), treatment COMM had a percentage value of 26 ± 1.5%, and the treatment FAST64/16 had the lowest percentage value for malformations in the haemal area (9 ± 12%), where only one of the three tanks of the treatment present larvae with malformations in this area.
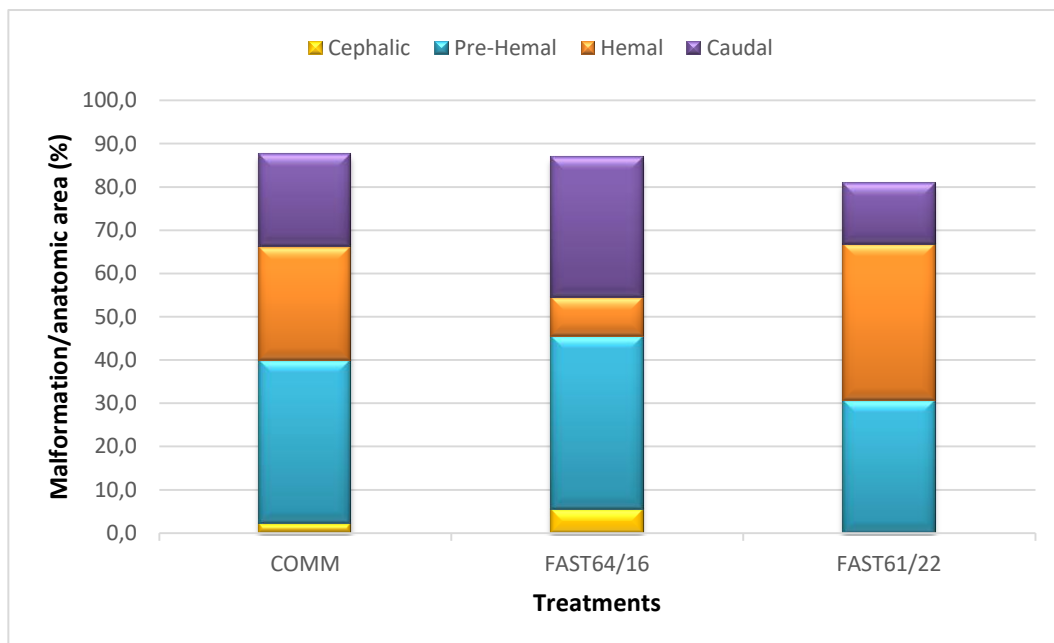


Figure 26 - Percentage of the malformations per anatomic area in gilthead seabream (*Sparus aurata*) fish depending if affects the cephalic, pre-haemal, haemal, or caudal area, fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), since 23 DAH until 58 DAH.

The percentage of malformations in the caudal area in the treatments COMM and FAST64/16 was only observed in two of the three tanks of the treatment, and in treatment FAST61/22 was only observed in one of the three tanks of the treatment. The mean percentage value for malformations in the caudal area was higher in treatment FAST64/16 (32 ± 22%), the treatment FAST61/22 had the lowest percentage value (14 ± 18.6%), and the treatment COMM had a percentage value of 21 ± 18.9%.

## 3.2.*Argyrosomus regius* rearing results

Meagre larvae pictures at 20, 31 and 46 DAH are show bellow, as an example of the larvae at that age.



Figure 27 - Meagre (*Argyrosomus regius*) larvae with 20 DAH.
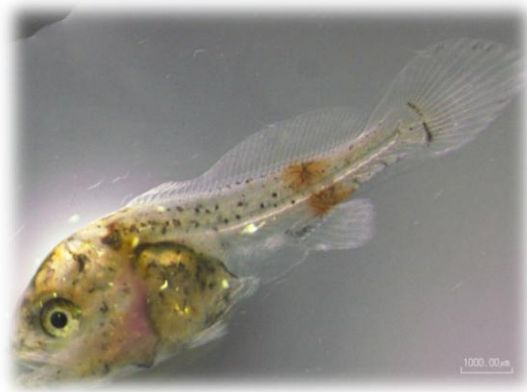


Figure 28 - Meagre (*Argyrosomus regius*) larvae with 31 DAH.



Figure 29 - Meagre (*Argyrosomus regius*) larvae with 46 DAH.

### 3.2.1. Growth performance

The different performances in meagre larvae in the three dietary treatments are represented in Figure 30. SGR (Specific Growth Rate) was similar between treatments, the best growth performance was observed in the larvae of treatment FAST61/22 with a SGR of $19.25 \pm 0.57\%$, while treatment FAST64/16 had the lowest SGR ($18.25 \pm 0.51\%$), and treatment COMM had a SGR of $18.61 \pm 0.23\%$.
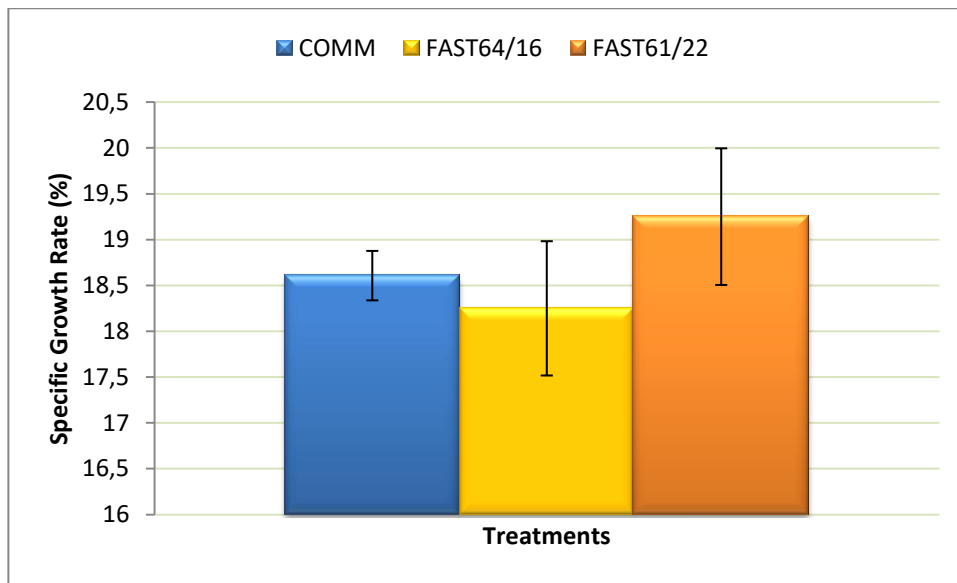


Figure 30 - Specific Growth Rate (in percentage) of meagre (*Argyrosomus regius*) larvae fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), since 20 DAH until 46 DAH. Standard deviations are represented by vertical bars.

The mean values of TL (Total length) at the different ages, in the three treatments, shows a exponential tendency (Figure 31). At the beginning of the trial (20 DAH), the mean TL of the larvae, in all treatments was the same. Passing 11days, at 31 DAH, the difference between treatments was very small, since the larvae were still fed with live food and microdiets. The treatments, at 31 DAH, had a TL of $8.6 \pm 0.2$mm, $8.8 \pm 0.2$mm and $10.1 \pm 0.3$mm (COMM, FAST64/16 and FAST61/22, respectively). Between 31 DAH and 46 DAH the larvae only resource of food was the dietary treatments, and is observed in the graph that at 31 DAH, are already differences in the size between the FAST61/22 and the other two treatments. Treatment FAST61/22, at 39 DAH, maintain higher mean values of TL ($18 \pm 0.4$mm), while treatment COMM demonstrated the lower

mean values (14.7 ± 0.4mm), and treatment FAST64/16 had a TL mean value of 16.3 ± 0.3mm. In one day of difference, was possible to observe an increase in TL mean values, treatment FAST61/22 maintain, at 40 DAH, the highest mean values (19.1 ± 0.6mm), treatment COMM maintain the lowest mean value (15.1 ± 0.7mm), and treatment FAST64/16 had a TL mean value of 16.3 ± 1.1mm.
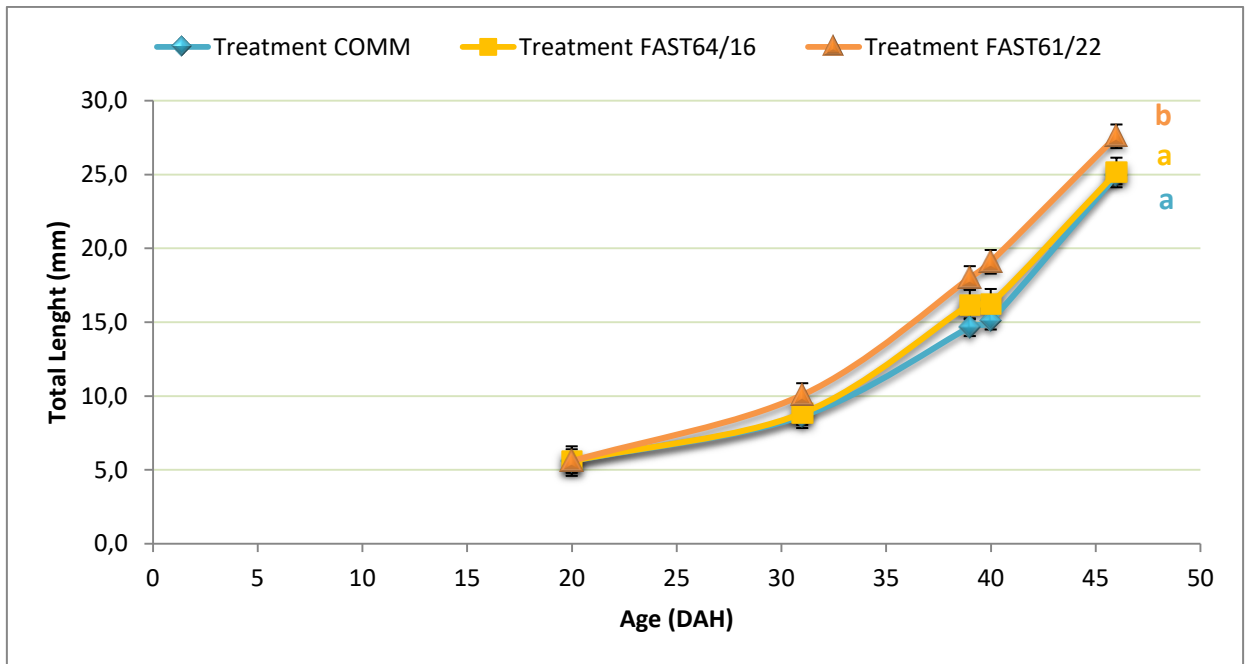


Figure 31 - Total Length mean values, in millimeters, of meagre (*Argyrosomus regius*) larvae fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), at 20, 31 and 46 DAH. Letters mean that differ significantly (p<0.05), and standard deviations are represented by vertical bars.

In the end of the experiment, at 46 DAH, TL is significantly different (p<0.05) between treatments, larvae from the treatment FAST61/22 had the highest TL mean values (27.6 ± 1.2mm), while treatment COMM had the lowest TL mean values (24.9 ± 0.7mm), and treatment FAST64/16 had a TL mean values of 25.1 ± 1.1mm.

### 3.2.2. Dry weight

The mean values of DW (Dry weight) at the beginning of the trial (20DAH) were similar in all treatments (0.22 ± 0.01mg). Passing 11 days, at 31 DAH, the difference

between treatments was very small, since the larvae were still fed with live food and microdiets, as previously mentioned. The treatments, at this age, had a DW of 0.85 ± 0.12mg, 1.04 ± 0.13mg and 1.6 ± 0.14mg (treatment COMM, FAST64/16 and FAST61/22, respectively). At 40 DAH, the treatment FAST61/22 present higher mean values of DW (10.51 ± 1.21mg), while treatment COMM had the lowest mean values (5.36 ± 0.88mg), the treatment FAST64/16 had a mean value of DW of 6.29 ± 1.11mg (Figure 32).
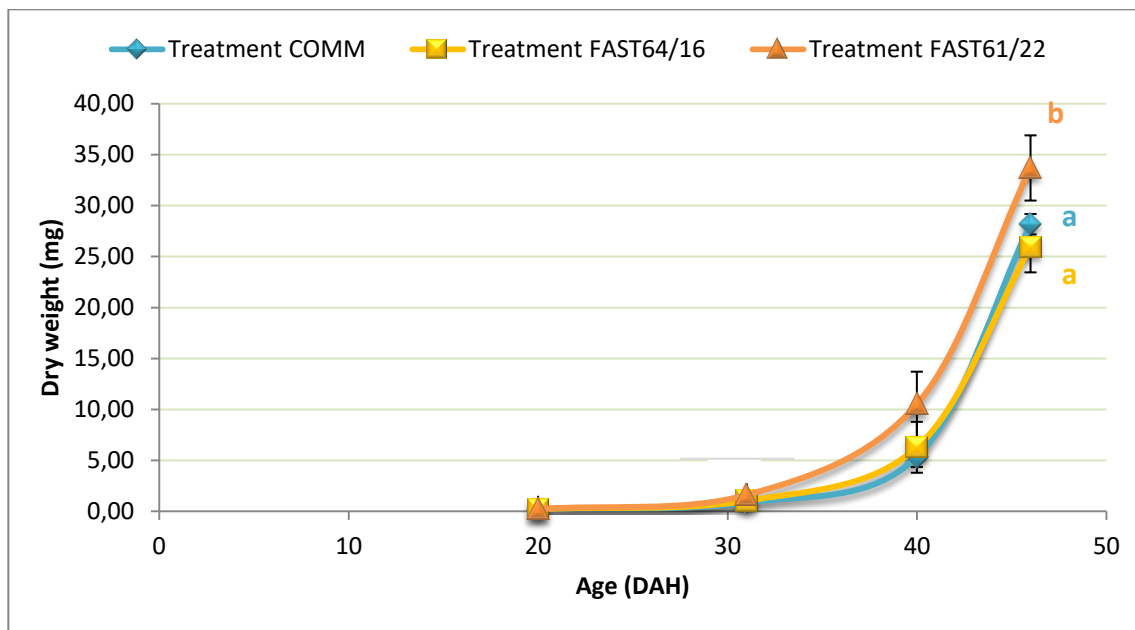


Figure 32 - Dry weight mean values, in milligrams, of meagre (*Argyrosomus regius*) larvae fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), at 20, 31 and 46 DAH. Letters mean that differ significantly ($p < 0.05$), and standard deviations are represented by vertical bars.

At 46 DAH, DW is significantly different ($p < 0.05$) between treatments, larvae from the treatment FAST61/22 maintain the highest DW mean values (33.7 ± 5.22mg), while treatment FAST64/16 had the lowest DW mean values (25.96 ± 3.3mg), and COMM treatment had a DW mean values of 28.18 ± 1.5mg.

### 3.2.3.Survival

In the rearing of meagre larvae, until 35 DAH the mortality was higher than expected ($6 \pm 0.33\%$, $5\pm 0.33\%$ and $5\%$, in the treatment COMM, FAST64/16, and FAST61/22, respectively), from 35 DAH until the end of the experiment, the mortality decrease to 0 or 1%, remaining constant in all tanks of the trial. The treatment FAST61/22 had, at the end of the trial the best survival on this experiment, with a survival of $21.2 \pm 3\%$ (Figure 33).



Figure 33 - Survival mean values, in percentage, of meagre (*Argyrosomus regius*) larvae fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), since 20 DAH until 46 DAH. Letters mean that differ significantly ($p<0.05$), and standard deviations are represented by vertical bars.

The treatment COMM had the lowest survival ($10.7\pm2.2\%$), and the treatment FAST64/16 had a survival of $16.5 \pm 0.5\%$. Between treatment the survival is significantly different ($p<0.05$).

### 3.2.4. Feeding behavior

Meagre adapts easily to captivity, nevertheless, some measures had to be taken since they react very easily to any sound (noises) and shades close to the tanks, the system rearing was in a close room with little movement to prevent stress. This larva prefers to feed mostly in the middle of the water column, so waits for the hydration of the food to dive towards the bottom to catch it. Since meagre fish possesses a voracious appetite, some cannibalism was observed, and the larvae seem to prefer to attack larvae with the size of the mouth, getting sometimes stuck in the gut and releasing the prey that goes to the bottom. In the bottom, the larvae does not seem to eat, neither the dead larvae neither the microdiet.

### 3.2.5. Water quality analysis

In meagre trial water samples were collected at 27, 34, 39 and 46 DAH, once per week for control of the water quality.

Ammonia concentration in COOM treatment increase from 27 to 34 DAH, keeping constant until the end of the trial. In FAST64/16 treatment ammonia concentration decrease from 27 to 34 DAH, and slightly increase in the next two weeks at 39 and 46 DAH. And in FAST61/22 treatment ammonia concentration is higher at 39 and 46 DAH. Ammonia values varies from 0.0010 until 0.3044 mg/L, being bellow the limit values recommended for ammonia (between 0.4 and 1 mg/L) for juvenile and adult fishes (Figure 34).
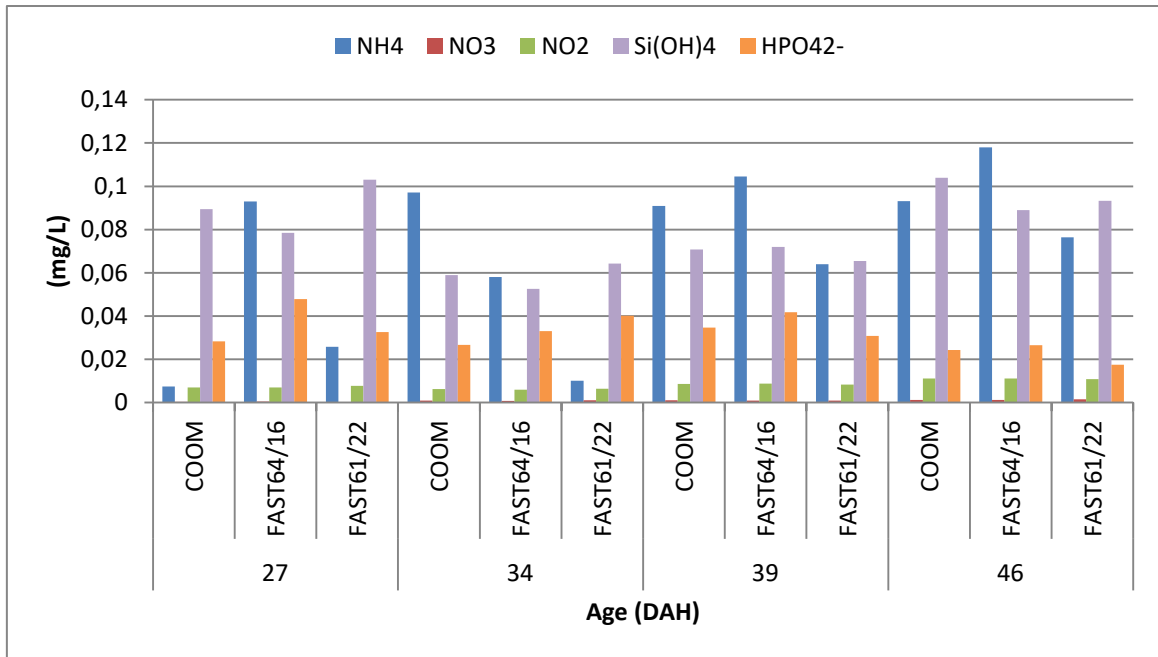
Figure 34 Variation of $NH_4^+$ (mg/L), $NO_3^-$ (mg/L), $NO_2^-$ (mg/L), $Si(OH)_4$ (mg/L) and $HPO_4^{-2}$(mg/L) concentrations, at 27, 34, 39 and 46 DAH, during meagre (*Argyrosomus regius*) larvae rearing fed with three different dietary treatments (COMM; FAST64/16; FAST61/22).

Nitrite and nitrate concentrations were constants during the trial, the values range between 0.0027 and 0.0122 mg/L, and between 0.0001 and 0.0020 mg/L, respectively, and being keeping bellow the limit values recommended (between 0.1 and 0.3 mg/L for nitrite and between 1 and 3 mg/L for nitrate).

Silicate concentrations were higher at 27 and 46 DAH and phosphates concentrations were lower at 46 DAH. The values of silicates and phosphates range from 0.0473 until 0.2246 mg/L, and from 0.0127 until 0.0840 mg/L, respectively, as shown in Annex B.

### 3.2.6.Malformations

The malformations observed in meagre larvae were mainly in the pre-haemal and haemal areas. An example of a normal caudal fin is shown in Figure 35, and an example of a lordosis malformation in a caudal fin is shown in Figure 36. Few malformations were found in the head (mandible) and in the caudal fin.
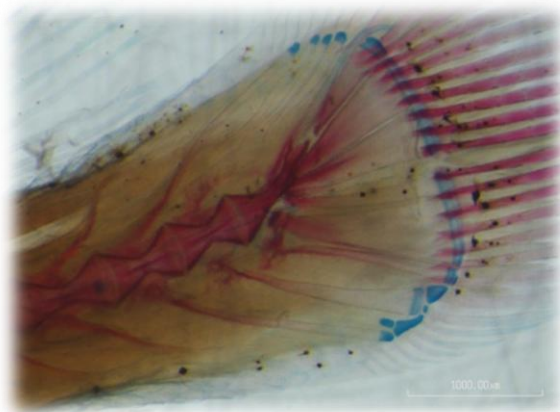
Figure 35 Exemple of a meagre's caudal fin without malformations.
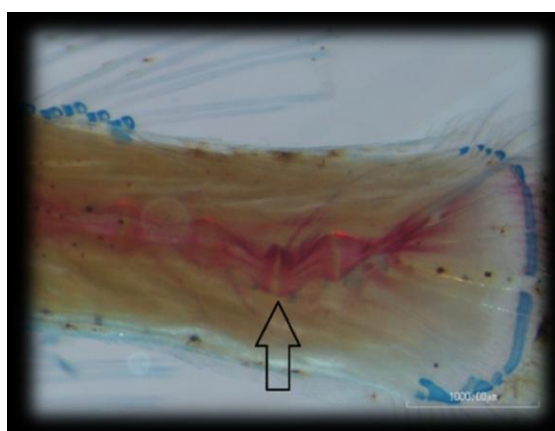


Figure 36 Example of a lordosis in a caudal area of a meagre larva.

To evaluate the malformations of the meagre larvae, were analyzed the incidence of malformations, the severity level, the effect of the malformations on body appearance, the number of malformations per fish and the malformations distribution for anatomic area.

All statistical analysis performed between the treatments COMM, FAST64/16, and FAST61/22 show no significant differences ($p > 0.05$).

### 3.2.6.1.   *Incidence of malformations*

In 60 meagre larvae taken for malformations analysis from each treatment, the highest percentages of malformed fish was observed in treatment FAST64/16, with 38 ±

9%, fallowed by treatment FAST61/22 with $30 \pm 6.7\%$, and treatment COMM had the lowest percentages of malformed fish ($28 \pm 2.3\%$), as demonstrated in Figure 37.
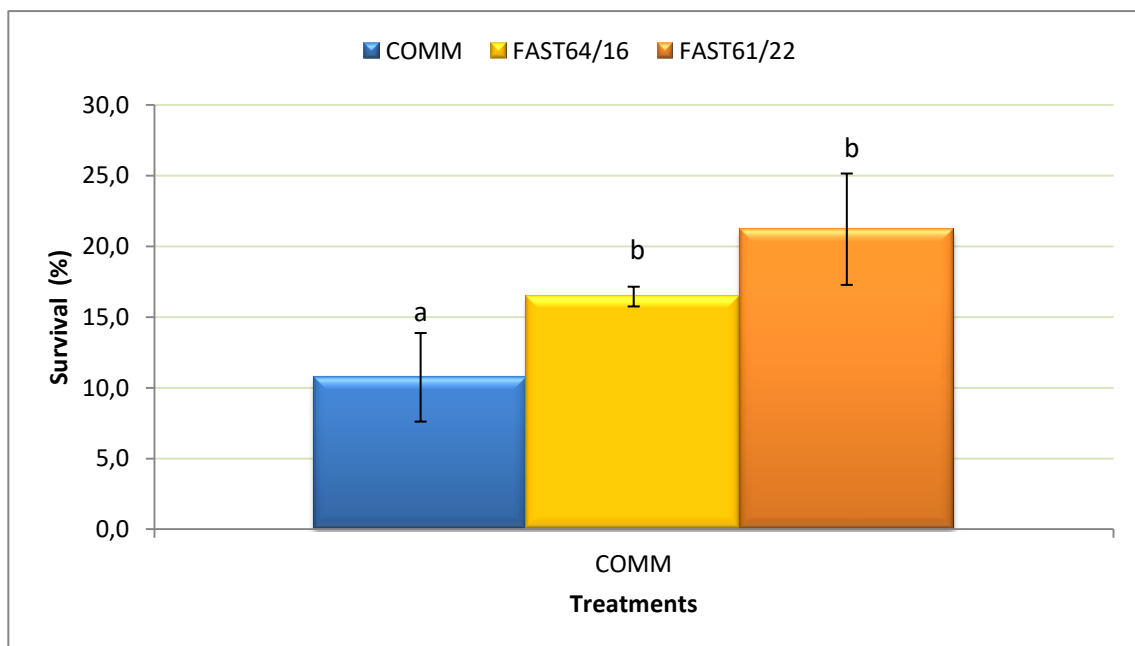


Figure 37 - Malformed fish mean values, in percentage, of meagre (*Argyrosomus regius*) larvae fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), since 20 DAH until 46 DAH. Standard deviations are represented by vertical bars.

### *3.2.6.2.* *Severity level*

The severity level analysis was according the levels described in the Table VI (Gavaia (Com Pess.)).

The percentage of malformations with severity level mean percentages are represented in Figure 38. Only treatment Fast64/16 had malformed fish with the severity level 1 ($4 \pm 5.5\%$), but only one of the three tanks in the treatment, present malformations with this level of severity.

The percentage of malformations with severity level 2 was higher in treatments COMM and FAST61/22 ($63 \pm 15.7\%$ and $63 \pm 24.3\%$, respectively), and the treatment FAST64/16 had a percentage value for malformations with severity level 2 of $54 \pm 9.5\%$.

The mean percentage value for malformations with severity level 3 was very similar between treatments, where the higher mean percentage were in treatment

FAST64/16 (32 ± 5.7%), followed by treatment COMM that had a percentage value of 31 ± 19.2%, and the treatment FAST61/22 had the lowest percentage value for malformations with severity level 3 (30 ± 20%).
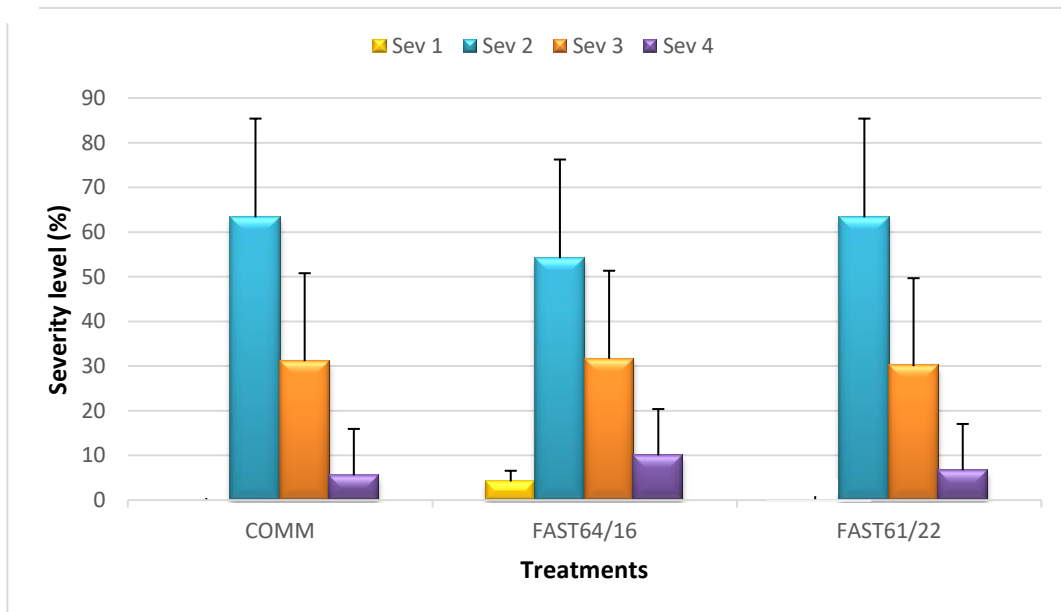


Figure 38 - Severity levels, in percentage, of the malformations in meagre (*Argyrosomus regius*) larvae fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), since 20 DAH until 46 DAH. Severity levels (Table IV), in this analysis according to Gavaia (Com Pess.), and standard deviations are represented by vertical bars.

The percentage of malformations with severity level 4 in the treatments COMM and FAST61/22 was only observed in one of the three tanks of the treatment, and in treatment FAST64/16 was only observed in two of the three tanks of the treatment. The mean percentage value for malformations with severity level 4 was higher in treatment FAST64/16 (10 ± 6.7%), the treatment COMM had the lowest percentage value (6 ± 7.6%), and the treatment FAST64/22 had a percentage value for severity level 4 of 7 ± 9%.

### *3.2.6.3.  Effect of the malformations on body appearance*

The Figure 39, represent the mean percentage of fish with malformations were the phenotype affect, or not, the phenotype of the fish.

The treatments COMM and FAST61/22 had the same mean percentage of fish with malformations were the phenotype was not affected (63 ± 16% and 63 ± 24%, respectively), followed by treatment FAST64/16 with a mean percentage of 58 ± 12%.
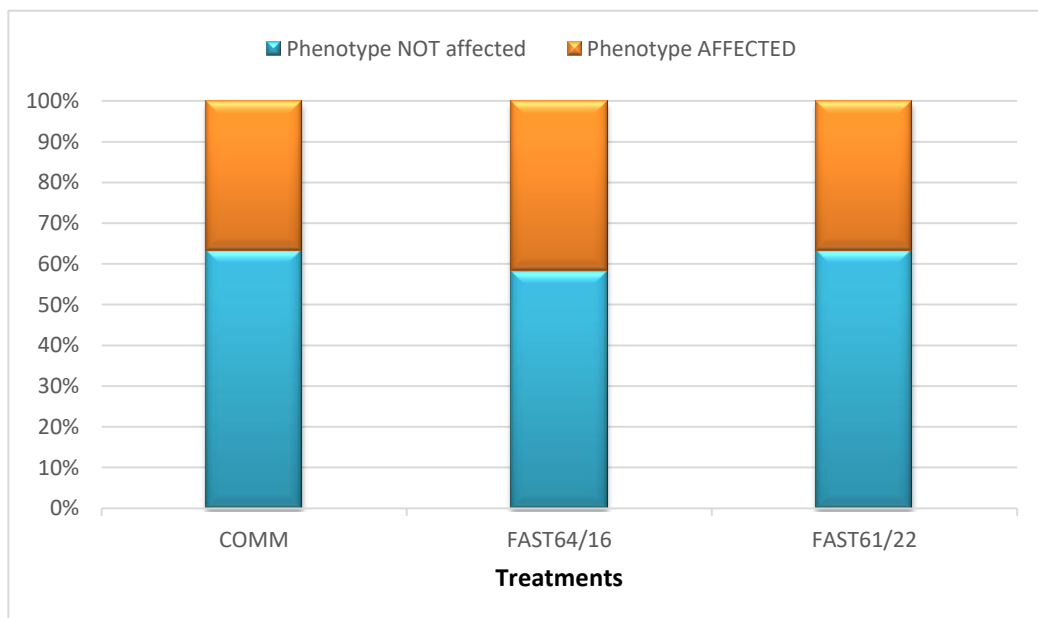


Figure 39 - Mean percentage of malformed meagre (*Argyrosomus regius*) fish depending if affects the phenotype or not, fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), since 20 DAH until 46 DAH.

The mean percentage of fish with malformations were the phenotype was affected, were higher in treatment FAST64/16 (42 ± 12%), the treatments COMM and FAST61/22 had a mean percentage of 37 ± 16% and 37 ± 24%.

### 3.2.6.4. *Malformations per fish*

The mean values of malformations per fish were very similar between treatments (Figure 40). The treatment COMM had the highest mean value of 1.2 ± 0.14 malformation/fish, followed by treatment FAST64/16 with a mean value of 1.1 ± 0.07 malformation/fish, and treatment FAST61/22 had a mean value of 1.07 ± 0.09 malformation/fish.
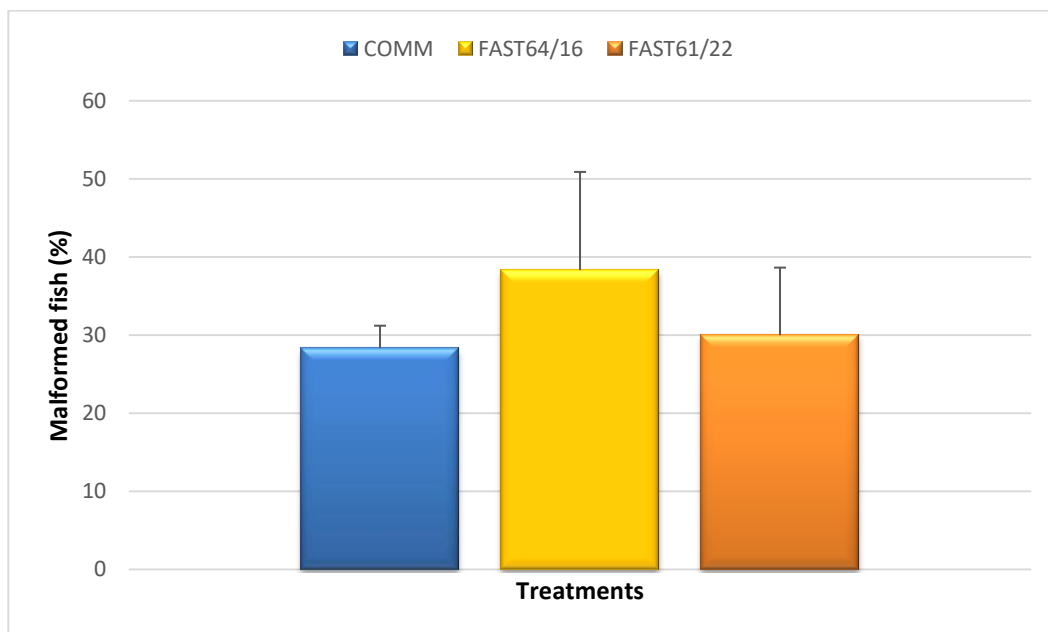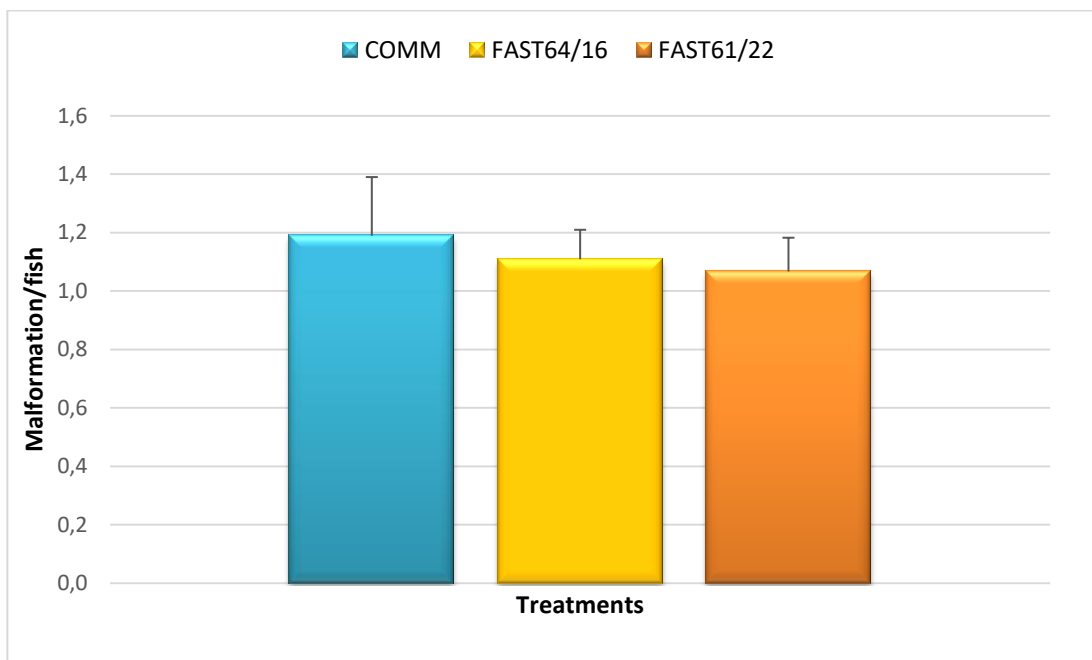


Figure 40 - Mean values of malformations per fish, in percentage, of meagre (*Argyrosomus regius*) larvae fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), since 20 DAH until 46 DAH. Standard deviations are represented by vertical bars.

Meaning that the majority of the analyzed fish only have one or two malformations.

### 3.2.6.5. *Malformations distributions in each anatomic area*

The mean percentage of malformations per anatomic area of the fish is represented in Figure 41. There are no malformations in the cephalic area in all the treatments COMM, FAST64/16, and FAST61/22.

The mean percentage of malformations in the pre-haemal area of the fish in the treatment FAST64/16, was only observed in two of the three tanks of the treatment. The mean percentage value for malformations in pre-haemal area was higher in treatment COMM (40 ± 8.1%), followed by treatment FAST61/22 with a percentage value for malformations in the pre-haemal area of 39 ± 3.57%, and the treatment FAST64/16 had the lowest percentage value of 23 ± 15.5%.
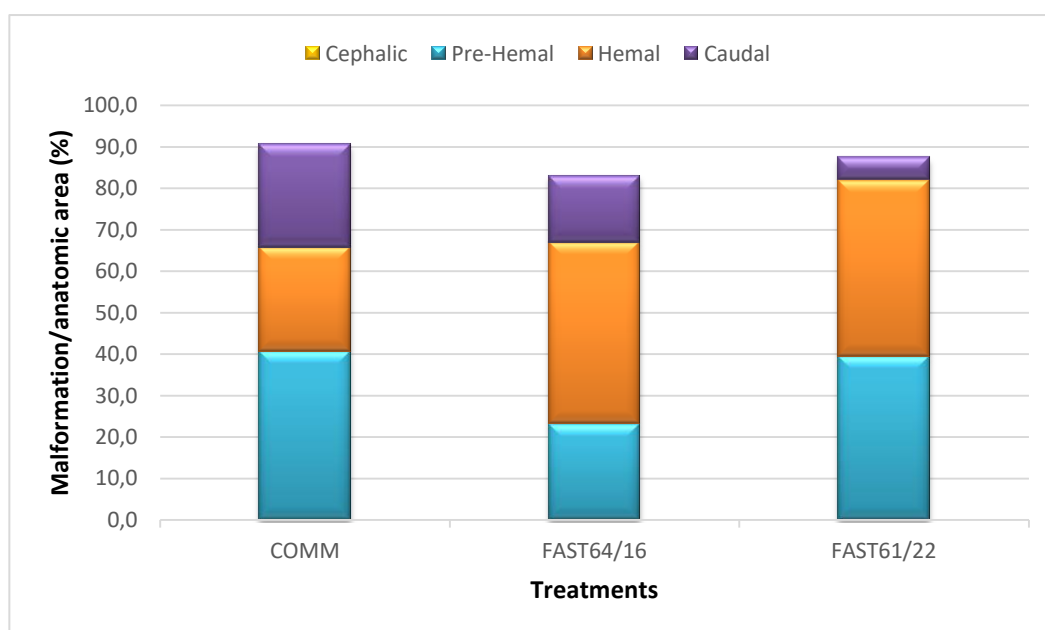


Figure 41 - Percentage of the malformations per anatomic area in meagre (*Argyrosomus regius*) fish depending if affects the cephalic, pre-haemal, haemal, or caudal area, fed with three different dietary treatments (COMM; FAST64/16; FAST61/22), since 20 DAH until 46 DAH.

The mean percentage value for malformations in the haemal area was higher in treatment FAST64/16 (44 ± 9.7%), followed by treatment FAST61/22 that had a

percentage value of 43 ± 5.17%, and the treatment COMM had the lowest percentage value for malformations in the haemal area (25 ± 7.53%).

The percentage of malformations in the caudal area in the treatment FAST61/22 was only observed in one of the three tanks of the treatment. The mean percentage value for malformations in the caudal area was higher in treatment COMM (25 ± 12.3%), the treatment FAST61/22 had the lowest percentage value (6 ± 7.57%), and the treatment FAST64/16 had a percentage value of 16 ± 3.2%.

# 4. Discussion and conclusions

In aquaculture the species success is based on the knowledge of the broodstock management and reproductive strategy (Soares *et al*., 2015), being important to good quality larvae, good larval development, and the improvements of knowledge regarding this subject. The improvements of the knowledge on dietary nutrient requirements of gilthead seabream larvae are increasing but not yet achieve the ideal. And there is a very limited knowledge on dietary nutrients requirements of meagre. The assessment of a optimum dietary protein:lipid ratio is essential to maximize production output and reduce costs. The improvement of the diets may also decrease malformations, as most malformations develop during skeletogenesis, therefore the larval period constitutes an important bottleneck in aquaculture since a great deal about physiological demands of fish larva remains to be elucidated (Darias *et al*., 2011).

To evaluate the impact of two new formulated microdiets on the development and quality of gilthead seabream (*Sparus aurata*) and meagre (*Argyrosomus regius*) larvae, comparing with a commercial diet, growth parameters were calculated and compared, to assess the efficacy of treatments. The meagre has been the subject of increasing interest, being a feasible candidate for the diversification of European aquaculture, which has promoted a number of studies regarding the optimization of its aquaculture production (Jiménez *et al.* 2005, Roo *et al.* 2010, Monfort 2010). And standard protocols have been established for gilthead seabream larval production and its availability makes this species an appropriate candidate to test new diets for larviculture (Monroig *et al*., 2006).

The experimental feeds had differences that may argue the more appropriate for the breading of gilthead seabream and meagre larvae.

Treatment FAST61/22 shown the best survival, the best growth performance, the highest total length mean values, and the highest dry weight mean values in the rearing of gilthead seabream and meagre larvae, when compared with treatments COOM and FAST64/16, meaning better larval development.

The best growth performance in gilthead seabream larvae was observed, as previous mentioned, in treatment FAST61/22 with a SGR of 11.03 ± 0.58% (Figure 14), superior

when compared with other study with seabream larvae growth by dietary vitamin E (±10%) (Atalah *et al*., 2012), while treatment COMM had the lowest SGR (8.28 ± 0.41%), unless superior when compared with another studies with seabream larvae fed with commercial diets (3.72 ± 0.13%) (Blasco *et al.,* 2015). SGR in meagre larvae was similar between treatments, the best growth performance was observed in the larvae of treatment FAST61/22 with a SGR of 19.25 ± 0.57% (Figure 30), superior when compared with larvae fed with enrich live prey (SGR between 13.32 and 6.51%)**,** with 31 DAH (Vallés and Estévez, 2015).

The mean values of TL at the different ages, in the three treatments, show an exponential tendency for both species (Figure 15 and 31). At the end of the trials with seabream and meagre larvae, TL is significantly different (p<0.05) between treatments (58 and 46 DAH, respectively). Treatment FAST61/22 had the highest TL mean values on both species (18.1 ± 1 mm; 27.6 ± 1.2 mm, respectively.

At the beginning of the trials the mean DW of the larvae (Figure 16 and 32), was very similar in all treatments, like in TL analysis, at 35 DAH, in seabream larvae, and at 31 DAH, in meagre larvae, the difference between treatments was very small, since the larvae were still fed with both live food and microdiets, as previously mentioned. After these ages, the larvae only resource of food was the dietary treatments, and at the end of the trials with seabream and meagre larvae DW is significantly different (p<0.05) between treatments (58 and 46 DAH, respectively). Treatment FAST61/22 had the highest DW mean values on both species (12.1 ± 2.3 mg; and 33.7 ± 5.22 mg, respectively), while treatment COMM and FAST64/16 had the lowest DW mean values, for seabream and meagre fish, respectively (4.6 ± 0.6 mg; 24.9 ± 0.7 mg, respectively). Showing that meagre grows much more than seabream in the first month and half of its life. This is one of the many reasons to improve rearing protocols and nutritional requirements for meagre larvae.

So, regarding larval growth, gilthead seabream and meagre larvae had a better growth with the microdiet FAST61/22, showing a good response to a feed with high protein/high lipid content.

The best survival on these experiments, in both gilthead seabream and meagre trials were with treatment FAST61/22, with a survival of 58.7 ± 5.6% (Figure 17) and 21.2 ±

3% (Figure 33), respectively. The treatment COMM had the lowest survival also in both seabream and meagre trials ($43.6 \pm 2.9\%$ and $10.7 \pm 2.2\%$, respectivly). Between treatment the survival is significantly different ($p<0.05$). The differences between the survivals of seabream and meagre larvae can be explained by the observation of cannibalism in meagre larvae as report by other authors (Vallés *et al.,* 2013; Roo *et al.,* 2010), but also can be associated with some swim bladder hyperinflation and be related with the density as already described in Roo *et al.* (2010), that larvae reared under low density showed lower survival than high density reared larvae, however low density reared larvae grow bigger than high density reared ones and Millán-Cubillo *et al.,* (2016) had that the effects of stocking density is clearly size and/or age-dependent in this specie. Nevertheless meagre larvae survival was similar when compared with other studies with similar rearing techniques (Saavedra et al., 2016), between $24.2 \pm 1.0\%$ and $26.4 \pm 0.7\%$, and higher than in Saavedra et al., (2015a), with a survival of $16.06 \pm 1.53\%$.

The water quality analysis (Figure 18 and 34) show that all variations in the nutrients concentrations stay below the standard values recommended, meaning that a good water renovation was performed during both trials.

In malformations analysis, with 60 larvae per treatment observed, the differences between treatments, in both species, are not significant. The highest percentages of malformed fish, in gilthead seabream rearing (Figure 22), was in treatment FAST61/22, with $44 \pm 10\%$, right fallowed by treatment COOM ($41 \pm 16\%$). Different than in meagre rearing (Figure 37), were treatment FAST64/16 had the highest mean percentage, with $38 \pm 9\%$. The lowest percentages of malformed fish were observed in treatment FAST64/16 had ($32 \pm 19\%$), in gilthead seabream larvae, and treatment COMM ($28 \pm 2.3\%$), in meagre larvae. Bone abnormalities are frequent in fish larviculture, can be severe and affect the quality of the fish or slight and not morphologically obvious. The detection of such abnormalities can be performed at very early developmental stages, thus allowing the aquaculturists to cost-effectively terminate the culture of larval populations with potentially high incidence of osteological malformations (Koumoundouros *et al.,* 1997).

The percentage of malformations severity levels (Table VI) mean percentages are represented in Figure 23 and 38**,** for seabream and meagre fish, respectively. Few malformations with severity level 1 were observed on both species. In treatments COMM and FAST61/22, the percentage of malformations with severity level 2 was superior,

followed by malformations with severity level 3, in both trials. In treatment FAST64/16 of gilthead seabream trial a superior percentage of malformations with severity 4 is found, caused by larvae with a sever lordosis in the pre-haemal area. According with Georgakopolou *et al.* (2010), the effect of water temperature on the development of haemal lordosis in *S. aurata* is significant, and also suggests that the combined effects of water currents and water temperature in pre- and post-metamorphosis could be the causative factors of haemal lordosis. Temperature remains constant during the trials, so water currents could not be the adequate for every larvae, since some larvae grows faster than others in the same tank. To avoid this problem, hatcheries can perform a selection by size during trials at selective ages.

The mean values of malformations per fish (Figure 25 and 40) were very similar between treatments in both species, where many of the analyzed malformed fish only have one or two malformations.

The mean percentage of malformations per anatomic area of the fishes is represented in Figure 26 and 41, for seabream and meagre, respectively. In both species, in general, the cephalic area is the less affected, followed by the caudal are, and the pre-haemal and haemal areas are more affected by malformations. Differing from the fact that during the intensive rearing of gilthead seabream, opercular deformities are the most commonly observed type of deformation (affecting up to 80% of the fisheries stock) (Verhaegen *et al.,* (2007), and, on the other hand, the fact that the caudal region is more susceptible than other regions to malformations, not been documented in seabass and other teleosts (Boglione *et al.,* 2001). But according with the study made by Gisbert *et al*. (2012), using juvenile meagre specimens radiography's images, by X-ray analysis, observing that was a certain regional variation in vertebral morphology along the spine, especially in the haemal region.

Skeletal deformities, such as those affecting neurocranium or head, vertebral column and appendicular skeleton, are the most significant deformities since they affect directly to production traits. Lordosis, scoliosis, kyphosis and vertebral fusion are the most frequent vertebral column deformities (reviewed by Boglione *et al.*, 2013) and affect fish appearance, but, in addition, they also lead to physiological alterations that result in a decrease of fish commercial traits value, a lower growth rate, a higher mortality during handling and an increased difficulty of filleting. That is the reason by which deformed

fish elimination prior to batch commercialization must be performed, significantly increasing the production costs (Negrín-Báez *et al.,* 2015). Andrades *et al.* (1996) showed that only a small fraction of gilthead seabream larvae affected by skeletal (lordotic) malformation can survive after the completion of larval development, leading to significant loss of money for the hatchery.

The effect of the malformations on body appearance (Figure 24 and 39, for gilthead seabream and meagre, respectively), is very important since deformities in commercially raised fish are a common source of downgrading of product value (Verhaegen *et al.*, 2007; Darias *et al*., 2011; Negrín-Báez *et al*., 2015). Others studies also affirm that skeletal deformities are a significant quality issue in the hatchery production of *S. aurata*, with a variety of different deformity types recorded under experimental or production scales (Andrades *et al.,* 1996; Koumoundouros *et al.,* 1997; Afonso *et al.,* 2000; Boglione *et al*., 2001; Verhaegen *et al.,* 2007; Fernandez *et al.,* 2008), in the hatchery production of *A. regius* few information is still known.

In this study the higher mean percentages where the phenotype of the fish was affected were in treatment FAST64/16 in both trials (61 ± 35.2%, 42 ± 12%, for seabream and meagre, respectively), however the differences between the treatments of meagre trial, were little. In gilthead seabream trial the lowest mean percentages of fish where the phenotype was affected, were in treatment FAST61/22 (34 ± 16.9%). These results suggest that microdiets with higher lipid content, when compared to what is currently available in the market, may decrease severe skeletal deformities in gilthead seabream fishes. These results also go in accordance with the study made by Cahu *et al.,* (2003), that suggest that among the different causative factors, larval nutrition have a key role in skeletogenesis. The knowledge of larval nutritional needs in the fish farming industry is limited due to the fast changing needs of the larval requirements during ontogeny (Darias *et al.,* 2011). Another important aspect that should be taken into account is the variety in ontogeny, feeding physiology and behavior even within the same family. Thus, species-specific findings for a process or function in a model species cannot be extrapolated directly to other teleosts and specific validation studies are essential (Rønnestad *et al.* 2013).

Meagre is a species of carnivorous marine fish, so the requirement of omega-3 fatty acids has to be higher than in other freshwater species (Emre, *et al.*, 2016), also high

protein requirement of meagre larvae was previously described by Saavedra *et al.,* (2016).

Production of meagre is carried using adapted rearing and feeding protocols of seabream, and in order to maximize survival and growth of meagre it is crucial to formulate specific and suitable diets. Due to economic considerations, diets for cultured species often have varying proportions of plant-based ingredients, although such feeds are effective for rising omnivorous and herbivorous species, they have provided limited success for carnivores, and this has been attributed to digestive limitations (Buddington *et al.*, 1997). Gilthead seabream larvae already exhibited differing capacities to break down different types of capsule in their guts, shown in the study made by Fernández-Diaz and Yúfera (1995), and add that there was also considerable individual variability within a single population feeding.

In conclusion, in the rearing of gilthead seabream the best growth and survival was observed with the microdiet FAST61/22, however this treatment also reveal highest percentage of malformed fish, while treatment FAST64/16 showed lowest percentage of malformed fish, not having statistical differences (in malformations analysis) between treatments. These results suggest that gilthead seabream require microdiets with higher lipid content, and possibly also higher protein content, when compared to what is currently available in the market.

In the rearing of meagre the best growth, survival was also observed with the microdiet FAST61/22. The larvae quality analysis not reveals statistical differences, however treatments FAST61/22 and COMM showed the lowest percentages of malformed fish. These results suggest that meagre have higher nutritional requirements compared to slower growing species, and require microdiets rich in both protein and lipids.

For future studies is recommended the used of microdiet FAST61/22, since in these experiments gives to the larvae the best growth performance and larval development. While meagre seem to require higher dietary protein and lipid, due to their much fast growth, for gilthead seabream a higher dietary lipid may be sufficient to guarantee maximum performance.

The requirements for microdiets with higher lipid content may be associated with a higher requirement for DHA as also mentioned by Vallés and Estévez (2015), important nutritionally (Das, 2006) and/or energy, mainly obtained from fish oils.

Hamre *et al*. (2013) affirms that are a urgent need for detailed studies on nutritional requirements in fish larvae This study showed that is possible to improve larval development through more nutritional microdiets, however more studies are necessary to achieve the right amount of protein:lipid ratio, as other nutrients for the larvae.

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**Annex A** Concentrations (mg/L) of $NH_4$, $NO^3$, $NO^2$, $Si(OH)_4$, $HPO_4^{2-}$, in gilthead seabream (*Sparus aurata*) rearing, between the different treatments (COMM, FAST64/16 and FAST61/22), at different ages (36, 43 and 46).

| Age (DAH) | Treatment | $NH_4$ | $NO^3$ | $NO^2$ | $Si(OH)_4$ | $HPO_4^{2-}$ |
|---|---|---|---|---|---|---|
| | | mg/L | | | | |
| 36 | COMM | 0,0013 | 0,0010 | 0,0035 | 0,0428 | 0,0189 |
| | FAST64/16 | 0,0015 | 0,0011 | 0,0028 | 0,0392 | 0,0198 |
| | FAST61/22 | 0,0258 | 0,0010 | 0,0035 | 0,0353 | 0,0220 |
| 43 | COMM | 0,1513 | 0,0011 | 0,0043 | 0,0370 | 0,0208 |
| | FAST64/16 | 0,0097 | 0,0010 | 0,0043 | 0,0482 | 0,0192 |
| | FAST61/22 | 0,0113 | 0,0011 | 0,0038 | 0,0451 | 0,0257 |
| 46 | COMM | 0,0088 | 0,0010 | 0,0038 | 0,0300 | 0,0316 |
| | COMM | 0,0141 | 0,0008 | 0,0036 | 0,0260 | 0,0211 |
| | FAST64/16 | 0,0062 | 0,0007 | 0,0042 | 0,0269 | 0,0171 |

**Annex B** Concentrations (mg/L) of $NH_4$, $NO^3$, $NO^2$, $Si(OH)_4$, $HPO_4^{2-}$, in meagre (*Argyrosomus regius*) rearing, between the different treatments (COMM, FAST64/16 and FAST61/22), at different ages (27, 34, 39 and 46), in triplicate.

| Age (DAH) | Treatment | $NH_4$ | $NO^3$ | $NO^2$ | $Si(OH)_4$ | $HPO_4^{2-}$ |
|---|---|---|---|---|---|---|
| | | | | mg/L | | |
| 27 | COOM | 0,00112 | 0,00014 | 0,00518 | 0,0776 | 0,02666 |
| | COOM | 0,00714 | 0,00014 | 0,0105 | 0,1061 | 0,02945 |
| | COOM | 0,01414 | 0,0007 | 0,00546 | 0,0846 | 0,02883 |
| | FAST64/16 | 0,00126 | 0,00014 | 0,00588 | 0,0854 | 0,02945 |
| | FAST64/16 | 0,01834 | 0,00014 | 0,0091 | 0,0633 | 0,03565 |
| | FAST64/16 | 0,25928 | 0,00098 | 0,00588 | 0,0868 | 0,07843 |
| | FAST61/22 | 0,04214 | 0,00014 | 0,00924 | 0,0991 | 0,03193 |
| | FAST61/22 | 0,0322 | 0,00014 | 0,0077 | 0,0776 | 0,03534 |
| | FAST61/22 | 0,00308 | 0,00028 | 0,0063 | 0,1324 | 0,03038 |
| 34 | COOM | 0,01792 | 0,0007 | 0,00616 | 0,0711 | 0,03162 |
| | COOM | 0,18326 | 0,00084 | 0,00616 | 0,0526 | 0,03162 |
| | COOM | 0,09016 | 0,00112 | 0,00644 | 0,0532 | 0,01674 |
| | FAST64/16 | 0,05376 | 0,00098 | 0,00574 | 0,0557 | 0,04402 |
| | FAST64/16 | 0,007 | 0,00056 | 0,00588 | 0,0526 | 0,02511 |
| | FAST64/16 | 0,11354 | 0,00084 | 0,00616 | 0,0493 | 0,02976 |
| | FAST61/22 | 0,01106 | 0,00056 | 0,00532 | 0,0496 | 0,02883 |
| | FAST61/22 | 0,01064 | 0,0007 | 0,00616 | 0,0571 | 0,04154 |
| | FAST61/22 | 0,0084 | 0,00182 | 0,00756 | 0,0862 | 0,04991 |
| 39 | COOM | 0,07518 | 0,00098 | 0,00812 | 0,0666 | 0,02945 |
| | COOM | 0,10668 | 0,00126 | 0,0091 | 0,0750 | 0,03999 |
| | FAST64/16 | 0,09366 | 0,00098 | 0,0084 | 0,0734 | 0,04154 |
| | FAST64/16 | 0,1155 | 0,00084 | 0,0091 | 0,0706 | 0,04185 |
| | FAST61/22 | 0,06412 | 0,00084 | 0,00798 | 0,0658 | 0,03038 |
| | FAST61/22 | 0,06384 | 0,00098 | 0,00868 | 0,0652 | 0,03131 |
| 46 | COOM | 0,0889 | 0,00126 | 0,0112 | 0,1249 | 0,02573 |
| | COOM | 0,09982 | 0,0014 | 0,01078 | 0,0868 | 0,02263 |
| | COOM | 0,09044 | 0,00112 | 0,01148 | 0,1002 | 0,02449 |
| | FAST64/16 | 0,14756 | 0,00126 | 0,01022 | 0,0846 | 0,02914 |
| | FAST64/16 | 0,11466 | 0,0014 | 0,01218 | 0,0994 | 0,02666 |
| | FAST64/16 | 0,09156 | 0,00112 | 0,01092 | 0,0829 | 0,02356 |
| | FAST61/22 | 0,08568 | 0,00196 | 0,0105 | 0,0949 | 0,01612 |
| | FAST61/22 | 0,0819 | 0,00126 | 0,01106 | 0,0952 | 0,01612 |
| | FAST61/22 | 0,06146 | 0,0014 | 0,01078 | 0,0899 | 0,02015 |