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IMPROVEMENT ON THE NUTRITIONAL QUALITY OF LARVAL FEEDS FOR GILTHEAD SEABREAM: ROTIFER FEEDS AND EARLY WEANING DIETS

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LIST OF ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
ANOVA	One-way analysis of variance
ARA	Arachidonic acid
BHT	Butylated hydroxytoluene
Ca	Calcium
CAT	Catalase
CCAP	The Culture Collection Algae and Protozoa
Cu	Copper
Cu/ZnSOD	Copper/zinc superoxide dismutase
DEPC	Diethyl pyrocarbonate
DPH	Days post hatched
DHA	Docosahexaenoic acid
EAA	Essential amino acids
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization
FM	Fish meal
FN	Fresh *Nannochloropsis oculata*
GALT	Gut-associated lymphoid tissue
GIA	Grupo de Investigación en Acuicultura
GI	Gastrointestinal tract
GPX	Glutathione peroxidase

HUFA	Highly unsaturated fatty acids
I	Iodine
INBY	Inactive Baker's Yeast
IUSA	Instituto Universitario de Sanidad Animal y Seguridad Alimentaria
LA	Linoleic acid
LNA	α-linoenic acid
Mn	Manganese
MnSOD	Manganese superoxide dismutase
MOS	Mannan oligosachharides
OC	Osteocalcin
P	Phosphorus
PAS	Periodic acid–Schiff
PL	Phospholipids
PP	Protein Plus
PUFA	Polyunsaturated fatty acids
SBM	Soybean meal
SDN	Spray-dried *Nannochloropsis oculata*
Se	Selenium
SOD	Superoxide dismutase
ULPGC	University of Las Palmas de Gran Canaria
Zn	Zinc

Chapter 1: Introduction

1. CURRENT STATUS OF AQUACULTURE

Aquaculture is an indispensable food production sector with the faster worldwide growth among both marine and inland food production systems. In fact, Aquaculture grows three times faster than agriculture, with an amazing rate of 8.3% per year from 1970 to 2006 (Diana, 2009). In the last decades, it has been growing at a rate higher than 5% per year (Izquierdo, 2005), with a global total aquaculture production that increased from 0.6 million metric tons in 1950 to 80 million tons in 2016 raising annual total world food fish production to 171 million tons (FAO, 2018) (Figure 1) (Table 1.1).

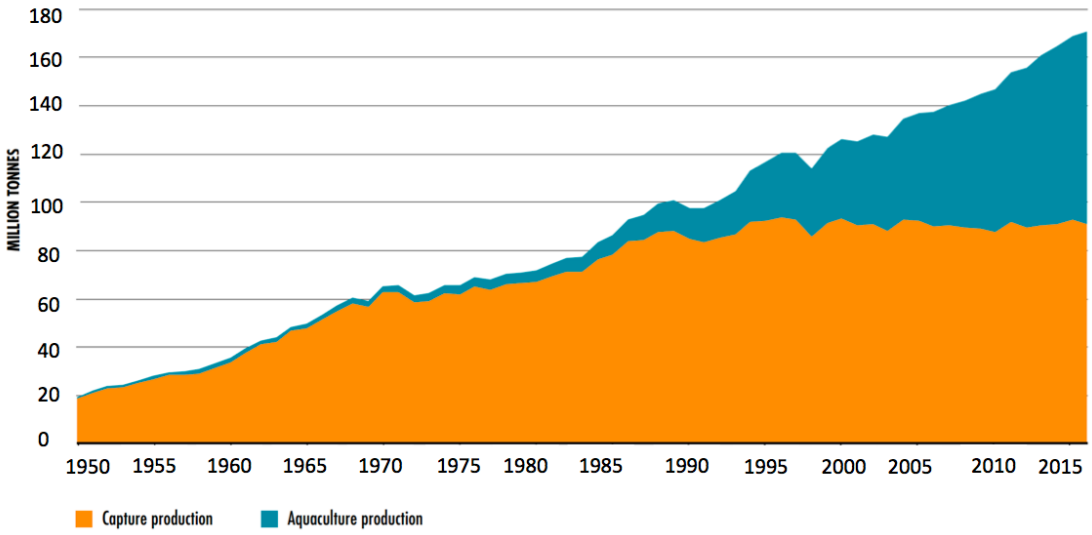

Figure 1. World captured fisheries and aquaculture production (FAO, 2018).

Table 1.1 World fisheries and aquaculture (fish) production (million tons) (from FAO, 2018)

Capture	2011	2012	2013	2014	2015	2016
Inland	10.7	11.2	11.2	11.3	11.4	11.6
Marine	81.5	78.4	79.4	79.9	81.2	79.3
Total Capture	92.2	89.5	90.6	91.2	92.7	90.9
Aquaculture						
Inland	38.6	42.0	44.8	46.9	48.6	51.4
Marine	23.2	24.4	25.4	26.8	27.5	28.7
Total aquaculture	61.8	66.4	70.2	73.7	76.1	80.0
Total World Fisheries	154.0	156.0	160.7	164.9	168.7	170.9

Worldwide, the growth of inland aquaculture outpaces mariculture and accounts for nearly 58% of aquaculture food fish production, likely reflecting its ease in development without a complex infrastructure (FAO, 2018). Crustacean aquaculture occurs in inland and mariculture environments. Crustaceans account for less than 10% of production by weight; nevertheless, their ratio of total by economic value is more than twice that amount, at 22.4% of the total. The economic input of world aquaculture was estimated at U.S. $138 billion (FAO, 2018) (Table 1.2).

Table 1.2 Aquaculture production by continents (data from FAO, Fishstat 2018)

	2000	**2002**	**2004**	**2006**	**2008**	**2010**	**2012**	**2014**	**2015**
Africa	400	453	559	755	942	1.286	1.484	1.711	1.772
Americas	1.423	1.801	2.143	2.369	2.470	2.514	2.990	3.347	3.273
Asia	28.422	32.361	36.895	41.780	47.001	52.452	58.956	65.506	68.393
Europe	2.051	2.043	2.173	2.193	2.327	2.523	2.827	2.929	2.975
Oceania	121	128	139	161	175	190	186	189	186
World	32.418	36.786	41.909	47.257	52.915	58.964	66.443	73.681	76.600

Aquaculture sector still has some problems that must be solved for sustainable production. Control of fish reproduction, rearing conditions, diet formulations and alternative feed ingredients for substitution fish meal and fish oil, all can lead to a more stabilized sector and promote a faster development at land and sea based aquaculture facilities (Figure 2, 3).

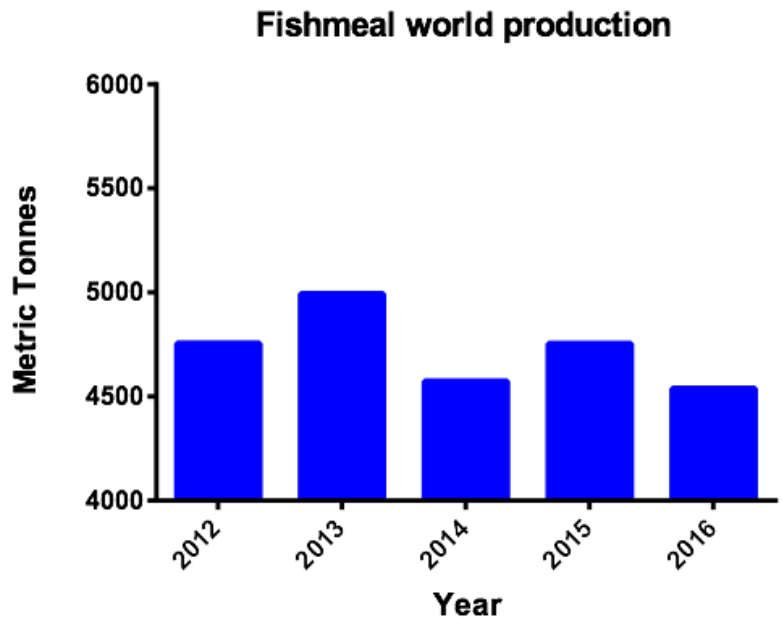

Figure 2. World fishmeal production (Source: Fishmeal and Fishoil. A summary of global trends. IFFO October 2017).

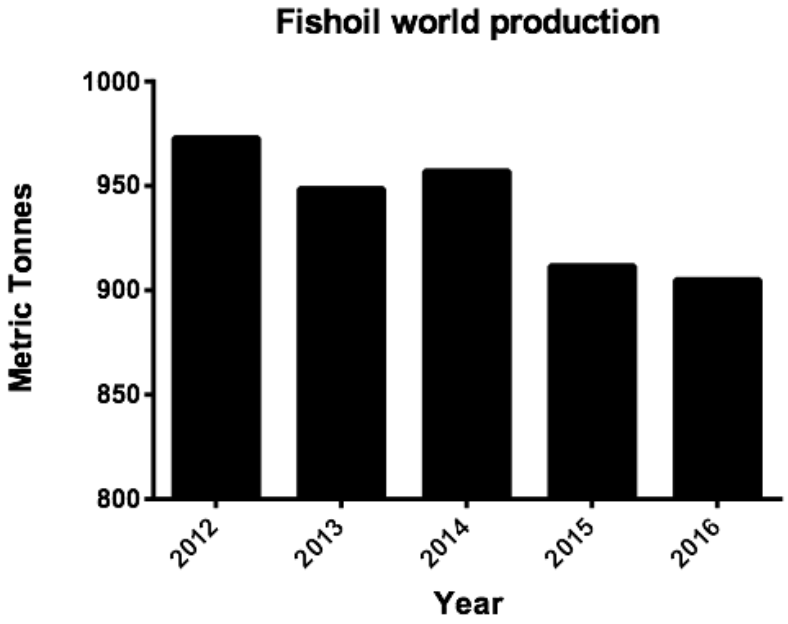

Figure 3. World fishoil production (Source: Fishmeal and Fishoil. A summary of global trends. IFFO October 2017).

The future success of the aquaculture sector is closely related to the increasing demand of food from marine origin that make this activity very attractive for

investors, as well as an alternative to other labor activities such as wild captures. However, the production success of marine fish species is limited by the quality and number of productions of fry (Watanabe *et al*., 1982; Yúfera *et al.*, 1999; Sargent *et al*., 1997; Izquierdo *et al*., 2000). Therefore, high survival rate should be obtained from larvae production to on-growing period. In order to execute this objective, the goals must be focused on: first of all, the improvement of management practices to create more efficient and diverse systems at every level of production intensity; secondly, an emphasis on local decision-making, human capacity development and collective action to generate productive aquaculture systems that fit into societal constraints and demands; thirdly, the development of risk management tools for all systems to reduce disease problems, eliminate antibiotic and drug and avoid invasive organism introduction into local waters; and last, the creation of systems to better differentiate and promote a more sustainably grown aquaculture products in the market and to individual consumers (Diana, 2009).

1.2 Importance of larval rearing for Mediterranean fish species

Gilthead seabream (*Sparus aurata)* and European sea bass (*Dicentrarchus labrax*) are still the most cultivated marine fish species in Europe, supporting the growth of European marine aquaculture over the last three decades (FEAP, 2015; Izquierdo, 2005). Demand for good quality fry has been increasing at a rate of 10% annually and the European annual fry production of seabream and sea bass respectively reached 599.9 and 492.0 millions in 2014 (FEAP, 2015).

The improved development of fry production is essentially affected by the effectiveness of first feeding regimes and the high nutritional quality of early weaning diets (Kolkovski *et al*., 1993; Sargent *et al*., 1997; Izquierdo *et al*., 2000). Rearing of marine fish larvae implies the administration of external nutrients by feeding firstly live preys for some days followed by early weaning diets. Nevertheless, both live preys and microdiets should meet the nutritional requirements of each species to ensure the best growth and survival rates (Kolkovski *et al*., 1993; Sargent *et al*., 1997).

Quality fish production can be only achieved by fulfilling nutritional requirements of

fish. Marine fish larvae require essential fatty acids (EFA), protein, vitamins and minerals after hatching. At present, in marine fish species, this can only be managed by first utilization of live preys. However these feeds are not able to completely covert high nutritional requirements of the fast growing first feeding larvae and, therefore, enrichment procedure of live preys should be done before they are used. Fish producers need to introduce microparticulate feeds as soon as possible in order to stabilize nutrition quality. Unfortunately, first feeding larvae are not capable of ingest and digest inert feeds.

Several strains of the rotifer *Brachionus plicatilis* constitute the first live prey fed to gilthead seabream due to its adequate size (90-130 μm). They are followed by Artemia *(*450-500-750 μm) and co-feeding with early weaning diets to fit larval mouth size. However, the extra effort in terms of manpower, infrastructure, time and energy to produce rotifers and Artemia species represents a significant cost (Kolkovski *et al.*, 2009). Moreover, the nutritional value of live preys varies notably according to its feed and environmental conditions such as light intensity and feeding regimes, temperature, salinity, etc., risking the optimal nutrition of the larvae (Jones *et al*., 1993; Kolkovski *et al*., 1993; Barnabé and Guissi, 1994; Rosenlund *et al*., 1997). Besides, from an experimental point of view, effective live prey enrichment is limited to few nutrients such as phospholipids (PL) or proteins (Rosenlund *et al*., 1997; Koven *et al*., 2001). For all these reasons, substitution of live prey by compound microdiets is crucial for sustaining cost effective production of high and constant quality juveniles. During the past three decades, extensive efforts have been made to develop microdiets as a complete or partial substitution for both rotifers and Artemia. The majority of the studies have focused on the larvae's nutritional requirements. Despite substantial achievements, complete replacement of live feeds at first feeding is not yet feasible for most marine species. A lower performance is commonly reported when inert diets are fed to larvae from the onset of exogenous feeding. This may be due to the composition, palatability or physical characteristics of dry feed (Person Le Ruyet *et al*., 1993). In addition to diet nutritional quality, important physical characteristics of diet particles within the water column such as sinking rates, nutritional stability, leaching rates and leachate profiles all contribute to the microdiets attractiveness that influence ingestion and digestion rates and can greatly affect larvae growth (Hamre *et al*., 2013).

Surprisingly, these factors have received very little attention. The production of microdiets could be a convenient and economic alternative to live feed, in spite of the problems of nutrient leaching and water stability normally encountered when producing microparticles (Baskerville-Bridges and Kling, 2000; Önal and Landgon, 2000; Pousão-Ferreira *et al.*, 2003). Therefore, it is important taking into account the physiology and ontogeny of the larval digestive system, feeding regime and technology as well as each species nutritional requirements in the process of microdiets preparation for marine fish larvae. In the last years, various studies have been conducted to determine the optimum lipid composition in early weaning diets for marine fish larvae, where more attention has been paid to study the highly unsaturated fatty acids (HUFA) and phospholipids requirements and its role in marine fish larvae development (Watanabe 1982; Izquierdo, 1996; Takeuchi, 1997; Sargent *et al.,* 1999; Izquierdo *et al.*, 2000; Montero *et al*., 2003).

Another rising problem is increasing high demand of crude material of feed ingredients which are mainly fish meal and fish oil as protein and lipid sources, respectively. In order to substitute these ingredients, terrestial and marine sources have been used in replacement of fish meal and fish oil (Turchini *et al*., 2009). For sustainable aquaculture, feed ingredients must be continuously produced. In order to improve weaning diets, addition of essential ingredients such as immune stimulants, vitamin, antioxidants and minerals should be also considered.

1.3 Early weaning diets for marine fish larvae

Larval survival is the most important issue when sustainable and efficient production is considered. The dependence on the availability of seeds (fry ready to be stock for on-growing) is one of the critical factors for the commercial success of the industrial marine fish production (FAO, 2018). There are two important crucial points during the first stages of larval development:

a) The period from the endogenous to the exogenous larvae feeding, which starts with supplying of live feeds such as rotifers from 3 and 4 days after hatching.

b) The moment of weaning or the transition from the ingestion of the live food to the early weaning inert food.

Microalgae are the first step in the chain of marine food web and they are used as feed for zooplankton such as rotifers, Artemia and copepods. They are not only given as a first feed but also used as ‘’green-water technique’’ in larvae culture tanks. Addition microalgae in rearing water have beneficial effects such as increased growth and survival of marine fish larvae (Reitan *et al*., 1997). This method also reduces stress, improves prey capture and increases appetite of larvae with shading effect on water light intensity in the rearing tank. It is also reported that microalgae can promote digestive process in larvae gut when they are presented by rotifers (Naas *et al*. 1992).

Most marine fish species need very small food organisms for first feeding, mostly with a diameter between 25%-50% of their mouth size (Hunter, 1981). Rotifers (*Brachionus plicatilis*) match that description with a suitable size for first feeding of most marine fish larvae. Rotifer cultures are sustained with microalgae culture. The high density of rotifer culture requires high-priced equipment and labor-intensive management. Those reasons make rotifer culture vulnerable during production. Even though, since the rotifer cultivation was settled down (Ito, 1960), this organism has been widely used for the cultivation of larvae of several species of marine fish. The current global shortage of Artemia cysts has urge the necessity for early replacement diets and a growing range of commercial microparticulate feeds is now offered to partially replace Artemia and to enable earlier weaning of fry (Shields, 2001).

Copepods are other candidates as live preys for marine fish larvae. They have been showed to be nutritionally superior to Artemia and rotifer (Van der Meeren *et al*., 2007; Ajiboye *et al*., 2011; Drillet *et al*., 2011). The culture of copepods at a commercial scale is difficult due to their required specific natural environmental conditions, lack of technical knowledge and sensitivity in manipulation. For these difficulties in copepod production, development of formulated diets has become more crucial in order to replace or substitute live preys during larval feeding period. Formulated feeds lead to cost effectiveness, availability and consistency of nutritional quality. In marine fish commercial production, there is not any solution for total replacement of live feeds by formulated diets fro first feeding. This is the main problem for sustainable marine larvae and fry culture (Howell *et al*., 1998). Beyond all that, live preys should be enriched with some commercial products before they are used in larval feeding. Rotifer and Artemia lack different nutrients, including essential

fatty acids as prey for marine fish larvae (Watanabe *et al.,* 1982). Enrichment procedure depends on quantity of live preys and the commercial company.

According to Hamre *et al*. (2013), there is some lack of knowledge in the development of techniques for larval rearing, including:

I. The lack of knowledge about the optimal environmental conditions and feeding requirements during the early life stages.
II. The unknown of the optimal nutritional requirements, which are difficult to assess in the absence of a suitable rearing technology.
III. The changes in specific husbandry and feeding practices from one species to another due to ontogenic changes and growth.
IV. The small size of the larvae and the need for small particle sizes, which imposes specific problems on feed technology.
V. Improvement of weaning diets species specific with different ingredients.

Marine fish larvae are very vulnerable during the first stages of development and have strict requirements for biotic and abiotic conditions to survive, develop and grow. Most researchs on larval nutrition have contributed to the knowledge necessary to estimate the nutrient requirements of larval fish. However, it is not easy to quantify the nutritional requirements of larval fish. Obviously, a good knowledge of the larval nutritional requirements throughout the larval development would contribute to optimize diets and feeding protocols. As marine fish species undergo dramatic morphological and physiological changes, including metamorphosis, during ontogenesis, their nutritional requirements may differ qualitatively and quantitatively from larvae to juvenile or adult stages.

Moreover, fish larvae grow rapidly, feed continuously and therefore, total ingestion of nutrients must be high. The larval nutritional requirements for a particular nutrient can be defined from a physiological point of view as the nutrient intake needed to fulfil a physiological role, but is frequently defined as the "requirement for maximal growth and/or survival" where the relation fish-diet-feeding has an important effect in the determination of the quantitative needs (Izquierdo and Lall, 2004). Enormous efforts have been made to improve microdiets efficiency to replace live feed, both rotifers

and Artemia, as complete or partial replacements for marine fish larvae (Koven *et al*., 2001; Kolkovski and Sakakura, 2004; Kolkovski *et al.*, 2009). There have been substantial achievements in the earlier weaning of larvae onto microdiets and partial replacement of live feeds, but microdiets still cannot completely replace live feeds for most species. The partial substitution of live food has been proven to be successful. The formulated microdiets supply with the live food (co-feeding) to enable early weaning, for instance, up to 90% substitution of rotifers with artificial diets has been achieved for red seabream (*Pagrus major*) and Japanese flounder (*Paralichthys olivaceus*) (Kanazawa *et al*., 1989), and gilthead seabream larvae (Liu *et al.,* 2002). Although weaning the larvae from Artemia onto a microdiet can be achieved at metamorphosis in many species (Foscarini, 1988; Hardy, 1989; Koven *et al*., 2001; Curnow *et al*., 2006a,b), the early introduction of formulated diets for completely replacement of live food has limited success (Kanazawa *et al*., 1989; Walford *et al*., 1991; Yúfera *et al.*, 1997; Rosenlund *et al*., 1997; Kolkovski *et al*., 2009). The addition of Artemia nauplii combined with a microdiet, enhances the peristaltic movement of the larval digestive tract enhancing microdiet ingestion and production of digestive enzymes (Kolkovski *et al*., 1995). The design and formulation of these early weaning diets requires meeting the optimal nutritional requirements to fulfill the larval physiological needs (Kolkovski *et al.*, 2009). The efficiency of utilization of feed particles by marine fish larvae is affected by many external and internal factors (Kolkovski, 2001; Koven *et al*., 2001). Microdiets have range of chemical substances and they can be grouped into classes according to constitutions, properties and functions (Figure 4 and 5).

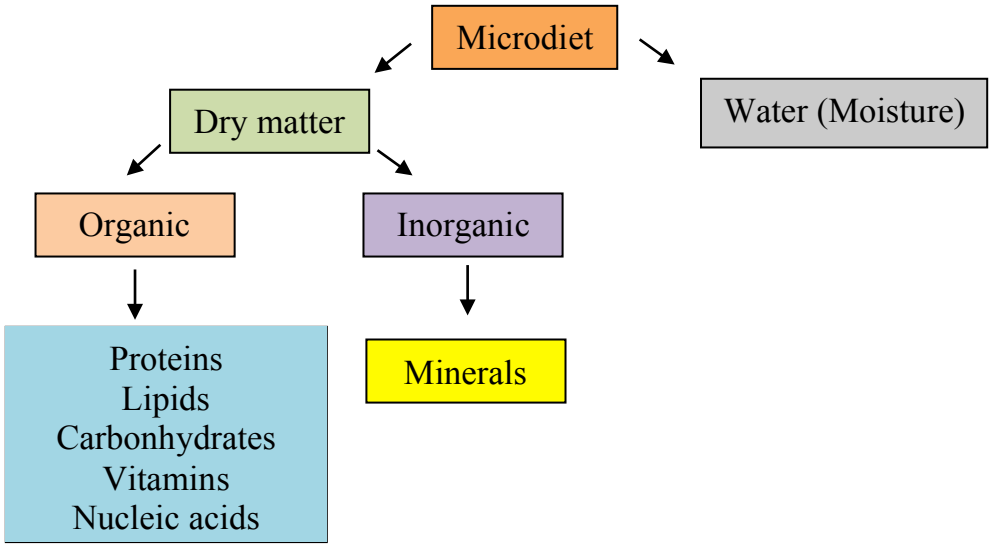

Figure 4. Major chemical components of fish feeds.

Microdiet should be acceptable and ingested in sufficient quantity by larvae, be properly digested and assimilated, meet all the nutritional requirements, be stable in water and not leach excessively (Howell *et al*., 1998). However, the studies of nutritional requirement measurements that use direct methods are scarce due to the difficulty of designing experimental diets with full control of nutritional composition and environmental factors in all the experimental tanks. Formulated diets have variable leakage and stability problems resulting in an unknown difference in nutrient composition between the formulated and ingested diet.

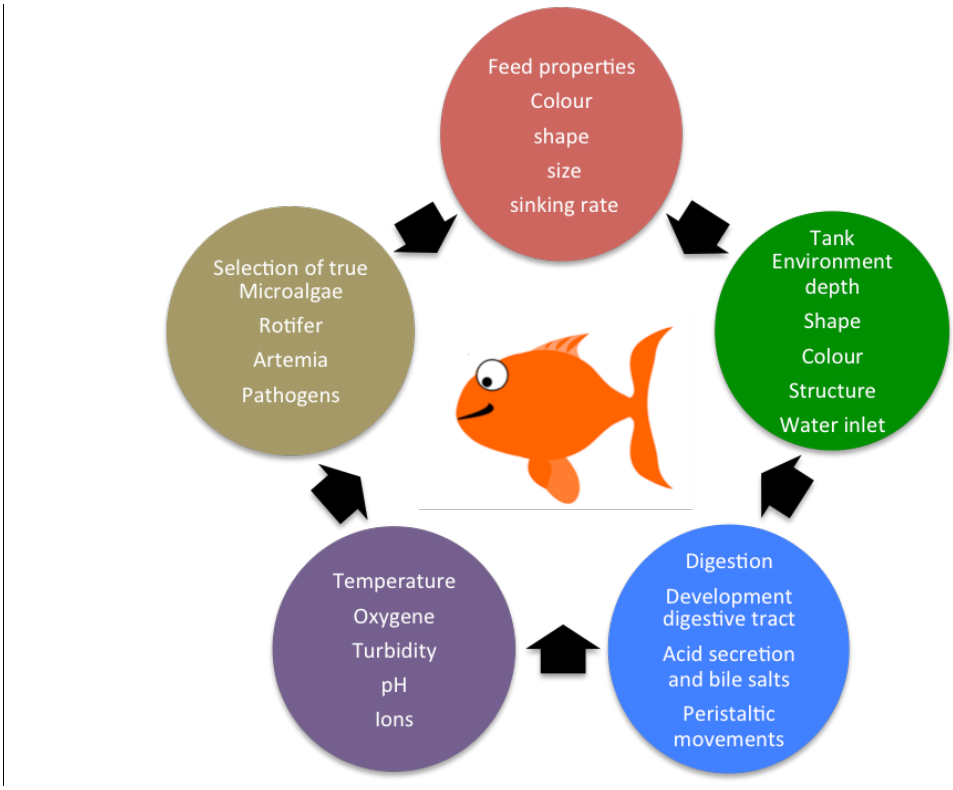

Figure 5. Factors affecting food particle utilization (Adapted from Kestemont and Baras, 2001; Kolkovski *et al*., 2009).

According to Kolkovski *et al*. (2009), microparticulated diets can be classified in three general types:

I. Microbound type is the simplest microdiet, where the powdered ingredients are microbound with a water stable matrix such as agar or carrageenan (López-Alvarado *et al*., 1994).

II. Microcoated type, its manufacturing process relied on coating the powdered ingredients with a glucidic (carraggeenan, alginate), proteic (gelatin, zein) or lipid binder to reduce leaching (Önal and Langdon, 2004).

III. Microencapsulated diets are made by encapsulating a solution, colloid or suspension of diet ingredients within a membrane, and it is produced with a cross linking agent (Yúfera *et al*., 1999; Kolkovski, 2009).

1.4 Important ingredients in microdiets for marine fish larvae

The important role of lipids, in particularly HUFA (long chain pollyunsaturated fatty acids), during larval development has been focused of many studies (Watanabe *et al*., 1982, Izquierdo, 1996). HUFA requirements of larvae can be summarized as (i) Fish

have to be fed n-3 PUFA and n-6 PUFA, (ii) freshwater fish are generally capable of converting 18:3n-3 and 18:2n-6 to longer HUFA such as 22:6n-3 and 20:4n-6. For that freshwater absolute need these 18C PUFA in their diet, (iii) the marine fish are not able to convert those 18C PUFA to longer HUFA. Therefore, marine fish larvae totally need HUFA such as DHA, EPA and ARA in their diet.

Tocopherols, caratenoids and ascorbic acids are main antioxidants used in diet. The vitamin E-type compounds, are not synthesized by animals and must be included and obtained from the diet, ultimately from higher plant and algal sources (Hess, 1993). Therefore, antioxidants should be included in diets along with elevating high lipid content. The dietary *n*-3 HUFA levels should be existed in diets. This occasion lead to increase unsaturation index of the diet. Therefore, it must be balanced by increasing dietary antioxidant content. It has been shown in gilthead sea bream, that the increase in dietary Vitamin E further improved growth in relation to the dietary n-3 HUFA levels denoting its protective role against oxidation (Saleh *et al*., 2014).

Carotenoid pigments occurred in the eggs of fish species, with pigment being mobilized from the flesh of salmonids and deposited in the ovarian tissues during sexual maturation (Torrisen *et al*., 1995; Hatlen *et al*., 1997). Among them, *β*-carotene, astaxanthin and canthaxanthin contribute to the antioxidant effect of Vitamine E (Bell *et al*., 2000). Similarly, selenium contributes to the antioxidant effect of Vitamine E and (Bell *et al*., 1985). According to those findings, mix utilization of antioxidants in diets is more efficient (Betancor *et al*., 2012).

Number of produced marine fish larvae is limited in aquaculture industry because of the larvae are very poorly developed when they are hatched out. One of the main reasons why microdiets fail to be as good as live preys for larvae is that feeding microdiets delays gut development and essential minerals would be important to skeletal deformities and modulation of gene expression (Kolkovski *et al.*, 2009; Izquierdo et al., 2015). Therefore, essential feed ingredients for health and quality should be established in fish larvae. Mineral studies in fish nutrition at larval stage are very limited. Therefore, determination of essential minerals in weaning diets at gilthead sea bream larvae can lead to open new perspective for alternative marine fish species.

Minerals can be defined as homogeneous inorganic substances. Fish body requires seven essential minerals such as calcium, sodium, manganese, potassium, phosphorus, chlorine and sulphur in large amounts. Other important minerals are cobalt, copper, iron, iodine, selenium, zinc and molybdenum in trace amount (Osborne and Voogt, 1978). The major minerals play important roles as structural components of tissues, cellular metabolism, osmoregulation, or acid-base balance (Jobling, 1998). It should be noted that fish cannot only take minerals through the diets but also are capable of absorbing some dissolved minerals from sea water (Lall, 2002).

Minerals include macrominerals such as sodium (Na), potassium (K), Magnesium (Mg) and Calcium (Ca) which are utilized in diets more than one gram amount; as well as microminerals are such as manganese (Mn), selenium (Se), zinc (Zn), iron (Fe) and copper (Cu) ,which very important for cellular metabolism in order to promote health and sustainable growth (Watanabe *et al.*, 1997; NRC, 2011).

Zn is an important mineral involved in enzyme functioning that catalyzes different metabolic pathways related to growth, reproduction, tissue development, vision or immunity (Watanabe *et al.,* 1997). Metabolism of carbohydrates, lipids and proteins is directly related to Zn-chained enzymes such as carbonic anhydrase, alkaline phosphatase or carboxypeptidase A. Zn also plays important roles in prevention of oxidative stress and reducing cellular damage (Watanabe *et al.*, 1997; Halver and Hardy, 2002).

Mn utilization in diet enhances growth, reproduction and prevention of skeletal abnormalities in terrestrial animals and fish. Mn is part of the many enzymes such as Mn superoxidase dismutase, pyruvate carboxylase and lack of this essential minerals lead to reduce growth in fish (Leach, 1976). Enrichment of live feeds with Mn reduces the incidence of bone anomalies gilthead sea bream larvae fed those live feeds (Nguyen *et al.*, 2008).

Se has antioxidant role in fish nutrition and appropriate level of Se in diets has positive effect on reproduction and larval development (Hamre *et al.*, 2008; Pacitti *et al.*, 2013). Se deficiency in diet resulted in low survival and growth (Le and Fotedar, 2013; Saleh *et al.*, 2014).

Cu is another important micromineral that plays essential functions in vital organs such as heart, eye, brain, liver or bone (Watanabe *et al.,* 1997; Lall and Lewis-McCrea, 2007), whereas proportion of copper should be well adjusted in diet since may negatively affect growth (Lin *et al.*, 2010) and cause toxicity that may induce necrosis in vital organs (Murai *et al.,* 1981; Woody and O'Neal, 2012). Deficiency of Cu may lead to Cu reduction in tissues and low growth (Tang *et al*., 2013). However, its importance in larval diets has not been sufficiently addressed.

Additionally, prebiotics are non-digestible food ingredients that beneficially affect the host by stimulating growth and/or health and promoting beneficial bacteria population in the gastrointestinal tract (GI) microbiota of poultry (Patterson & Burkholder 2003), terrestrial livestock (Flickinger *et al.*, 2003) and humans (Gibson & Roberfroid 1995). For instance, mannan oligosachharides (MOS) may be highly important for marine fish nutrition. Probiotic products have been studied mainly in juvenile and adult fish trials (Torrecillas *et al.*, 2007; Torrecillas *et al.*, 2011; Torrecillas *et al*., 2013), but few studies have been able to evaluate in larval weaning diets. These aspects have not been studied and no information has been published about the effect of microalgae, immune stimulants and minerals on gilthead seabream larvae.

OBJECTIVES

IMPROVEMENT ON THE NUTRITIONAL QUALITY OF LARVAL FEEDS FOR GILTHEAD SEABREAM: ROTIFER FEEDS AND EARLY WEANING DIETS

Since adequate larval nutrition still constitutes one of the main bottlenecks for the reliable production of sufficient amounts of high quality gilthead sea bream juveniles, the main aim of this thesis was to improve the nutritional quality of larval feeds. To achieve this aim the thesis targeted two well differentiated and critical phases of seabream larval production: first feeding and weaning to dry diets. Since rotifers must be used during first feeding of sea bream larvae, the first specific aim was to study the effect of different rotifers feeds on rotifer production and its nutritional quality under the point of view of their biochemical composition. For improvement of the nutritional quality of weaning diets the inclusion of microalgae rich in essential fatty acids for marine fish larvae, as alternatives to fish oil were considered, together with the supplementation of probiotics or minerals.

Therefore, to achieve the main goal the following objectives were formulated:

1- To evaluate the effect of different commercial rotifer feeds on growth performance, fatty acid and amino acid composition of rotifers (Chapter 3).

2- To determine the nutritional value of single cell oils (microalgae oils) rich in DHA ARA or EPA as replacements to fish oil in early weaning diets for gilthead sea bream larvae (Chapters 4 and 5).

3- To determine the potential positive effect of mana oligosaccharides addition into early weaning diets on gilthead sea bream larvae growth performance, fatty acid composition, stress resistance and intestinal morphology.

4- To evaluate the effect of inclusion of different minerals in early weaning diets for seabream (*Sparus aurata*) larvae on growth performance, mineral deposition and molecular markers of oxidative status and bone development.

CHAPTER 2: MATERIALS AND METHODS

2.1. LARVAE

2.1.1. Gilthead seabream larvae

Gilthead seabream larvae were obtained from natural spawnings from Grupo de Investigación en Acuicultura (GIA, Las Palmas de Gran Canaria, Spain). Larvae previously fed rotifers (*Brachinous plicatilis*) enriched with DHA Protein Selco® (INVE, Dendermond, Belgium) until 16 dph, were randomly distributed in experimental tanks at a density of 2100 larvae $tank^{-1}$ and fed one of the diets tested in triplicate.

2.2. EXPERIMENTAL CONDITIONS

The experimental tanks had a volume of 200 L and a cylinder shape with a conical bottom and were made of light grey color painted fiberglass. Tanks were supplied with filtered seawater (37 ppm salinity) at an increasing rate of 0.4-1.0 L min^{-1} to assure good water quality during the entire trial. Water entered from the tank bottom and exited from the top to ensure water renewal and maintain high water quality. Temperature, pH and water quality were daily tested. Seawater was continuously aerated (125 ml min^{-1}). Photoperiod was kept at 12h light: 12h dark, by fluorescent daylights that provided a light intensity of 1700 lux (digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia).

2.3. DIETS AND FEEDING

2.3.1. Rotifers

Rotifers (*Brachionus plicatilis*) were produced in 1700 L cylindrical containers provided with a central aeration through a porous stone. The culture medium included 80% seawater and 20% freshwater. Rotifers from the *Brachionus plicatilis* strain S-1 (150-250 μm lorica length) were inoculated at an initial density of 100 ind ml^{-1}. They were fed daily with 0.4 g fresh yeast extract for 10^6 ind. divided in two doses. Every four days rotifers were carefully filtered through a 64-μm mesh, rinsed in water, the container washed and the culture re-suspended in clean water at culture densities of 100-250 ind. ml^{-1}. The rotifers fed to seabream larvae when green water was used in the larval rearing were previously enriched for 6 h at densities of 300 rotifers ml^{-1} with emulsified DHA Protein Selco® (INVE, Dendermond, Belgium) at a ration of 0.125 g l^{-1} ind. divided in two daily doses. Rotifers were used to feed seabream larvae during green water culture and during the first two days of trial as co-feeding with the

experimental microdiets.

2.3.2. Microdiets

2.3.2.1. Microdiets formulation

The experimental microdiets (pellet size 250-500 µm) included krill oil (Qrill, high PL, Aker BioMarine, Fjordalléen, Norway) or soybean lecithin (Acrofarma, Barcelona, Spain) as sources of PL. Vitamin E, in the tocopheryl acetate form, was obtained from Sigma-Aldrich (Madrid, Spain). An organic form of selenium extracted from yeast (Sel-Plex, Alltech Inc, Lexington, KY) was employed. The desired lipid content was completed with a non-essential fatty acid source, oleic acid (Merck, Darmstadt, Germany). The protein source used was squid meal (Riber & Son, Bergen, Norway), which in some experimental diets was defatted (3 consecutive times with a chloroform:meal ratio of 3:1) to allow a better control of the microdiet lipid content.

2.3.2.2. Microdiets preparation

The microdiets were prepared by mixing squid powder, attractants mixture which prepared according to Kanazawa *et al.* (1989), and the hydro and lipo-soluble vitamins mixture and minerals mixture which prepared according to Teshima *et al*. (1982) (Table 2.1). The squid powder and water-soluble components of attractants and hydro-soluble vitamins were mixed well in mortar, and then the lipids and fatsoluble vitamins were combined to obtain a homogeneous mix, which was afterwards merged with the powder mix. Finally, gelatine dissolved in warm water was added to the previously mixed ingredients to form a paste that was compressed pelleted (Severin, Suderm, Germany) and dried in an oven at 38ºC for 24 h (Ako, Barcelona, Spain). Pellets were ground (Braun, Kronberg, Germany) and sieved (Filtra, Barcelona, Spain) to obtain several particle sizes between 250 and 500 µm. Diets were prepared and analyzed for proximate and fatty acid composition at GIA laboratories.

Table 2.1 Mix of attractants, minerals and vitamins of the experimental microdiets

Vitamins	mg $100g^{-1}$ DW
Hydro-soluble vitamins	
Cyanocobalamin	0.030
Astaxanthin	5.000
Folic Acid	5.440
Pyridoxine-HCl	17.280
Thiamine-HCl	21.770
Riboflavin	72.530
Calcium Pantothenate	101.590
p-aminobenzoic acid	145.000
Ascorbic polyphosphate	180.000
Nicotinic acid	290.160
myo-Inositol	1450.900
Subtotal	2289.700
Lipo-soluble vitamins	
Retinol acetate	0.180
Ergocalciferol	3.650
Menadione	17.280
α-Tocopherol acetate	150.000
Subtotal	171.110
Choline chloride	2965.800
Total	5426.610

Attractants	mg $100g^{-1}$ DW
Inosine 5-monophosphate	500
Betaine	660
L-Serine	170
L-Tyrosine	170
L-Phenilalanine	250
DL-Alanine	500
L-Sodium aspartate	330
L-Valine	250
Glycine	170
Total	3000
Minerals	**mg $100g^{-1}$ DW**
NaCl	215.133
$MgSO_4 7H_2O$	677.545
$NaH_2PO_4 H_2O$	381.453
K_2HPO_4	758.949
$Ca(H_2PO_4) 2H_2O$	671.610
FeC_6H_5O	146.884
$C_3H_5O_3 1/2Ca$	1617.210
$Al_2(SO_4)_3 6H_2O$	0.693
$ZNSO_4 7H_2O$	14.837
$CuSO_4.5H_2O$	1.247
$MnSO_4 H_20$	2.998
KI	0.742
$CoSO_4 7H_2O$	10.706
Total	4500.007

2.3.3. Feeding procedures

Diets were manually supplied every 45 min from 8:00 to 19:00. Larvae were fed with rotifers twice a day during the first two days of experimental feeding at a density of 1 ind. ml^{-1}. To guarantee feed availability, daily dry supply was initially 2.5 g and increased 0.5 g each week. Larvae were observed under the binocular microscope to determine feed acceptance. If apparent feed intake differences were observed along different experimental diets, diet acceptance ratio was determined calculating the percentage of gut occupation of the larvae by image analysis program. For that reason 30 larvae from per tank were sampled (Leica Wild M3Z, Optotek, California, USA).

2.4. SAMPLING PROTOCOLS

In order to get growth performance data, standard length (cm) and weight (g) were recorded by measuring and weighing all the fish. All experimental fish were not fed

during 24 h before sampling. Fish were caught and introduced into an anaesthetic tank containing clove oil (2ml/100ml) (Guinama S.L.U., Valencia, Spain) to reduce stress and improve handling during sampling. Measured fish were returned to the tanks with high aeration and water flow until symptoms of recovery appeared (recuperation of verticality and sense of equilibrium, normal movement and response to external stimuli). Sampled fish were sacrificed with excess clove oil and ice.

2.5. BIOLOGICAL PARAMETERS

2.5.1. Activity test and survival

At the end of the experiments an activity test was conducted by handling 20 larvae $tank^{-1}$ out of the water in a scoop net for 1-1.5 min. and, subsequently, allocating them in another tank supplied with clean seawater and aeration, to determine survival after 24 h. Final survival was calculated by individually counting all the alive larvae $tank^{-1}$ at the end of the experimental trials.

2.5.2 Growth and productive parameters

Growth was determined by measuring total length and dry body weight. Total length of 30 anesthetized larvae from each tank was measured in a Profile Projector (V-12A Nikon, Nikon Co., Tokyo, Japan) at each sampling point. Whole body weight was determined by 3 replicates of 10 starved larvae washed with distilled water and dried in a glass slide at an oven at 110ºC, for approximately 24 h, followed by 1 h periods until constant weight was reached.

2.6. BIOCHEMICAL ANALYSES

To analyze biochemical composition, all the remaining larvae in each tank, after a starving period of 12 h, were collected, washed with distilled water and kept at -80ºC in air free labelled plastic sampling bags until analysis. Prior to the beginning of the dietary experiments, seabream larvae samples were taken to analyze initial biochemical composition.

2.6.1. Crude protein

Proteins were estimated from the total nitrogen present in the sample, using the Kjeldhal method (A.O.A.C, 1995). After digestion of the sample (250 mg) with concentrated sulphuric acid at a temperature of 420ºC, nitrogen was distillate and

determined by colorimetric methods. Then total nitrogen content was converted to total crude protein value by multiplying by the empirical factor 6.25.

2.6.2. Crude lipids

Lipids were extracted following the method of Folch *et al.* (1957). The method starts taking a sample amount between 50-200 mg and homogenizing it in an Ultra Turrax (IKA-Werke, T25 BASIC, Staufen Germany,) during 5 min in a solution of 5 ml of chloroform: methanol (2:1) with 0.01% of butylated hydroxytoluene (BHT). The resulting solution was filtered by gravimetric pressure through glass wool and 0.88% KCl added to increase the water phase polarity. After decantation and centrifugation at 2000 rpm during 5 min the watery and organic phases were separated. Once the watery phase was eliminated, the solvent was dried under nitrogen atmosphere and subsequently total lipids weighed.

2.6.3. Ash content

Ash content was determined in 200 mg samples by complete combustion in an oven at a temperature of 450ºC (A.O.A.C., 1995).

2.6.4. Dry matter content

Dry matter content was determined by oven drying until constant weight at 110ºC, with a first 24 h drying period, followed by 1 h periods until weight was not reduced any further. Sample weight (approximately 100 mg) was recorded before drying and after each drying period, following the cooling until room temperature in a desiccator. Dry matter was expressed as a percentage of the weight according to Official Methods of Analysis (A.O.A.C., 1995), using the following equation:

$$\%DW = \frac{(c-a) \times 100}{b-a}$$

Where:

a = Weight of empty flask

b = Weight of wet sample + flask

c = Weight of dry sample + flask

2.6.5. Fatty Acid Methyl Esters (FAMEs)

Fatty acid methyl esters (FAME) were obtained by acid transmethylation of total lipid

with 1% sulphuric acid in methanol following the method of Christie (1982). The reaction was conducted in dark conditions under nitrogen atmosphere for 16 h at 50ºC. Afterwards, fatty acid methyl esters were extracted with hexane:diethyl ether (1:1, v/v) and purified by adsorption chromatography on NH_2 Sep-pack cartridges (Waters S.A., Massachussets, USA) as described by Christie (1982). Fatty acid methyl esters were separated by GLC (GC-14A, Shimadzu, Tokyo, Japan) in a Supercolvax-10-fused silica capillary column (length:30 mm, internal diameter: 0.32 mm; Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180ºC for the first 10 min, increasing to 215ºC at a rate 2.5º C min^{-1} and then held at 215ºC for 10 min, following the conditions described in Izquierdo *et al.* (1992). Fatty acid methyl esters were quantified by FIED and identified by comparison with external standards and well-characterized fish oils (EPA 28, Nippai, Ltd. Tokyo, Japan).

2.6.6. Mineral composition

With the aim of understanding how the different diets modulated the mineral composition of the fish, fish were taken at the beginning and end of the trials, and several tissues were evaluated, including whole body, liver and vertebrae. Samples were homogenized, lyophilized and stored at -80ºC prior to analysis. The analysis consisted of a digestion of the samples in nitric acid and the posterior evaluation of the mineral content by means of an inductively coupled plasma mass spectrometry (ICP-MS).

2.6.6.1. Digestion

Digestions of samples were conducted in a Milestone Microwave Support System (Milestone Srl, Bolonia, Italy). The samples were added distilled water, HNO_3 and H_2O_2 and submitted to a pressure of 50 Bar. Microwaves were absorbed by the molecules/ions in the solution and put into rotary motion. The rotation leads to increase in the kinetic energy, which in turn gives rise in temperature. This results in the decomposition of the molecules/ions in the solution. The tests concluded on UW using a program that increases the temperature stepwise up to 260ºC. The program lasts 62 minutes: 37 minutes were used to perform the digestion of the samples (Table 2.2) and 25 minutes to cool the samples until they can be safely handled.

Table 2.2 Temperature program for sample digestion

Temperature program:
10 min increase to 80ºC
2 min at 80ºC
5 min increasing temperature up to 105ºC
5 min at 105ºC
10 min increase temp to 260ºC
5 min at 260ºC

2.6.6.2. Mineral analysis

An inductively coupled plasma mass spectrometry (ICP-MS) "Thermo ScientificiCAPQ" (Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA) was used to determine mineral content. The conditions for the analysis are described in Table 2.2.

Table 2.3 Conditions for mineral analysis with ICP-MS

Parameter Measure
Software Qtegra y LIMS 1
Nebulizer gas flow 0.9 l/min
Radio frequency 1200 W
Cool gas flow 13 l/min
Auxiliary gas flow 0.8 l/min
Helium gas collision cell flow

2.6.7. Amino acid composition

Total amino acids analyses were conducted at the The Scientific and Technological Research Council of Turkey (Gebze, MAM, Turkey). From each sample, 5 mg were added to a glass ampoule together with 5 ml lithium citrate loading buffer. Ampoules were sealed and placed in an oven (115°C) for 2 h to facilitate hydrolysis. The samples were then removed, allowed to cool and filtered with a 0.45 PTFE syringe filter. Samples were diluted as necessary to fall within the detectable range for the assay. Amino acids were measured with a Biochrom 30 amino acid analyzer (Biochrom, Holliston, MA, USA) (Hawkyard *et al.*, 2016).

2.7. HISTOLOGY

At the end of the feeding trial, ten larvae from each tank were fixed in 10% neutral-buffered formalin and embedded in paraffin. Whole larvae 4-µm sections were stained with H&E for morphological studies and with Alcian blue/periodic acid– Schiff (PAS) for mucus production evaluation (Martoja and Martoja, 1970; Vandewalle *et al.*, 1998). Sections were evaluated under light microscopy with 50x magnification (Olympus CX41 microscope). Micrographs were taken at a final magnification of 400x using an Olympus DP50 (Olympus Optical, Shinjuku-ku, Tokyo, Japan) camera. The number of goblet cells stained for specific acid mucin staining by unit of fold area was determined using an ANALYSIS® (Image Pro Plus®, Media Cybernetics, Silver Spring, MD, USA) (Torrecillas *et al.,* 2013).

2.8. GENE EXPRESSION

At the beginning and final sampling points, around 100 mg of unfed seabream larvae were collected, washed in Diethyl pyrocarbonate (DEPC) water and conserved in 500 µl of RNA Later (Sigma-Aldrich, Madrid, Spain) overnight at 4^0C, then RNA Later was removed and samples kept at -80^0C until RNA extraction. Molecular biology analysis was carried out at the GIA laboratories in Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA, ULPGC, Las Palmas, Spain). Total RNA from larvae samples (average weight per sample 60mg) was extracted using the Rneasy Mini Kit (Qiagen). Total body tissue was homogenised using the TissueLyzer-II (Qiagen, Hilden, Germany) with QIAzol lysis reagent (Qiagen). Samples were centrifuged with chloroform for phase separation (12000g, 15min, 4ºC). The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into an RNeasy spin column where total RNA bonded to a membrane and contaminants were washed away by RW1 and RPE buffers (Qiagen). Purified RNA was eluted with 30ml of RNase-free water. The quality and quantity of RNA were analysed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Synthesis of complementary deoxyribonucleic acid (cDNA) was conducted using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Primer efficiency was tested with serial dilutions of a cDNA pool (1, 1:5, 1:10, 1:15,1:20 and 1:25). The product size of the real-time q PCR amplification was checked by electrophoresis analyses using PB322 cut with HAEIII as a standard.

Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using b-actin as the house-keeping gene in a final volume of 20ml per reaction well, and 100ng of total RNA reverse transcribed to complementary cDNA. Each gene sample was analyzed once per gene. The PCR conditions were the following: 95ºC for 3min 30 sec followed by 40 cycles of 95ºC for 15 sec, 61ºC for 30 sec, and 72ºC for 30 sec; 95ºC for 1min, and a final denaturing step from 61ºC to 95ºC for 10sec. Data obtained were normalized and the Livak method (2–""Ct) used to determine relative mRNA expression levels. Gilthead seabream specific gene primers were designed after searching the NCBI nucleotide database and using the Oligo 7 Primer Analysis software (Molecular Biology Insights, Cascade, CO, USA). Detailed information on primer sequences and accession numbers is presented in Table 2.3.

Table 2.4 Sequences of forward and reverse primers (5´-3´) for real-time quantitative-PCR of seabream genes

Gene	Primers	Accesion no
Catalase (*cat*)	Forward primer: 5′-ATGGTGTGGGACTTCTGGAG-3′ Reverse primer: 3′-AGTGGAACTTGCAGTAGAAAC-5′	FJ860003
Superoxide dismutase (*CuZnsod*)	Forward primer: 5′-AAGAATCATGGCGGTCCTACTGA-3′ Reverse primer: 3′-TGAGCATCTTGTCCGTGATGTCT -5′	AJ937872
Manganese superoxide (Mn*sod*)	Forward primer: 5'-AGTGCCTCCTGATATTTCTCCTCTG-3' Reverse primer: 5'-CCTGACCTGACCTACGACTATGG-3'	JQ3088331
Glutathione peroxidase (*gpx*)	Forward primer: 5′-TCCATTCCCCAGCGATGATGCC-3′ Reverse primer: 3′-TCGCCATCAGGACCAACAAGGA-5′	DQ524992
Osteocalcin (*oc*)	Forward primer: 5′-AGCCCAAAGCACGTAAGCAAGCTA-3′ Reverse primer: 3′-TTTCATCACGCTACTCTACGGGTT-5′	AF048703.1

2.9. STATISTICAL ANALYSIS

All data were statistically analysed using SPSS software v21 (IBM Corp., Chicago, IL, USA) and SD and means were calculated for each parameter measured. Data were tested for normality with the one-sample Kolmogorov-Smirnov test. For normally distributed data, one-way analysis of variance (ANOVA) was used to determine the effects of the different diets. Data were tested for homogeneity and post-hoc analysis was carried out using Tukey and Duncan test if variances were the same or different.

Significant differences were considered for $p<0.05$, and are expressed in the tables with superindexes. When data did not follow a normal distribution, logarithmic or arcsin transformations were carried out or non-parametric tests, such as Kruskal-Wallis, were used. Quadratic and linear regressions were used to establish a relation between levels of micronutrients and their effect on the different biomarkers.

CHAPTER 3

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Nutritional value and production performance of the rotifer *Brachionus plicatilis* Müller, 1786 cultured with different feeds at commercial scale

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3.1. ABSTRACT

The rotifer *Brachionus plicatilis* is the first live feed in larviculture of marine fish species. Rotifers are commonly fed commercial diets and freshly cultured microalgae species. These diets differed in both their biochemical composition and physical properties and production technology and feeding protocols largely varied among facilities. The objective of the present study was to determine the effects of three different forms of *Nannochloropsis oculata* and commonly used commercial diets on growth performance and biochemical compositions of rotifers produced under commercial conditions. This study was performed in Akvatek Marine Fish Culture Company (İzmir, Turkey), where microalgae were cultured in tubular photobioreactors and microalgae biomass was stored as spray-dried or fresh paste forms. Rotifers were fed one of six different types of food: Algome® (dried *Schizochytrium sp.*), ProteinPlus® (PP), inactive baker's yeast® (INBY), spray-dried *N. oculata* (SDN) or freshly cultured *N. oculata* (FN). Rotifers were fed the experimental diets 4 times a day during 14 days of batch or semi-continuous culture. Initial stocking density of rotifers was 600 ind/ml^{-1}. During the experiment, total rotifer biomass, egg ratio and egg carried female numbers were daily counted. Proximate composition, fatty acids and amino acids profiles of rotifers, diets and biomass were also analyzed. The highest growth was obtained in rotifers fed SDN (p<0.05), whereas rotifers fed PP showed highest ∑n-3, ARA, EPA and DHA contents (p<0.05). Amino acid profiles of rotifers were enhanced by utilization of both INBY and SDN diets. Overall, the results indicated that spray dried *N. oculata* was the best food for long-term biomass production of rotifers. However, its nutritional profile was markedly improved by feeding PP, INBY and SDN diets.

3.2. INTRODUCTION

Rotifer (*Brachionus plicatilis*) and *Artemia nauplii* (Artemia) are the most common live preys used in commercial marine fish hatcheries, their use being dependent on larval mouth size (Rainuzzo *et al.*, 1997; Reitan *et al.*, 1997; Conceição *et al.*, 2010; Hawkyard *et al.*, 2016). Rotifers are still indispensable in larvae culture due to its suitable size and relatively simple production techniques. However, their nutritional quality and reliable production markedly depends on the type of feed used, and the stable production of high quality rotifers is crucial for commercial marine fish

hatcheries. For instance, in comparison to copepods, natural preys for many marine fish larvae, rotifers are low in essential fatty acids and certain amino acids, vitamins and minerals (Hamre *et al.*, 2013). Commercial rotifer diets and enrichment products have different nutritional compositions and this variation could affect the quality and quantity of produced rotifers and, eventually, their effected on fish larvae. Therefore, adequate feed and enrichment protocols are important to sustain the desirable nutritional properties of live feeds for marine fish larvae (Hamre *et al.*, 2008; Matsunari *et al.*, 2012).

Microalgae are still widely used in larval production as a rotifer feed (Eryalçın *et al.*, 2013; Haas *et al.*, 2016) or in green water production (Tendencia *et al.*, 2015), either in fresh (Patil *et al.*, 2007), spray-dried (Harel *et al.*, 2002), freeze-dried (Tibaldi *et al.*, 2015) or paste forms (Schwarz *et al.*, 2008). Several studies on antibacterial effects of microalgae show that microalgae addition in culture water of marine fish larvae decrease the incidence of bacterial diseases (Salvesen *et al.,* 2000; Sharifah and Eguchi, 2011) and play an important role in fish larvae intestine development, either directly or through rotifers (Ringø *et al.*, 2014). Fresh microalgae production expenditures in commercial marine fish hatcheries may count up to 30-40% of total production costs (Norsker *et al.*, 2011). Therefore, many hatcheries are interested in the use of commercial microalgae products, despite their price may be elevated and depend on their quality and purpose of utilization (Borowitzka, 2013). Among them, fresh microalgae has beneficial antibacterial effect on larvae culture tank against to pathogens such as *Vibrio sp.* (Shields and Lupatsch, 2012; Taniguchi *et al.*, 2011). Microalgae biomass can be also concentrated in paste form which presents a high cell density. However, without frozen process paste forms should be utilized within a week. Freeze-dried microalgae forms have several advantages such as easy utilization at any time with maintained original cell shape and texture, it is available with preserved biochemical properties such as essential fatty acids and amino acids profile (Lubzens *et al.*, 1995; Pedro and Fernandez-Diaz, 2001). On the other hand, spray-dried microalgae can also be alternative biomass. However, within this process microalgae cells get smaller and decrease product quality (Ryckebosch *et al.*, 2011).

Several commercial rotifer feeds such as concentrated suspensions, frozen biomass, microencapsulated and yeast-based diets are available and their effect on rotifer

production may differ (Srivastava *et al.*, 2006; Dhert *et al.*, 2014; Hamre, 2016). This variation is mainly dependent on the nutritional value and physic-chemical properties of rotifer feeds and rotifer culture conditions and protocols. For instance, micro-encapsulated diets contain certain amount of vitamins, minerals and highly unsaturated fatty acids (HUFA). For example, Culture Selco® has an optimum HUFA composition when it compared to most commonly utilized rotifer live feeds such as microalgae or baker's yeast. Besides, biochemical properties of paste form mainly depend on cultured microalgae species and culture conditions. Therefore, it is more difficult to sustain certain amount of nutrients by fresh microalgae or paste (Dhert *et al.*, 2001).

The type of culture also effects the rotifer production success. Batch, semi-continuous and high density culture methods are applied depend on needs of facilities, tank volumes and feeds utilized in rotifer production. In batch culture, harvested rotifers are used for new inoculation for rotifer cultures and as life preys for cultured species. In this method, high density commercial feeds are delivered to the culture tanks after only 3-4 days of culture. This may lead to undesirable water parameters. Partly harvesting and washing rotifer cultures are known as semi-continuous culture that is frequently longer due to the periodic harvesting. High-density culture is also managed by utilization concentrated microalgae biomass. However, water quality and stability are still main problems in this method. In order to eliminate those problems, in high-density culture methods, protein skimmers, filtering and partly renewing water techniques could be applied (Yoshimatsu and Hossain, 2014).

N. oculata has high phototrophic growth potential (Spolaore *et al.*, 2006; Hemaiswarya *et al.*, 2011) and is also rich in eicosapentaenoic (20:5n-3) and arachidonic (20:4n-6) acids, essential fatty acids for marine fish larvae (Izquierdo and Koven, 2011). Therefore, different forms of this microalgae species were prepared and investigated for rotifer culture. The aim of this study was to compare the performance of different forms of *N. oculata* and different commercial rotifers feeds in either batch or semi-continuous culture of rotifers during 14 days.

3.3. MATERIALS AND METHODS

3.3.1. Microalgae culture

N. oculata from CCAP (The Culture Collection Algae and Protozoa (Strain number: CCAP 849/1), Scotland, UK) was cultured in f/2 medium previously sterilized at 121 ^{o}C for 15 min. All sub-cultures were maintained at 27 ^{o}C and salinity of 32 PSU under a 12L:12D photoperiod. N. *oculata* culture volume was up-scaled from 50 ml test tubes to 250 ml erlenmeyer flasks, followed by 1 L, 5 L and 300 L culture containers. The population growth was daily determined by cell counting using Neubaeur chambers. Inoculation to a larger volume followed population growth reaching to late log-phase.

3.3.2. Spray-dried microalgae production

N. oculata was spray dried at 90 ^{o}C processing heat. From 1 L fresh *N. oculata* 160 gram dry cell biomass was maintained. The spray-dried *N. oculata* biomass was stored at -80 ^{o}C after produced.

3.3.3. Rotifer batch culture

Prior to the beginning of the trial, rotifers (*Brachionus plicatilis*, L-type strain, lorica size 200-250 μm) were produced in 1000 L circular tanks with baker's yeast (*Saccharomyces cerevisiae*). Stock cultures were maintained in Erlenmeyer flasks with 30 rot/ml density and cultivated semi-continuously. Salinity, oxygen and temperature were measured during experiment.

3.3.4. Experimental design and diets

Initial rotifer stock density was 600-700 rot/ml in all experimental culture tanks. Rotifers were fed for 10 days semi-continuously with the experimental diets. The rotifer feeds used were: Algome® (containing dried *Schizoctyrium sp.*), Protein-Plus (PP; 0.3-1 g /10^6, Algamac®), Inactive Beaker's Yeast (INBY; 0.3-0.4 g/ml), spray-dried *N. oculata* (SDN; 0.5-0.6 g/10^6 rotifers) and freshly cultured *N. oculata* (FN; density of 10^7 cell/ml^{-1} / 10^6 rotifers). Each diet was tested in triplicate.

3.3.5. Rotifer growth determination and sampling procedures

Each experimental feed was tested in triplicate and applied at a daily ration of 0.8 g dry weight (DW) 10^6 rotifer/day. During the experiment, egg number, egg carrying

female number and total rotifer biomass were daily calculated. Three 1 ml samples were collected from each rotifer culture and Lugol solution was added in order to settle rotifers for counting. Dry weight was determined in 5 ml of microalgal culture or 100 ml of rotifer culture samples filtered on previously weighed precombusted Whatman GF/C fiberglass filters. Microalgae biomass was washed three times with 5 ml of 0.5 M ammonium formate in order to remove salts, whereas rotifers samples were rinsed with distilled water. Filters were dried overnight at 80°C and dry weight determined gravimetrically. Microalgal biomass (25-50 ml) was sampled by centrifugation and immediately frozen at -80°C. For proximate, fatty acid and amino acid composition 150 ml of rotifer culture was filtered on a sieve (45 μm mesh size), rinsed with distilled water and frozen at -80°C until analysis.

3.3.6. Proximate composition

Moisture (A.O.A.C., 1995), protein (A.O.A.C., 1995) and crude lipid (Folch *et al.*, 1957) contents of rotifer were analyzed (Table 3.1).

Table 3.1 Proximate compositions (g 100 g^{-1} dry weight) of experimental rotifer diets

Diets	Algome®	PP®	INBY®	SDN	FN
Crude protein	16.75±0.01	42.01±0.03	45.24±0.04	58.54±0.03	42.04±0.07
Crude lipids	0.41±0.02	3.44±0.04	1.01±0.02	1.06±0.05	5.91±0.02
Crude ash	24.35±0.59	6.93±0.04	4.61±0.01	10.27±0.11	5.55±0.09
Moisture	96.33±0.04	93.84±0.10	94.89±0.37	97.24±0.16	97.12±0.07

3.3.7. Fatty acid analysis

Fatty acid methyl esters in microalgae, diets and experimental rotifers were obtained by transmethylation with 1% sulphuric acid in methanol (Christie, 1982). Fatty acid methyl esters were separated by GC (GC-14A; Shimadzu, Tokyo, Japan) in a Supercolvax-10- fused silica capillary column (constant pressure with 100KPa, length: 30 m; internal diameter: 0.32 mm; 0,25 i.d (Ref.: 24080-U) Supelco, Bellefonte, PA, USA) using helium as a carrier gas. Column temperature was 180 °C

for the first 10 min, increasing to 220 °C at a rate of 2 °C min^{-1} and then held at 220 °C for 15 min. Fatty acid methyl esters were quantified by FID following the conditions described in Izquierdo *et al.* (1990) and identified by comparison with external standards and well characterized fish oils (EPA 28, Nippai, Ltd Tokyo, Japan). Fatty acid profiles of experimental rotifer diets are shown in Table 3.2.

Table 3.2 Main fatty acid compositions of experimental rotifer (*Brachionus plicatilis*) feeds (% total fatty acids)

	Algome®	**PP®**	**INBY®**	**SDN**	**FN**
12:0	0.22±0.00	0.23±0.01	0.16±0.00	0.05±0.00	0.16±0.00
13:0	0.04±0.00	n.d.	n.d.	n.d.	n.d.
14:0	6.07±0.00	8.94±0.01	0.60±0.02	1.05±0.02	1.43±0.00
14:1	n.d.	n.d.	0.11±0.00	n.d	n.d.
15:0	2.60±0.01	0.34±0.00	0.26±0.01	0.50±0.00	0.49±0.02
16:0	52.02±0.04	25.61±0.04	18.80±0.21	28.45±0.08	25.94±0.08
16:1	n.d.	0.24±0.01	28.76±0.18	1.65±0.02	0.95±0.03
17:0	0.82±0.00	0.08±0.01	0.23±0.02	0.11±0.01	0.17±0.00
18:0	1.22±0.01	0.75±0.01	14.38±0.05	2.11±0.02	4.51±0.00
18:1n-9	1.55±0.00	0.95±0.00	28.21±0.13	4.83±0.02	15.71±0.02
18:2n-6	3.20±0.01	3.88±0.01	0.46±0.01	19.71±0.03	23.98±0.05
18:3n-3	0.08±0.00	0.36±0.00	n.d.	17.29±0.06	10.97±0.03
18:3n-6	0.03±0.00	1.12±0.21	n.d.	n.d.	n.d.
20:0	n.d.	0.09±0.01	0.14±0.01	0.14±0.01	0.95±0.01
20:1	n.d.	n.d.	0.09±0.01	0.18±0.02	0.42±0.01
20:2n-6	0.10±0.01	0.23±0.00	n.d.	0.10±0.01	0.09±0.00
20:1n-9	n.d.	n.d.	n.d.	0.98±0.04	n.d.
20:3n-3	0.30±0.00	0.99±0.01	0.24±0.00	n.d	0.03±0.01
20:3n-6	0.06±0.00	0.29±0.00	n.d.	n.d.	n.d.

20:4n-6	0.04±0.01	0.28±0.01	n.d.	n.d.	0.10±0.00
20:5n-3	0.24±0.01	0.74±0.00	n.d.	n.d.	0.05±0.01
22:0	0.05±0.01	0.06±0.01	0.06±0.01	0.08±0.00	0.30±0.01
22:5n-6	n.d.	0.21±0.01	n.d	0.09±0.01	0.08±0.01
22:6n-3	24.84±0.01	34.88±0.01	0.09±0.02	0.10±0.08	0.04±0.00
23:0	n.d.	n.d.	n.d.	n.d.	0.11±0.01
24:0	0.04±0.00	0.22±0.01	0.16±0.01	0.24±0.00	0.46±0.00
24:1	0.04±0.00	0.17±0.01	n.d.	n.d.	n.d.
Σ Saturated	63.07±0.05	36.29±0.05	34.79±0.18	32.71±0.07	34.63±0.04
Σ Monounsaturated	0.04±0.00	0.41±0.02	28.96±0.16	1.82±0.04	1.37±0.04
Σ n-3	25.45±0.01	36.96±0.00	0.33±0.02	17.39±0.02	11.08±0.03
Σ n-6	3.33±0.02	1.16±0.05	0.46±0.01	19.80±0.04	24.15±0.06
Σ n-9	1.55±0.00	0.95±0.00	28.21±013	5.81±0.02	15.71±0.02
Σ n-3 HUFA	25.37±0.01	36.60±0.00	0.33±0.02	0.10±0.08	0.11±0.00
EPA/ARA	6.83±1.18	2.65±0.13	n.d.	n.d.	0.45±0.07
DHA/EPA	105.73±3.15	47.13±0.01	n.d.	0.15±0.00	0.90±0.14
DHA/ARA	724.33±146.14	124.71±6.27	n.d.	0.15±0.00	0.40±0.00
n-3/n-6	7.65±0.04	32.00±1.41	0.71±0.02	0.88±0.00	0.46±0.00

3.3.8. Amino acid analysis

Total amino acids analyses were conducted at the The Scientific and Technological Research Council of Turkey (Gebze, MAM, Turkey). From each sample, 5 mg were added to a glass ampoule together with 5 ml lithium citrate loading buffer. Ampoules were sealed and placed in an oven (115°C) for 2 h to facilitate hydrolysis. The samples were then removed, allowed to cool and filtered with a 0.45 PTFE syringe filter. Samples were diluted as necessary to fall within the detectable range for the assay. Amino acids were measured with a Biochrom 30 amino acid analyzer (Biochrom, Holliston, MA, USA) (Hawkyard *et al.*, 2016) (Table 3.3).

Table 3.3 Free amino acid compositions of experimental rotifer feeds (mg g^{-1} dry weight, mean±SD)

	Algome®	**PP®**	**INBY®**	**SDN**	**FN**
Aspartic acid	5.48±0.07^{b}	13.27±0.09^{a}	2.77±0.03^{c}	1.98±0.04^{c}	0.46±0.016^{d}
Glutamic acid	8.65±0.03^{b}	12.01±0.01^{a}	4.53±0.03^{c}	4.49±0.03^{c}	0.83±0.06^{d}
Serine	2.18±0.01^{a}	1.83±0.02^{b}	1.51±0.01^{c}	1.74±0.02^{b}	0.25±0.014^{d}
Glycine	1.60±0.02^{a}	1.47±0.04^{b}	1.54±0.010^{b}	1.53±0.01^{b}	0.38±0.02^{c}
Histidine	1.31±0.07^{a}	0.79±0.01^{b}	0.71±0.03^{b}	0.80±0.08^{b}	0.06±0.012^{c}
Arginine	2.34±0.09^{a}	1.29±0.01^{c}	0.83±0.02^{d}	1.66±0.08^{b}	0.37±0.00^{e}
Threonine	2.25±0.04^{a}	0.40±0.00^{d}	1.13±0.08^{c}	1.77±0.09^{b}	0.36±0.04^{d}
Alanine	3.19±0.01^{a}	2.47±0.06^{b}	2.06±0.09^{c}	1.98±0.01^{c}	0.52±0.06^{d}
Proline	1.53±0.01^{bc}	1.30±0.03^{c}	1.89±0.02^{b}	3.28±0.09^{a}	0.81±0.05^{d}
Tyrosine	1.67±0.01^{a}	1.48±0.00^{b}	1.56±0.07^{b}	1.04±0.01^{c}	0.26±0.07^{d}
Valine	3.05±0.05^{a}	1.88±0.01^{c}	2.72±0.05^{b}	1.77±0.08^{c}	0.41±0.04^{d}
Methionine	0.56±0.02^{a}	0.12±0.01^{c}	0.53±0.01^{b}	0.12±0.00^{c}	-

Isoleucine	2.36±0.00[a]	1.35±0.04[c]	2.00±0.01[b]	1.12±0.04[c]	0.25±0.03[d]
Leucine	3.00±0.02[a]	2.52±0.06[c]	2.84±0.03[b]	2.20±0.01[c]	0.52±0.06[d]
Phenylalanine	2.14±0.02[a]	1.70±0.03[b]	2.17±0.07[a]	1.59±0.09[b]	0.41±0.02[c]
Lysine	3.75±0.02[a]	3.83±0.014[a]	3.38±0.01[b]	3.55±0.01[b]	0.51±0.03[c]
Free amino acid	45.09±0.05[a]	47.73±0.03[a]	32.19±0.02[b]	30.65±0.07[b]	6.46±0.04[c]
Essential amino acid	18.42±0.07[a]	12.59±0.05[c]	15.48±0.04[b]	12.92±0.03[c]	2.52±0.08[d]
Non-essential amino acid	26.27±0.02[b]	35.14±0.01[a]	16.71±0.04[c]	17.73±0.04[c]	3.94±0.02[d]

*Different letters shows significant differences among groups

3.3.9. Statistical analysis

All data were statistically analyzed using a SPSS Statistical Software System 15.0 (SPSS Inc., Chicago, IL, USA). The significant level for all the analysis was set at 5%, and results were given as mean values and standard deviation. All values presented as percentage were arcsine-transformed. Also, all variables were checked for normality and homogeneity of variance, using the Kolmogorov–Smirnoff and the Levene's test respectively. Means were compared using one-way ANOVA or a Kruskal–Wallis test.

3.4. RESULTS

3.4.1. Rotifer growth performance

Rotifers fed SDN showed the significantly highest total biomass production during the first 11 days of feeding (p<0.05) (Figure 6). There were no significant differences among the rotifers fed PP, SDN, INBY, FN and those 4 groups showed higher biomass production than rotifers fed Algome® diet at 12 and 13 days of culture. FN showed significantly increasing potential of total rotifer biomass last 3 days of feeding trial (p<0.05) (Figure 6).

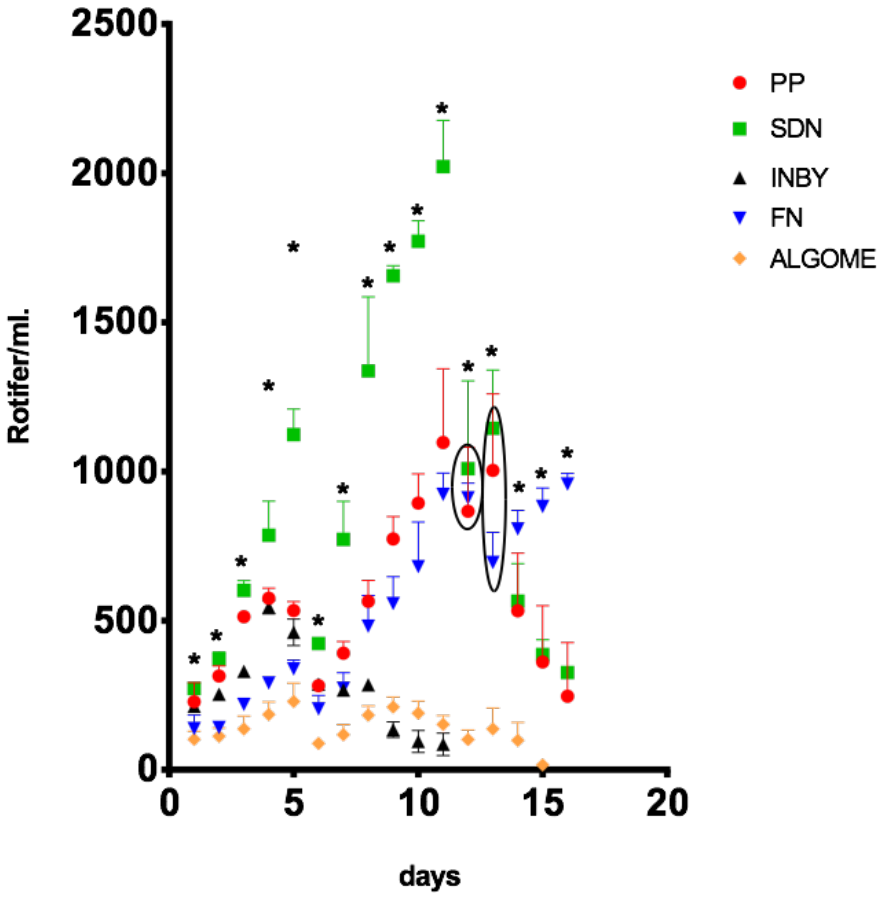

Figure 6. Density of rotifers fed the different feeds during 16 days (PP; Protein Plus, SDN; spray-dried *Nannochloropsis oculata*, INBY; Inactive Baker's Yeast, FN; fresh *Nannochloropsis oculata*, Algome®; spray-dried *Schizoctyrium* sp.).

Rotifer fed SDN showed higher egg production in per ml during first 11 days of experiment ($p<0.05$) (Fig. 2). PP group rotifers significantly contained higher number of eggs at 12 and 15 days of experiment ($p<0.05$) (Figure 7).

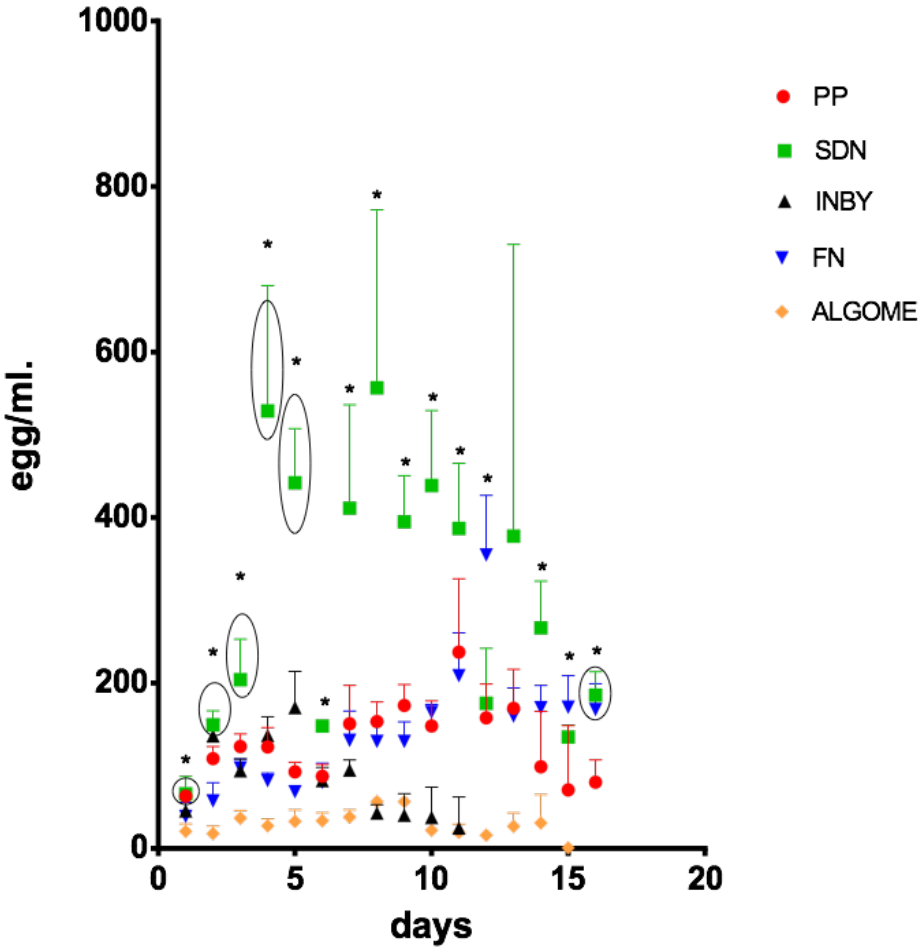

Figure 7. Rotifer egg density (Egg/ml, mean±SD) fed different feeds during 16 days experiment (PP; Protein Plus, SDN; spray-dried *Nannochloropsis oculata*, INBY; Inactive Baker's Yeast, FN; fresh *Nannochloropsis oculata*, Algome®; spray-dried *Schizoctyrium* sp.).

Rotifers fed SDN showed significantly higher egg carrying female number from the day 1 until day 12 of culture (p<0.05) (Figure 8). Rotifers fed FN showed the highest value of egg carrying female number at 12 days of culture. At day 13 of the culture period, only rotifer group fed Algome® showed lowest value. Rotifers fed SDN and FN showed significantly higher egg carrying female numbers for the last 3 days of culture (p<0.05). Female rotifers were only observed after 11 days of culture in INBY group.

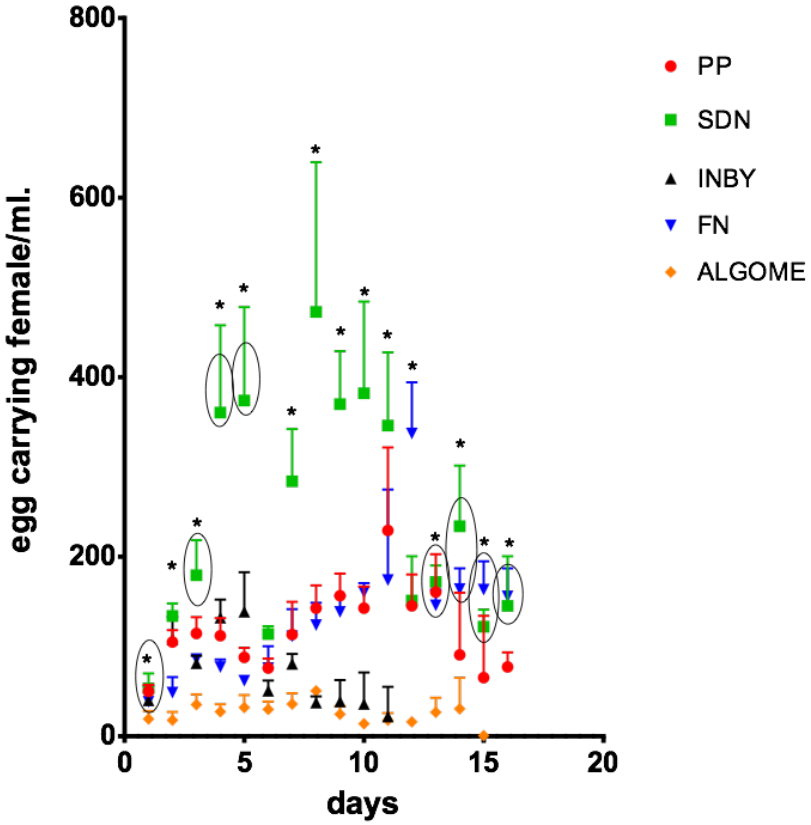

Figure 8. Density of egg carrying female (egg carrying female/ml, mean±SD) fed different feeds during 16 days (PP; Protein Plus, SDN; spray-dried *Nannochloropsis oculata*, INBY; Inactive Baker's Yeast, FN; fresh *Nannochloropsis oculata*, Algome®; spray-dried *Schizoctyrium* sp.).

3.4.2. Proximate composition of rotifers

At the end of the feeding trial, rotifer fed Protein Plus diet presented significantly (p<0.05) higher crude protein content than the other groups (Table 3.4). Similarly, rotifer fed Protein Plus diet showed significantly (p<0.05) higher crude lipid content than the other groups.

Table 3.4 Proximate compositions (g 100 g^{-1} dry weight) of rotifer (*Brachionus plicatilis*) fed different diets at the end of the experiment. Different letters within a line denote significant differences (p<0.05). Values expressed in mean ± SD (n =3 tanks/diet); different letters shows significant differences among groups

	Initial	Algome®	PP®	INBY®	SDN	FN
Crude Protein	37.07±0.01	42.25±0.02^{b}	45.04±0.07^{a}	40.03±0.06^{c}	40.01±0.02^{c}	38.02±0.08^{d}
Crude Lipid	0.44±0.08	0.60±0.13^{d}	1.43±0.18^{a}	0.89±0.12^{c}	1.29±0.48^{b}	1.41±0.05^{a}
Crude Ash	1.15±0.01	1.67±0.03^{b}	1.21±0.02^{d}	1.23±0.01^{d}	1.33±0.03^{c}	1.94±0.05^{a}
Dry matter	14.48±1.04	10.20±0.14^{d}	12.23±0.02^{b}	12.98±0.15^{a}	11.64±1.17^{c}	8.67±0.11^{e}

3.4.3. Fatty acid composition of rotifers

At the end of the feeding, arachidonic acid (AA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3), ∑n-3 and ∑n-3 HUFA were increased in rotifers by feeding Protein Plus (p<0.05). Similarly, DHA/EPA ratio and n-3/n-6 ratio were higher in rotifers fed Protein Plus (p<0.05). However, oleic acid level was found higher in rotifer fed Inactive Beaker's Yeast (p<0.05). Moreover, linoleic acid (LA; 18:2n-6) was found higher in rotifers fed spray-dried *N. oculata*. α-linoenic acid (LNA; 18:3n-3) was enhanced by both diet spray-dried *N. oculata* and fresh *N. oculata* (p<0.05) (Table 3.5).

Table 3.5 Fatty acid compositions of rotifers fed different diets (% dry weight, mean ± SD, n = 3, different superscripts denote significant differences among rotifers fed different diets in selected fatty acids)

	Initial	Algome®	PP®	INBY®	SDN	FN
10:0	0.09±0.01	n.d.	n.d.	0.11±0.01	n.d.	0.04±0.00
12:0	1.37±0.03	0.21±0.01^{b}	0.22±0.00^{b}	0.60±0.01^{a}	0.10±0.00^{c}	n.d.
13:0	n.d.	n.d.	n.d.	n.d.	n.d.	0.04±0.01
14:0	1.95±0.02	4.48±0.04^{b}	5.38±0.01^{a}	3.02±0.06^{c}	2.49±0.07^{e}	3.22±0.01^{d}

14:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15:0	0.37±0.02	1.89±0.02^{a}	0.62±0.00^{e}	0.90±0.01^{d}	1.21±0.04^{b}	1.14±0.03^{c}
16:0	10.99±0.04	22.74±0.12^{b}	16.16±0.00^{d}	11.08±0.02^{e}	23.10±0.06^{a}	21.30±0.01^{c}
16:1	16.68±0.06	6.26±0.04^{b}	2.37±0.02^{c}	16.61±0.06^{a}	2.07±0.02^{d}	1.87±0.01^{e}
17:0	0.23±0.01	0.62±0.03^{a}	0.28±0.01^{c}	0.58±0.00^{b}	0.29±0.01^{c}	0.33±0.01^{d}
18:0	5.04±0.07	4.17±0.01^{b}	2.49±0.01^{e}	5.88±0.02^{a}	2.92±0.02^{c}	2.73±0.01^{d}
18:1n-9	32.03±0.13	8.38±0.00^{b}	4.26±0.10^{e}	23.76±0.08^{a}	6.41±0.01^{c}	4.82±0.01^{d}
18:2n-6	3.45±0.08	6.20±0.07^{d}	11.67±0.05^{c}	2.76±0.10^{e}	22.56±0.16^{a}	21.76±0.01^{b}
18:3n-3	0.51±0.02	0.26±0.07^{c}	1.21±0.01^{b}	n.d.	12.49±0.03^{a}	12.40±0.03^{a}
18:3n-6	n.d.	n.d.	1.13±0.03	n.d.	n.d.	n.d.
20:0	0.14±0.01	0.20±0.00^{b}	0.13±0.00^{d}	0.24±0.00^{a}	0.12±0.01^{d}	0.17±0.02^{c}
20:1	2.66±0.03	0.72±0.06^{d}	0.63±0.01^{e}	2.25±0.02^{a}	1.11±0.02^{c}	1.43±0.01^{b}
20:2n-6	n.d.	0.30±0.01^{c}	1.35±0.07^{d}	n.d.	1.93±0.01^{a}	1.72±0.06^{b}
20:1n-9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:3n-3	n.d.	0.15±0.04^{c}	0.88±0.00^{b}	0.10±0.00^{d}	1.13±0.01^{a}	n.d.
20:3n-6	0.31±0.02	0.33±0.00^{c}	1.51±0.00^{a}	n.d.	0.89±0.01^{b}	0.91±0.01^{b}
20:4n-6	0.70±0.04	0.78±0.30^{b}	1.75±0.01^{a}	n.d.	n.d.	0.34±0.04^{c}
20:5n-3	0.32±0.00	2.02±0.01^{b}	2.54±0.01^{a}	0.21±0.05^{d}	0.15±0.01^{d}	0.28±0.01^{c}
22:0	0.13±0.01	0.23±0.00^{b}	0.15±0.01^{c}	0.26±0.01^{a}	0.12±0.01^{d}	0.11±0.00^{e}

22:5n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d..
22:1n-9	0.92±0.01	0.23±0.01	0.28±0.00	0.70±0.01	0.32±0.04	n.d.
22:5n-6	n.d.	1.13±0.08^{a}	1±0.00^{b}	n.d.	n.d.	n.d.
22:6n-3	n.d.	9.78±0.27^{b}	21.74±0.19^{a}	n.d.	n.d.	n.d.
23:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
24:0	n.d.	0.28±0.01^{a}	0.18±0.01^{d}	0.24±0.00^{b}	0.21±0.01^{c}	0.14±0.00^{e}
24:1	0.59±0.04	n.d.	n.d.	0.67±0.11^{a}	0.42±0.35^{b}	0.31±0.03^{c}
Σ Saturated	20.29±0.00	34.81±0.17^{a}	25.60±0.02^{d}	22.98±0.04^{e}	30.54±0.16^{b}	29.20±0.01^{c}
Σ Monounsaturated	19.93±0.01	6.97±0.03^{b}	2.99±0.01^{d}	19.53±0.04^{a}	3.59±0.40^{c}	3.61±0.05^{c}
Σ n-3	0.83±0.02	12.20±0.17^{d}	26.36±0.21^{a}	0.31±0.05^{e}	13.77±0.01^{b}	12.68±0.04^{c}
Σ n-6	4.45±0.10	8.43±0.16^{d}	16.05±0.03^{c}	2.76±0.10^{e}	23.45±0.18^{a}	23.00±0.02^{b}
Σ n-9	32.95±0.15	8.61±0.01^{b}	4.54±0.10^{e}	24.46±0.08^{a}	6.73±0.02^{c}	4.82±0.01^{d}
Σ n-3 HUFA	0.32±0.00	11.94±0.24^{b}	25.16±0.21^{a}	0.31±0.05^{d}	1.28±0.02^{c}	0.28±0.01^{d}
EPA/ARA	n.d.	2.81±1.10^{a}	1.46±0.00^{b}	0.31±0.05^{b}	0.15±0.01^{b}	0.84±0.05^{b}
DHA/EPA	n.d.	4.85±0.12^{b}	8.56±0.03^{a}	n.d.	n.d.	n.d.
DHA/ARA	n.d.	13.60±4.99	12.46±0.06	n.d.	n.d.	n.d.
n-3/n-6	0.19±0.00	1.45±0.01^{b}	1.64±0.01^{a}	0.11±0.02^{e}	0.59±0.00^{c}	0.55±0.00^{d}

*Different letters shows significant differences among groups

3.4.4. Amino acid composition of rotifers

At end of the feeding trial, essential amino acid compositions of rotifers showed that Histidine, Threonine, Tyrosine, Valine, Methionine, Isoleucine, Leucine, Phenylalanine, Lysine were significantly higher in rotifers fed INBY (p<0.05). On the contrary, Aspartic acid, Glutamic acid, Serine, Glycine, Alanine and Proline were

higher in rotifers fed spray-dried *N. oculata* (p<0.05). Arginine contents of rotifers were increased by feeding fresh *N. oculata* (p<0.05) (Table 3.6)

Table 3.6 Free amino acid compositions of rotifer biomass fed different feeds (mg g^{-1} dry weight, mean±SD, n=3 different superscripts denote significant differences among groups)

	Initial	**Algome®**	**PP®**	**INBY®**	**SDN**	**FN**
Aspartic acid	0.57±0.11	0.07±0.15^{d}	1.15±0.03^{b}	1.10±0.04^{c}	1.32±0.02^{a}	1.14±0.09^{bc}
Glutamic acid	0.67±0.01	1.33±0.16^{d}	1.66±0.14^{c}	1.71±0.07^{b}	1.85±0.07^{a}	1.65±0.01^{c}
Serine	0.23±0.01	2.36±0.01^{d}	0.24±0.02^{c}	0.32±0.02^{a}	0.32±0.02^{a}	0.25±0.00^{b}
Glycine	0.23±0.07	0.23±0.14^{d}	0.27±0.00^{c}	0.29±0.01^{b}	0.29±0.02^{a}	0.26±0.00^{c}
Histidine	0.10±0.03	0.11±1.41^{c}	0.10±0.02^{c}	0.16±0.01^{a}	0.14±0.05^{b}	0.11±0.02^{c}
Arginine	0.37±0.14	0.38±0.05^{d}	0.47±0.0^{b}	0.47±0.04^{b}	0.45±0.03^{c}	0.62±0.02^{a}
Threonine	0.23±0.00	0.12±0.00^{e}	0.15±0.02^{c}	0.21±0.00^{a}	0.20±0.02^{b}	0.13±0.02^{d}
Alanine	0.33±0.00	0.23±0.28^{d}	0.29±0.05^{c}	0.33±0.03^{b}	0.36±0.05^{a}	0.29±0.01^{c}
Proline	0.28±0.07	0.22±0.01^{c}	0.25±0.01^{b}	0.30±0.012^{a}	0.31±0.01^{a}	0.28±0.01^{b}
Tyrosine	0.26±0.07	0.12±0.03^{e}	0.27±0.07^{d}	0.32±0.04^{a}	0.28±0.02^{c}	0.28±0.00^{b}
Valine	0.32±0.02	0.24±0.4^{d}	0.33±0.02^{c}	0.4±0.015^{a}	0.36±0.03^{b}	0.31±0.02^{c}
Methionine	0.07±0.00	0.004±0.00^{d}	-	0.05±0.01^{a}	0.02±0.00^{c}	0.03±0.00^{b}
Isoleucine	0.30±0.03	0.21±0.02^{d}	0.30±0.02^{c}	0.38±0.04^{a}	0.32±0.03^{b}	0.29±0.02^{c}
Leucine	0.45±0.07	0.31±0.02^{e}	0.44±0.00^{d}	0.53±0.00^{a}	0.52±0.00^{b}	0.46±0.00^{c}

Phenylalanine	0.32±0.07	0.20±0.2^{e}	0.32±0.00^{d}	0.38±0.04^{a}	0.36±0.02^{b}	0.33±0.01^{c}
Lysine	0.81±0.01	0.49±0.07^{d}	0.82±0.01^{c}	1.18±0.03^{a}	1.06±0.08^{ab}	1.03±0.00^{b}
Free amino acid	5.61±0.01^{d}	5.18±0.02^{d}	7.06±0.04^{c}	8.15±0.01^{a}	8.17±0.05^{a}	7.49±0.07^{b}
Essential amino acid	2.6±0.06^{c}	1.72±0.04^{d}	2.46±0.08^{c}	3.61±0.04^{a}	2.98±0.01^{b}	2.69±0.09^{c}
Non-essential amino acid	3.01±0.01^{e}	3.46±0.02^{d}	4.6±0.02^{bc}	4.54±0.05^{c}	5.19±0.09^{a}	4.8±0.01^{b}

3.5. DISCUSSION

The microalgae *Nannochloropsis oculata* is commonly used in larviculture as greenwater (Skiftesvik *et al.*, 2003; Rocha *et al.*, 2008), for rotifer production (Ferreira *et al.*, 2009) or as feed ingredients in early weaning diets for marine fish larvae (Van der Meeren *et al*., 2007; Eryalçın *et al.*, 2013; Cavonius *et al.*, 2015; Eryalçın *et al.*, 2015). Due to their high nutritional value, suitable size and high productivity potential, this type of small size microalgae is still highly required for rotifer and marine fish larvae culture (Reitan *et al.*, 1997; Shields and Lupatsch, 2012). Marine microalgae have a high nutritional value in terms of lipid, protein and pigments contents (Borowitzka, 2013). They are processed in several ways and used as enrichment for rotifer and Artemia (Ma and Qin, 2014), feeds for copepods (Øie *et al.*, 2011; Knuckey *et al.*, 2005; Rasdi and Qin, 2016) and alternative protein and lipids ingredients in larval microdiets (Ganuza *et al.*, 2008; Walker and Berlinsky, 2011; Ju *et al.,* 2012; Patterson and Gatlin, 2013; Eryalçın *et al.*, 2013; Eryalçın *et al.*, 2015). However, the difficulty to optimize the nutritional value of fresh microalgae is still an issue to overcome depending on culture medium, harvesting time, labor talent and the physical and chemical conditions of culture water (Cho *et al.*, 2007). Therefore, several dried commercial rotifer feeds are widely used due to their high nutritional value and long-lasting duration in high biomass culture (Vigani *et al.*, 2015). Nevertheless, these products are expensive and commercial hatcheries still look for alternative feeding materials. In this study, although Protein Plus® diet increased essential fatty acids levels such as ARA, EPA and DHA in rotifer biomass, these results were not reflected in a higher growth of rotifer. On the other hand, spray-

dried *N. oculata* diet increased linoleic and alfa-linolenic acid levels in rotifer and rotifer led to a significantly higher growth performance.

Marine fish larvae require sufficient amount of essential fatty acids for their growth and survival (Izquierdo and Koven, 2011). Requirements of HUFAs in marine fish larvae such as EPA and DHA should be between 0.3 g kg^{-1} and 39 g kg^{-1} in weaning diets (Izquierdo, 1996). Those essential compounds should be delivered via live feeds to larvae. Proper ratio of n-3 to n-6 fatty acids is also important for optimal growth in marine fish larvae. In this study, we obtained the highest n-3/n-6 ratio (1.64) in rotifer fed Protein Plus®, with values similar to those in enriched rotifer (Bransden *et al.*, 2005). However, this feed was not as good as spray-dried *N. oculata* for rotifers growth. According to the FA profile, cultivation with Protein Plus® does not require fatty acid enrichments for rotifers with containing 25.16% of total n-3 HUFA levels. Commonly used microalgae *N. oculata* is known to be rich in EPA but with reduced DHA (Durmaz, 2007). In this study experimental, rotifers reflected the fatty acid profiles of the diets. Especially, essential fatty acids (ARA, EPA and DHA) and total n-3 levels were increased by feeding Protein Plus®. Fatty acid profiles of rotifers is related to what they ingest and digest. However, rotifer fed both *Nannochloropsis* feeds (spray-dried and fresh) did not contain sufficient amount of essential fatty acids (1.18% and 0.28%, respectively) compared to those fed Protein Plus® (25.16%) or Algome® (11.94%). One of the reason of this results could be related to different FA conversion metabolism in rotifers compared to copepods. Copepods apparently have the ability to convert 18:3n-3 into 20:5n-3 and 22:6n-3 (Nanton and Castell, 1999) this event is related to the desaturating capacity of selected copepod species, cultured temperature and given feeds. In the present study, rotifers were not synthetizing these fatty acids.

Rotifer culture is important for marine fish hatcheries and larval nutrition still depends on culture of rotifers in sufficient amount and high quality (Lubzens *et al.*, 1989; Lubzens *et al.*, 1995; Mæhre *et al.*, 2013). Success of rotifer culture in hatcheries not only depends on quantitate production but also on the quality of rotifer biomass. Therefore, rotifer culture management should be established in good quantitative and qualitative terms for successful marine fish larvae production in commercial hatcheries. In the present study, rotifers fed spray dried *N. oculata* diet resulted in

significantly higher rotifer biomass for 16 days of batch and semi-continuous culture. Although the spray-dried *N. oculata* supported high values of rotifer biomass, egg production and egg carrying female, essential fatty acid composition was enhanced by utilization of Protein Plus®. Rotifer is a filter feeding organism and the ingestion rate of feeds is directly related to the size of feeds (Dhont *et al.*, 2013). After ingestion, nutritional compounds play important roles for rotifer growth in batch culture (Rothhaupt, 1995; Cheng *et al.*, 2011). Therefore, selection of food type and size in rotifer feeding is essential to ensure ingestion of feeds by rotifers.

The present work shows that spray-dried *N. oculata* results in high egg number production and egg carrying female rotifers during 16 days of experiment. Additionally, rotifers fed spray-dried *N. oculata* showed high total biomass for the first 11 days of experiment. These results confirm that the experimental spray-dried *N. oculata* can be totally replaced by other commercial rotifer diets such as Protein Plus. However, when nutritional properties are considered, rotifers fed spray-dried *N. oculata* were not appropriate for nutrition of marine fish larvae. These results demonstrate that although spray-dried *N. oculata* is a perfect feed for rotifer cultivation, before being fed to marine fish larvae these rotifers would require a previous enrichment with EFA. Therefore, it would be preferable to use a great rotifer food, followed by an enrichment product that optimizes the use of n-3 HUFA.

Essential amino acids (EAA) pool of rotifers and formulated diets has been reported in other studies (Aragão *et al.*, 2004; Li *et al.*, 2009; Mæhre *et al.*, 2013). In this study, Algome® diet seems to be good enough amino acid composition including 18.42% of EAA for larval requirements. However, rotifer fed Algome® diet did not reflected high EAA accumulation (1.72%), whereas, rotifer fed INBY and spray-dried *N. oculata* showed a better essential amino acids (EAA) profile (3.61% and 2.98%, respectively) resulting in higher protein content and higher growth than those fed Algome®. These improved EAA profiles are contributed to inclusion of living organisms such as *Saccharomyces cerevisiae* and *N. oculata*. In free amino acids, histidine, arginine, glycine and alanine play important roles in survival, growth and physiological functions such as protection against pH change, neurological function and development, cell signaling, blood flow, appetite of larval and juvenile marine fish (Conceição *et al.*, 2003; Li *et al.*, 2009). According to the EAA composition of

rotifer diets, Algome® seems to be the best diet for amino acid sources for larvae (Srivastava *et al*., 2006). Algome® includes dried *Schizocytrium* sp., which is known for its high DHA content (Ganuza *et al.*, 2008). However, rotifers fed with Inactive baker's yeast and spray-dried *N. oculata* showed significantly higher amount of free amino acid values at the end of the experiment.

From the point of view of commercial marine fish hatcheries, high growth rate and high nutritional value of rotifer should be enhanced in order to sustain both quantity and quality of culture marine fish species (Ma *et al.*, 2014; Maisashvili *et al.*, 2015). For that reason, several artificial diets for rotifers are commonly used to simplify rotifers production. Among them, macroalgal detritus are also being investigated for rotifer culture (Yin *et al.*, 2013). It is suggested that rotifers fed fresh *Nannochloropsis* sp. enhanced rotifer population compared to macroalgal detritus. On the other hand, utilization of half and half both macroalgae detritus and fresh microalgae could give the same result in growth rotifer fed only fresh microalgae. Similarly, flocculated and paste form of *N. oculata* also performed much better than commercial products for juvenile seahorse culture (Sales *et al.*, 2016). In our study, one of the reasons that not well performed fresh *N. oculata* as good as spray-dried could be related to higher ash content in fresh form and this different physicochemical properties that make the nutrients less available than those of spray dried microalgae. Therefore, processed microalgae meal is more convinient for widespread utilization in hatcheries. Enhancing the good results of Protein Plus®, although not so good as spray-dried *N. oculata* most probably related to good protein and lipid contents than Baker´s yeast and Algome®. Better amino acid profile, i.e. lower Iso and Phe than Baker´s yeast and Algome®, but similar to spray dried group. More similar in Val, Iso and Phe to the spray dried *N. oculata*. Moreover, inactive bakers yeast and Algome® did not supported rotifer growth. Algome® has a very low protein and different amino acid profile than the other experimental feeds. Baker's yeast contain high protein but the profile different to the spray-dried *N. oculata* and indeed produced rotifers with lower protein denoting that the amino acid profile was not very good for rotifers, besides it is too high in 18:1n-9, but low in 18:2n-6 or 18:3n-3.

3.6. CONCLUSIONS

In conclusion, hatchery produced spray-dried *Nannochloropsis oculata* was the best option for longer rotifer cultivation performance since produced a high number of rotifers. Freshly culture *Nannochloropsis oculata* can be cultured and spray-dried form can be used as alternative for rotifer feeds and desirable EFA composition should be maintained by enrichment once a high production number is achieved. Further study should focus on combined utilization of both rotifer diets, Protein Plus (EFA source) and inactive baker's yeast (EAA source), to provide rotifers with the best nutritional properties during their production. Recent studies also focused on the effect of the mineral compositions in rotifers, such as selenium, zinc, copper, manganese and iodine levels, on larval fish requirements. It is also need to investigate on the mineral and vitamin contents of rotifers fed with different commercial rotifer feeds and microalgae diets.

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CHAPTER 4

Aquaculture Research

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Fish oil replacement by different microalgal products in microdiets for early weaning of gilthead sea bream (*Sparus aurata*, L.)

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4.1. ABSTRACT

The aim of this study was to determine if algal products rich in DHA or ARA are able to completely replace fish oil in microdiets for marine fish larvae, gilthead seabream and if extra supplementation with EPA may further enhance larval performance. For that purpose, 20 day-old gilthead seabream larvae of 5.97±0.4 mm mean total length and 0.12±0.001 mg mean dry body weight were fed with five microdiets tested by triplicate: a control diet based on sardine oil; a diet containing AquaGrow® DHA (diet DHA) to completely substitute the sardine oil; a diet containing AquaGrow® ARA (diet ARA); a diet containing both products, AquaGrow® DHA and AquaGrow® ARA to completely substitute the fish oil; and, a diet containing both products, AquaGrow® DHA and AquaGrow® ARA, together with an EPA source. Temperature, air and salinity activity tests were also performed to detect larval resistance to stress. At the end of the experiment, final survivals did not differ among groups. The microorganism produced DHA was able to completely replace fish oil in weaning diets for gilthead seabream without affecting survival, growth or stress resistance, whereas the inclusion of microorganism produced ARA did not improve larval performance. Moreover, addition of EPA to diets with total replacement of fish oil by microorganism produced DHA and ARA, significantly improved growth in terms of body weight and total length. The results of this study denoted the good nutritional value of microorganisms produced DHA as a replacement of fish oil in weaning diets for gilthead seabream, without a complementary addition of ARA. However, dietary supplementation of EPA seems to be necessary to further promote larval performance.

4.2. INTRODUCTION

Successful rearing of marine fish larvae is greatly affected by highly unsaturated fatty acids (HUFA) profiles of live prey and microdiets. Traditional HUFA sources derive from fish oil (Izquierdo, 1996), but the high demand of this limited resource obtained from wild catches markedly increases its price and constrains its use in the fast developing aquaculture industry (Izquierdo *et al.*, 2005). There are also some other limitations of the fish oil use, such as the difficulty of maintaining high oxidative stability of polyunsaturated fatty acids in fish oil. The quality of fish oil depends on the fishing season and the species it is obtained from. Besides, fish oil may be contaminated by environmental pollution. Some PCBs (polychlorinated biphenyls)

and organochlorine chemicals such as chlordane, dioxins and dichloro-diphenyltrichloroethane have been reported in fish (Mac *et al.*, 1979). In addition to these constraints, purification of specific polyunsaturated fatty acids from marine fish oil such as DHA, EPA and ARA is difficult because of the several fatty acids with varying lengths and degrees of unsaturation (Vazhappilly and Chen, 1998; Sijtsma and Swaaf, 2004). Therefore, the suitability of alternative HUFA sources is being investigated to completely replace fish oil in aquafeeds (Turchini *et al.*, 2009). Docosahexaenoic (DHA, 22:6n-3), eicosapentaenoic (EPA, 20:5n-3) and arachidonic (ARA, 20:4n-6) acids are essential polyunsaturated fatty acids with important roles for fish larvae and culture performance (Izquierdo and Koven, 2011). Among different HUFA, DHA is the most important essential fatty acid to promote marine fish larvae growth (Watanabe *et al.*, 1989; Izquierdo and Koven, 2011). DHA is found in high contents in olfactory nerve, retina and neural system at the first developing stages (Tocher and Harvie, 1988). EPA is also important for marine fish larval growth, survival and stress resistance in diets with low (Watanabe *et al*., 1989) or high (Liu *et al.*, 2002) content of DHA. However, excess EPA content or unbalanced EPA/DHA ratios may lead to mortalities on marine fish larvae (Toyota *et al.*, 1991; Rodriguez *et al.*, 1994). ARA (22:4n-6) is another important essential fatty acid, which may improve growth and survival (Bessonart *et al.*, 1999), stress resistance (Ganga *et al.*, 2005; Van Anholt *et al.*, 2004; Xu *et al.*, 2010) and flatfish malpigmentation (Estevez *et al.*, 1999; Lund *et al.*, 2010). ARA is a preferred substrate for eicosanoids production such as 2-series prostanoids and 4-series leukotrines. ARA and EPA compete with each other as substrates for cyclo-oxygeneases and lipooxygenases and, therefore, an adequate ratio of EPA/ARA should be maintain in the diet to obtain better survival, growth, pigmentation and complete metamorphosis (Bell and Sargent, 2003). Thus, complete fish oil replacement in diets for marine fish requires the use of other HUFA sources to fulfill the requirements for those three fatty acids. Several studies have been focused on HUFA production from single-cell microorganisms (Ratledge, 2001; de Swaaf, 2003; Pulz and Gross, 2004; Spolaore *et al.*, 2006; Ganuza and Izquierdo, 2007). Marine microalgae are a basic step of the aquatic food chain and the prime organisms for HUFA production. In aquaculture, they are used to produce molluscs, crustaceans, marine fish larvae and as feeds for live prey of fish larvae such as rotifer (*Brachionus plicatilis*). They have crucial effects on rotifers during the enrichment process in

terms of delivering HUFA, vitamins or pigments to live prey (Muller-Fuega, 2000). HUFA can be produced either phototrophically or hetetrophically from these microalgae. Appropriate species selection together with suitable culture techniques can lead to a high production of HUFA. Microalgae have been used as DHA sources to replace fish oil in gilthead seabream larvae microdiets (Atalah *et al.*, 2007). However, such replacement may lead to reduced dietary contents of an essential fatty acid, EPA, and this could reduce the larval growth potential (Ganuza *et al.*, 2008). Therefore, the aim of this study was to determine if algal products rich in DHA or ARA are able to completely replace fish oil in microdiets for marine fish larvae, gilthead seabream and if extra supplementation with EPA may further enhance larval performance.

4.3. MATERIALS AND METHODS

4.3.1. Experimental diets

Five experimental microdiets (pellet size <250 μm) with different sources of DHA, ARA and EPA were prepared (Table 4.1): a control diet (diet Control) based on sardine oil and squid protein, which had been successfully used in other studies to wean gilthead seabream larvae; a diet containing AquaGrow® DHA (diet DHA) to completely substitute the sardine oil and increase DHA contents; a diet containing AquaGrow® ARA (diet ARA) to increase the total dietary ARA level; a diet containing both products, AquaGrow® DHA and AquaGrow® ARA to completely substitute the fish oil with high levels of both dietary ARA and DHA (diet DHA+ARA); and finally, a diet containing both products, AquaGrow® DHA and Aqua-Grow®ARA, together with an EPA source to completely substitute the fish oil with high levels of DHA, EPA and ARA (diet DHA+ARA+EPA). The desired lipid content was completed with a non-essential fatty acid source, oleic acid. Fatty acid composition of total lipids (% dry weight) in the experimental diets is given in Table 4.2. The ingredients were mixed in a mortar and then combined with gelatin dissolved in water at 80°C. The paste obtained was compressed pelleted and dried in an oven at 40°C for 24 h. The pellets were ground in a Sample Mill (Braun KSM 2) and sieved to obtain a particle size of 150–250 μm (Atalah *et al.*, 2011).

Table 4.1 Lipid ingredients and proximate composition of the experimental diets

	Control	DHA	ARA	DHA+ARA	DHA+ARA+ EPA
Ingredients (g kg^{-1} of diet)					
Squid Powder [a]	75.60	69.50	72.75	67.47	68.10
DHA Aquagrow® [b]	-	11.00	-	11.00	6.00
ARA Aquagrow® [c]	-	-	3.03	3.03	2.50
EPA 50[d]	-	-	-	-	3.40
Fish oil[e]	5.90	-	5.72	-	-
Gelatin	3.00	3.00	3.00	3.00	3.00
Oleic acid	-	1.00	-	-	1.50
Soy Lecithin	2.00	2.00	2.00	2.00	2.00
Vitamin Mix[g]	6.00	6.00	6.00	6.00	6.00
Mineral Mix[h]	4.50	4.50	4.50	4.50	4.50
Attractant	3.00	3.00	3.00	3.00	3.00
Proximate composition ([i]% dry weight)					
Crude Lipid	18.36	18.38	20.85	19.75	19.51
Crude Protein	63.97	64.60	61.68	59.09	59.61
Ash	6.16	6.80	7.87	8.56	7.49

[a]Squid meal (Riber & Son, Bergen, Norway).
[b]Aquagrow DHA, ABN Advanced Bionutrition, Columbia, USA.
[c]Aquagrow ARA, ABN Advanced Bionutrition, Columbia, USA.
[d]EPA 50 (CRODA, East Yorkshire, England, UK).
[e]Fish oil (Capelin oil,Denofa, Fredrikstad, Norway).
[f]Oleic acid Vegetable, Merch, Darmstadt, Germany.
[g]The vitamin mixture contained (mg 100 g^{-1} diet): choline chloride (2965.8), inositol (1450.9), nicotinic acid (290.16), ascorbyl polyphosphate (180), a-tocopherol (150), p-aminobenzoic acid (145), calcium panthotenate (101.59), riboflavin (72.53), thiamin HCl (21.77), piridoxyne HCl (17.28), menadione (17.28), astaxanthin (5) cholecalciferol (3.65), retinol acetate (0.18), cyanocobalamin (0.03).
[h]Teshima, Kanazawa and Sakamoto (1982).
Squid Powder (Crude protein: 82%, Crude lipid: 17%)
DHA Aquagrow (Crude protein: 13%, Crude lipid: 35%).
ARA Aquagrow (Crude Protein: 15%, Crude lipid: 33%).

Table 4.2 Fatty acid compositions of the experimental diets (% dry weight, n=3)

Fatty Acid (%)	C	DHA	ARA	DHA +ARA	DHA +ARA +EPA
14:0	0.31	0.93	0.38	1.07	0.55
15:0	0.06	0.04	0.06	0.05	0.04
16:0ISO	0.06	0.05	0.06	0.05	0.04
16:0	3.23	3.32	3.65	3.70	2.71
16:1n-7	0.30	0.18	0.32	0.20	0.13
16:2n-6	0.03	0.01	0.04	0.01	0.01
16:2n-4	0.08	0.06	0.09	0.07	0.06
17:0	0.07	0.01	0.04	0.01	0.02
16:3n-3	0.02	0.02	0.02	0.03	0.02
16:3n-1	0.05	0.04	0.05	0.04	0.04
18:0	0.64	0.49	0.79	0.64	0.52
18:1n-9	2.61	2.83	2.84	2.02	2.81
18:1n-7	0.33	0.15	0.36	0.16	0.18
18:1n-5	0.03	0.02	0.03	0.02	0.02
18:2n-6	3.53	1.65	3.91	1.65	1.63
18:2n-4	0.01	n.d.	0.01	n.d.	0.03
18:3n-6	0.01	0.00	0.09	0.08	0.07
18:3n-4	0.01	0.00	0.02	0.01	0.01
18:3n-3	0.49	0.14	0.51	0.14	0.16
18:4n-3	0.05	0.01	0.06	0.01	0.18
20:0	0.03	0.02	0.04	0.03	0.03

20:1n-9+n7	0.65	0.36	0.65	0.37	0.35
20:1n-5	0.03	0.01	0.03	0.01	0.02
20:2n-6	0.06	0.03	0.07	0.04	0.04
20:3n-6	0.01	n.d.	0.07	0.06	0.07
20:4n-6	0.14	0.14	0.77	0.79	0.70
20:3n-3	0.08	0.06	0.08	0.06	0.05
20:4n-3	0.06	0.01	0.06	0.01	0.10
20:5n-3	1.34	1.08	1.37	1.15	3.54
22:1n-11	0.24	0.02	0.23	0.01	0.04
22:1n-9	0.07	0.04	0.07	0.04	0.03
22:4n-6	0.03	0.01	0.03	0.01	0.08
22:5n-6	0.04	0.03	0.03	0.03	0.04
22:5n-3	0.16	0.09	0.17	0.09	0.14
22:6n-3	3.48	6.46	3.76	7.02	4.95
∑ Saturates	4.30	4.82	4.97	5.50	3.87
∑ Monounsaturated	4.26	3.62	4.56	2.84	3.59
∑ (n-6)	3.84	1.87	5.01	2.66	2.63
∑ (n-3)	5.70	7.88	6.06	8.53	9.16
∑ (n-3) HUFA	5.12	7.70	5.44	8.34	8.78
EPA:DHA	0.39	0.17	0.36	0.16	0.72
EPA:ARA	9.37	7.95	1.77	1.46	5.06

Dietary fatty acids less then <0.03% are not shown in the table (14:1n-5, 14:1n-7, 15:1n-5, 16:1n-5, 16:0, 16:3n-4, 16, 4n-1, 16:4n-3, 18:2n-9, 18:3n-1, 18:4n-1, 20:2n-9, 20:3n-9).

4.3.2. Larval feeding

Thirty one thousand and five hundred 20 day-old gilthead seabream larvae (5.97 ± 0.4 mm total length, mean ± SD; 0.12 ± 0.001 mg dry body weight) were distributed (2100 individuals per tank) in 15 fibreglass cylindrical tanks (200 litres) filled with

filtered sea water at a temperature of 18.3 ± 0.2°C. Tanks were provided with a photoperiod of 12 h artificial light. Larvae were fed the experimental diets for 21 days at a rate of 2.0-2.5 g day^{-1} by manual feeding every 45 min during light periods. The first 4 days of experiment, larvae were fed with non-enriched rotifers twice daily at 12:00 and 16:00 hours to obtain a rotifer density of 2 $ind.ml^{-1}$ in rearing tanks. The tanks were siphoned daily to remove uneaten feeds and faeces. Following feeding larvae were observed under a binocular microscope to determine feed acceptance. If apparent feed intake differences were observed along different experimental diets, diet acceptance was determined calculating the percentage of gut occupation by the microdiet using image analysis of larvae pictures. For such study, 30 larvae per tank were taken, their abdominal cavity was observed in a stereoscope (Leica Wild M3Z, Optotek, California, USA) and the area of the gut occupied by digested matter was measured on the optic micrographs taken at a magnification of 25 µm, using Image ProPlus® (Media Cybernetics Inc., Silver Springs, MD, USA) semiautomatic image analysis system. Larval growth was determined by measuring the total length of 30 larvae at 20-27-34 and 41 days post hatched (dph) using a profile projector (Mitutoyo Profile Projector PJ-A3000). At the end of the experiment, survival rate was determined by counting all live larvae and air exposure; salinity or temperature stress resistance tests were applied to groups of 25 alive larvae in each tank. In air exposure test, larvae were held out of the water for 1 min 30 sec. in a net and then moved into 2-L beaker containing aerated seawater. For salinity stress test, larvae were put into freshwater for 1 min and then placed in seawater again. For temperature stress test, 25 larvae removed from their tank where the water temperature was about 18.3°C were put in 2-L beaker filled with 15°C seawater for 1 min. They were then removed into 2-L tanks of initial temperature, where they were kept for 24 h. After 24 h, survival was determined by counting all the remaining alive and dead larvae. At the end of the feeding trial, all larvae in the tanks and microdiets were kept in -80°C until analysed. Moisture (A.O.A.C 1995), crude protein (A.O.A.C 1995) and crude lipid (Folch *et al.*, 1957) contents of larvae and diets were analysed. Fatty acid methyl esters were obtained using transmethylation of crude lipids as described by Christie (1982) separated using GLC, quantified using FID (GC-14A, Shimadzu, Tokyo, Japan) under the conditions described in Izquierdo *et al.* (1990) and identified by comparison to previously characterized standards and GLC-MS.

4.3.3. Statistical analysis

All data were treated using one-way ANOVA and means were compared by Duncan's test (p<0.05) using SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).

4.4. RESULTS

After 7 days of feeding, microphotography of larval digestive system showed a high degree of gut occupation, regardless the diet used. Along the feeding trial, no significant differences were found in body weight of fish fed DHA, ARA or DHA+ARA diets in comparison with Control diet (Figure 9). However, larvae fed DHA+ARA+EPA diet showed a body weight significantly (p<0.05) higher than that of DHA, ARA or DHA+ARA fed larvae even after only 7 or 15 days of feeding. Similarly, after 15 and 21 days of feeding, larvae fed DHA+ARA+EPA diet had a significantly (p<0.05) higher total length than the larvae fed any other experimental diet (Figure 10).

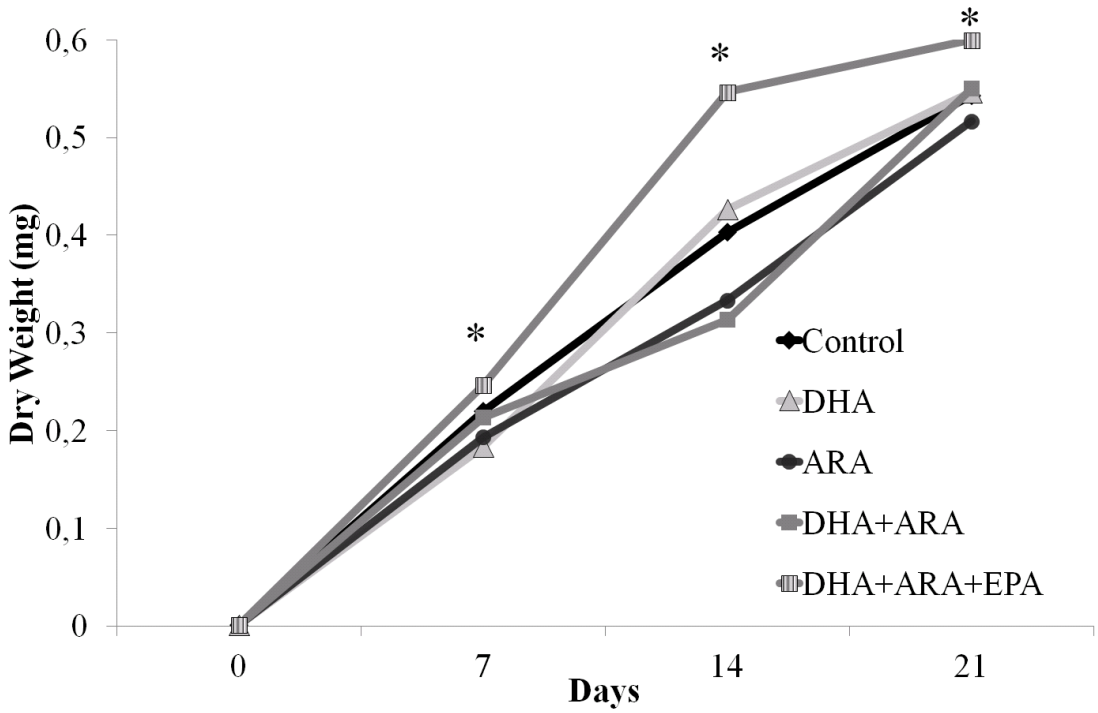

Figure 9. Progress of body weight of gilthead seabream larvae fed diets with different lipid sources along the feeding trial *(p<0.05).

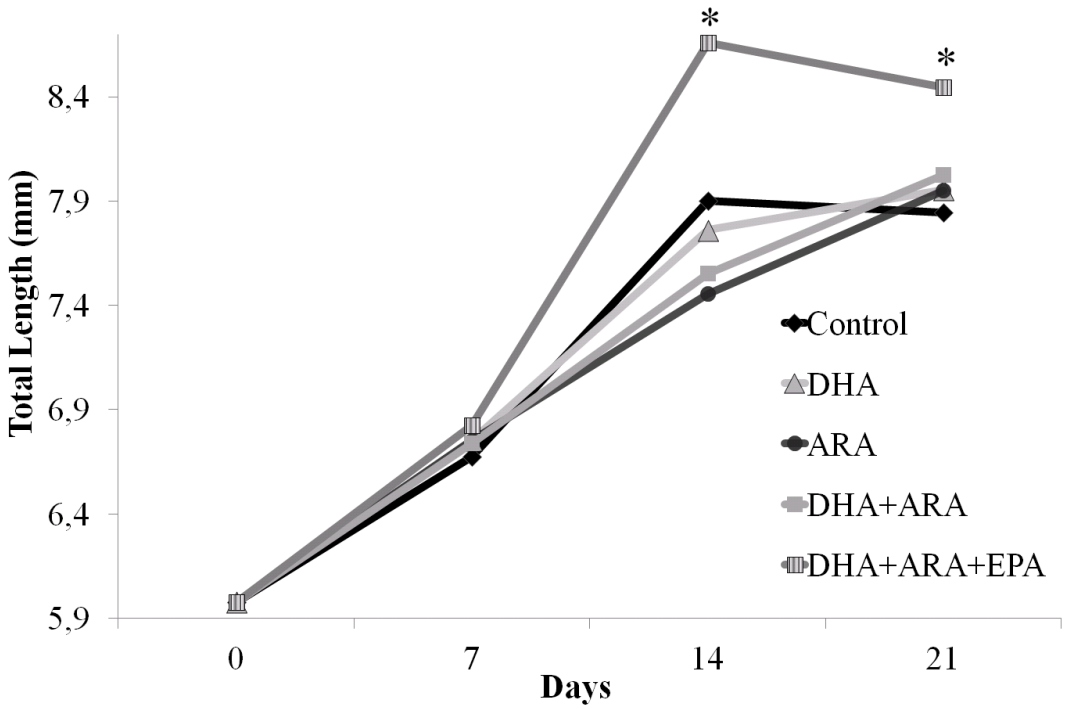

Figure 10. Progress of total length of gilthead seabream larvae fed diets with different lipid sources along the feeding trial *(p<0.05).

At the end of the study, no significant differences were found in protein and lipid composition of larvae fed the different experimental diets (Table 4.3). Analysis of fatty acid profiles of experimental fish (% dry body weight) showed that feeding all the experimental diets reduced the content of saturated fatty acids (mainly 16:0 and 18:0). Only DHA, DHA+ARA, DHA+ARA+EPA microdiets showed reduction in monounsaturated fatty acids in comparison with the initial larvae, whereas n-3 fatty acids content, particularly that of DHA, was increased for all diets (Table 4.3). On the contrary, except for the larvae fed diet DHA+ARA+EPA, EPA contents in larvae fed the experimental diets were lower than in the initial larvae. Fish fed the control diet showed the highest linoleic (18:2n-6) and linolenic (18:3n-3) acids contents. Feeding DHA (DHA, DHA+ARA & DHA+ARA+EPA diets) particularly increased DHA contents in the larvae, whereas feeding ARA (ARA, DHA+ARA & DHA+ARA+EPA diets) increased larval ARA content. Addition of EPA (DHA+ARA+EPA diet) significantly increased the larval EPA contents in comparison with fish fed the other diets, which also had lower EPA than the initial larvae. Thus, fish fed diets DHA, DHA+ARA & DHA+ARA+EPA showed a significantly (p<0.05) higher n-3 HUFA content. EPA/DHA was significantly higher in fish fed DHA+ARA+EPA diets.

Table 4.3 Fatty acid compositions of gilthead sea bream after feeding 21 days with diets containing different lipid sources (% dry weight, mean ± SD, n = 3, different superscripts denote significant differences among larvae fed different diets in selected fatty acids)

Fatty Acid (%)	Initial	C	DHA	ARA	DHA +ARA	DHA +ARA +EPA
Crude Lipid	16.11	19.61±1.64	18.55±1.42	19.02±1.17	20.66±1.74	18.87±1.07
Crude Protein	60.7	77.3±0.54	78.95±0.14	74.81±0.48	77.02±0.82	77.1±0.75
14:0	0.15	0.14±0.06	0.28±0.11	0.15±0.05	0.29±0.15	0.18±0.08
14:1n-7	0.04	0.02±0.05	0.01±0.01	0.01±0.02	0.02±0.01	0.02±0.02
15:0	0.09	0.06±0.03	0.05±0.03	0.06±0.02	0.05±0.02	0.05±0.02
16:0	3.57	3.87±3.53	3.74±0.50	3.97±3.46	4.19±2.16	3.51±2.20
16:1n-7	0.7	0.26 ± 0.26^{a}	0.19 ± 0.03^{b}	0.21 ± 0.15^{ab}	0.22 ± 0.09^{ab}	0.17 ± 0.06^{ab}
16:1n-5	0.11	0.06±0.07	0.05±0.04	0.05±0.03	0.06±0.00	0.06±0.02
16:2n-6	0.07	0.10±0.02	0.10±0.02	0.10±0.03	0.08±0.32	0.06±0.25
16:2n-4	0.21	0.13±0.05	0.11±0.00	0.12±0.09	0.12±0.04	0.12±0.07
17:0	0.09	0.04±0.05	0.03±0.00	0.04±0.02	0.04±0.04	0.04±0.03
16:3n-3	0.01	0.02±0.01	0.05±0.01	0.02±0.01	0.06±0.02	0.04±0.01
16:3n-1	0.17	0.08±0.04	0.08±0.02	0.09±0.04	0.10±0.03	0.08±0.02
16:4n-3	0.03	0.04±0.02	0.06±0.03	0.05±0.01	0.06±0.02	0.06±0.00

18:0	2.46	1.48±1.16	1.33±0.26	1.61±1.92	1.62±0.85	1.38±1.11
18:1n-9	1.44	2.21±0.74	2.21±0.32	2.04±1.03	1.93±0.58	2.14±0.46
18:1n-7	0.58	0.49±0.06	0.30±0.08	0.40±0.22	0.36±0.23	0.35±0.13
18:1n-5	0.06	0.04±0.05	0.03±0.01	0.04±0.02	0.03±0.01	0.03±0.01
18:2n-6	0.55	2.25±0.72^{c}	1.00±0.08^{a}	1.88±1.12^{b}	1.00±0.25^{a}	0.87±0.11^{a}
18:3n-6	0.02	0.04±0.06	0.03±0.01	0.05±0.04	0.05±0.01	0.05±0.02
18:3n-4	0.01	0.02±0.05	0.02±0.01	0.03±0.01	0.02±0.00	0.02±0.03
18:3n-3	0.05	0.20±0.08^{c}	0.05±0.01^{a}	0.15±0.12^{b}	0.05±0.01^{a}	0.05±0.03^{a}
18:4n-3	0.01	0.02±0.01	0.01±0.00	0.02±0.03	0.01±0.03	0.05±0.05
20:0	0.04	0.03±0.03	0.03±0.01	0.04±0.04	0.03±0.02	0.03±0.02
20:1n-9+n7	0.16	0.41±0.13	0.24±0.04	0.34±0.18	0.25±0.06	0.21±0.05
20:1n-5	0.08	0.03±0.03	0.02±0.00	0.03±0.02	0.03±0.01	0.02±0.01
20:2n-6	0.09	0.16±0.10	0.08±0.00	0.13±0.03	0.09±0.02	0.06±0.02
20:3n-6	0.06	0.02±0.03	0.01±0.00	0.05±0.01	0.05±0.01	0.04±0.01
20:4n-6	0.39	0.26±0.12^{a}	0.22±0.04^{a}	0.83±0.26^{c}	0.92±0.12^{c}	0.76±0.24^{b}
20:3n-3	0.01	0.06±0.03	0.04±0.00	0.05±0.02	0.04±0.01	0.05±0.01
20:4n-3	0.06	0.04±0.01	0.01±0.00	0.03±0.02	0.01±0.00	0.04±0.02
20:5n-3	1.23	1.14±0.50^{b}	0.81±0.09^{c}	0.87±0.53^{b}	0.78±0.14^{c}	1.94±0.23^{a}

22:1n-11	0.02	0.10±0.05	0.01±0.01	0.09±0.06	0.03±0.13	0.04±0.19
22:1n-9	0.06	0.11±0.05	0.07±0.04	0.08±0.03	0.04±0.12	0.03±0.11
22:4n-6	0.02	0.02±0.01	0.01±0.03	0.04±0.11	0.07±0.02	0.05±0.03
22:5n-6	0.07	0.05 ± 0.02^{a}	0.04 ± 0.00^{b}	0.05 ± 0.02^{a}	0.04 ± 0.01^{b}	0.05 ± 0.01^{a}
22:5n-3	0.50	0.28±0.15	0.17±0.02	0.24±0.09	0.18±0.06	0.31±0.13
22:6n-3	2.41	5.24 ± 1.32^{a}	7.02 ± 0.98^{c}	5.03 ± 1.55^{a}	7.67 ± 1.45^{c}	5.84 ± 1.86^{b}
∑ Saturates	6.4	5.63±4.75	5.45±0.86	5.86±5.38	6.23±3.07	5.19±3.36
∑Mono unsaturated	3.21	3.74 ± 1.88^{c}	3.15 ± 0.27^{bc}	3.28 ± 1.71^{bc}	2.97 ± 0.91^{a}	3.09 ± 0.74^{ab}
∑ (n-6)	1.26	2.90 ± 0.95^{a}	1.49 ± 0.15^{c}	3.12 ± 1.46^{a}	2.30 ± 0.67^{b}	1.94 ± 0.56^{b}
∑ (n-3)	4.32	7.05 ± 2.09^{b}	8.22 ± 1.05^{a}	6.46 ± 2.33^{b}	8.86 ± 1.68^{a}	8.37 ± 2.14^{a}
∑ n-3 HUFA	4.22	6.76 ± 1.99^{a}	8.05 ± 1.08^{b}	6.22 ± 2.19^{a}	8.68 ± 1.65^{b}	8.16 ± 2.16^{b}
EPA:DHA	0.51	0.22 ± 0.01^{ab}	0.12 ± 0.00^{a}	0.17 ± 0.01^{ab}	0.10 ± 0.00^{a}	0.33 ± 0.01^{b}
EPA:ARA	3.19	4.43 ± 0.51^{a}	3.61 ± 0.18^{b}	1.05 ± 0.06^{d}	0.84 ± 0.03^{d}	2.57 ± 0.10^{c}

*Fatty acids of experimental fish less then <0.03% are not shown in the table (14:1n-5, 15:1n-5, 16:1n-5, 16:3n-4, 16:0ISO, 16:4n-1, 18:2n-9, 18:3n-1, 18:4n-1, 20:2n-9, 20:3n-9).

No significant differences were found in survival after activity test of air exposure (94.4±4.49%, mean ± SD) and temperature (94±3.96%, mean ± SD) among larvae fed the different experimental diets. However, after the salinity test, larvae fed DHA+ARA+EPA diet had a significantly lower survival than larvae fed control and ARA diets (p<0.05) (Figure 11). Final survival was not significantly different among all groups, being between 37% and 48% in larval feeding stage (Figure 12).

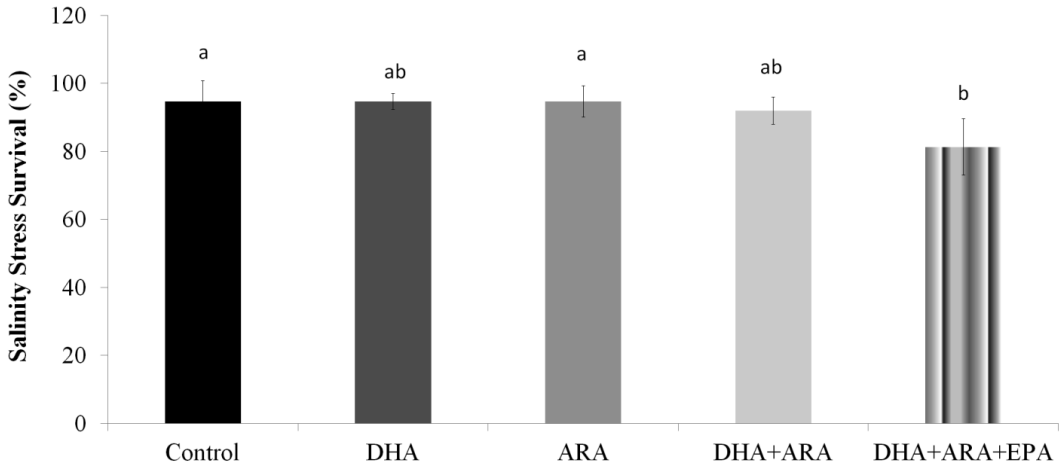

Figure 11. Salinity stress survival of gilthead sea bream larvae (%, mean ± SD).

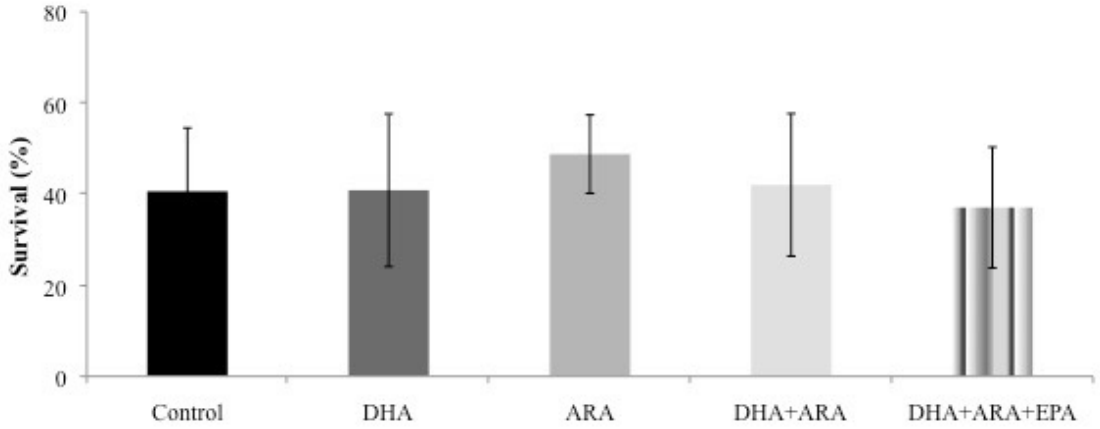

Figure 12. Survival of gilthead sea bream larvae at the end of the experiment (%).

4.5. DISCUSSION

In this study, Aquagrow DHA® provided DHA from the marine dinoflagellate *Crypthecodinium cohnii*, which can be cultivated heterotrophically. The microorganism is characteristic of good DHA production, high biomass, and high growth rate and without occurrence of EPA (Vazhappilly and Chen, 1998; Swaaf *et al.*, 1999). Aquagrow ARA® is a spray-dried nutrition product that provides a high level of ARA. The microorganism produced DHA was able to completely replace fish oil in weaning diets for gilthead seabream without affecting survival, growth or stress resistance. Certain microorganisms such as *Schizochytrium* sp. and *Crythecodinium cohnii* can constitute interesting sources of DHA (Navarro and Sarasquete, 1998; Lazo *et al.*, 2000; Harel *et al.*, 2002; Atalah *et al*., 2007; Miller *et al.*, 2007; Ganuza *et al*., 2008; Li *et al.*, 2010). For instance, fish oil substitution by *Cryptecodinium cohnii*

in diets for gilthead seabream resulted in good survival and a very good growth performance, in relation to a higher proportion of dietary DHA (Atalah *et al*., 2007; Ganuza *et al*., 2008). Survival rate of gilthead sea bream larvae fed microdiets varies largely in different studies depending on the larval age and the co-feeding with live feed or not. Survival rates vary from 7 to 25% for larvae fed with inert diets from mouth opening to day 21 (Robin and Vincent, 2003), whereas feeding from 4 to 15 days old survival ranges from 62 to 73% (Yufera *et al.*, 1999). In this study, larval survival rates of 37% to 48% for 20–41-day-old larvae are comparable to the values (45–60%) obtained for 16–31-day-old larvae (Ganuza *et al*. 2008) without co-feeding with live feeds. Despite the efforts to obtain DHA production by heterotrophic marine organisms that accumulate HUFA, the number of microorganisms used is very limited (Barclay *et al.*, 1994; Kyle, 1996; Ratledge, 2001; de Swaaf, 2003). However, these organisms can be an excellent alternative to fish oil use in aquafeeds, if production techniques for this organisms are improved to reduce their cost and increase their availability.

The content of DHA in diets where fish oil was completely substituted by microorganism produced DHA (over 3% in dry weight basis) was higher than the optimum dietary levels previously suggested for gilthead seabream (0.8% in dry weight basis, Izquierdo *et al*., 2005). In other species, such as red seabream (*Pagrus major*) (Izquierdo *et al.*, 1989), striped trumpeter (*Latris lineata*) (Bransden *et al.*, 2005) or common dentex (*Dentex dentex*) (Mourente *et al.*, 1999), the minimum DHA requirement for optimum growth has been reported to be 1.2, 2.3, and 2.0% dw DHA respectively. Therefore, even lower contents of DHA Aquagrow® in weaning diets for gilthead seabream may be adequate to promote culture performance. Nevertheless, the high DHA contents used in this study did not have a negative impact in larval production in agreement with the higher dietary levels used in these types of diets by other authors (Liu *et al*., 2002; Izquierdo *et al.*, 2010). Despite in this study microalgae produced DHA was found to be a good source of DHA for gilthead seabream weaning diets, its content in EPA, another essential fatty acid for marine fish larvae may be insufficient, in view of the improved growth obtained when this product was complemented with an EPA source. Thus, in this study, addition of EPA to diets with total replacement of fish oil by microorganism produced DHA and ARA significantly improved growth in terms of body weight and total length. Previous

studies have shown that even when the DHA requirements are fulfilled in the diet, low dietary EPA can negatively affect growth, survival and resistance to stress (Liu *et al*., 2002). EPA is an essential fatty acid for marine fish larvae, as its synthesis is very limited in these fish, despite its important roles in fish metabolism (Izquierdo and Koven, 2011). EPA is important regulator of eicosanoid synthesis and a good substrate for both cycloxygenases and lipoxygenases (Ganga *et al*., 2005). Although some eicosanoids derived from EPA can have a lower biological activity than those derived from ARA, others are equally active. Besides being abundant in the natural preys, EPA is a preferred substrate for digestive lipolytic enzymes (Iijima *et al.*, 1990; Izquierdo *et al.*, 2000) and a potential important energy reserve as it is a preferential substrate over DHA for mitochondrial beta-oxidation (Sargent *et al.*, 2002). Also, it is neccesary to complete flatfish development (Copeman *et al.*, 2002). Thus, dietary EPA as a sole HUFA improves larval performance, in terms of survival, stress resistance and growth in red seabream (Watanabe *et al*., 1989), flatfish (Furuita *et al.*, 1998) or yellowtail (Furuita *et al.*, 1996). Its requirement has been estimated to be between 0.7% and 1.6% for different species (Izquierdo and Koven, 2011). Despite dietary protein is also determinant for larval growth, in this study, variation in crude protein in experimental microdiets from 59.09% to 64.60% was not correlated with fish growth. Moreover, the highest growth in terms of both body weight and total length was obtained with the diet containing an increase in EPA and one of the lowest protein contents (59.61%).

On the contrary, the inclusion of a microorganism produced ARA source (total dietary content of about 0.8% in dry weight) did not improve larval performance neither in diets containing fish oil nor in those containing the microrganism produced DHA. Probably, the ARA levels contained in both the fish oil used or the microrganism produced DHA (about 0.2% dry weight) provide sufficient ARA amounts to cover the essential fatty acid requirements of gilthead seabream larvae. In agreement with this, the elevation of dietary ARA contents from 0.2% to 0.5% or 1.8% did not affect growth, respectively, in Senegalese sole (*Solea senegalensis*) (Villalta *et al.*, 2005; Lund *et al.*, 2008), gilthead seabream (Koven *et al.*, 2001) or summer flounder (*Paralicthys dentatus*) (Willey *et al.*, 2003). In contrast, ARA elevation up to 1% improved growth in gilthead seabream (Bessonart *et al*., 1999), whereas they did not have any effect in growth if dietary EPA was low (Atalah *et al*., 2011). Indeed,

dietary ARA requirements could depend on the dietary levels of EPA.

In summary, the results of this study denote the good nutritional value of microorganisms produced DHA as a replacement of fish oil in weaning diets for gilthead seabream, without a complementary addition of ARA. However, dietary supplementation of EPA seems to be necessary to further promote larval performance. As the level of DHA in the fish oil-based diet was more than enough to cover DHA requirements, probably it is possible to reduce the content of the used microorganism produced DHA source down to half of the level used in this study without affecting larval performance. The rest of dietary lipids were covered by a cheaper lipid source. Nevertheless, to keep adequate levels of dietary EPA, it would be necessary to complement the microorganism produced DHA source with EPA rich oil, for instance derived from photosynthetic algae.

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CHAPTER 5

Hidrobiológica 2015, 25 (2): 30-40

Nannochloropsis gaditana and Crypthecodinium cohnii Two microalgae as alternative sources of essential fatty acids in early weaning for gilthead seabream

Nannochloropsis gaditana y Crypthecodinium cohnii Dos microalgas como fuentes alternativas de ácidos grasos esenciales en el destete precoz de la dorada

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5.1. ABSTRACT

The heterotrophic dinoflagellate *Crypthecodinium cohnii* and the photoautotrophic eustigmataceae *Nannochloropsis gaditana* were cultured and tested as total or partial replacements of fish oil in gilthead sea bream weaning diets. Twenty day old fish larvae were fed with four microdiets: a control diet based on fish oil, two diets including *N. gaditana* (11%) or *C. cohnii* (8%) to substitute completely fish oil (diets N and C, respectively), and a diet including both *N. gaditana* (5.5%) and *C. cohnii* (5.5%) together with fish oil (5.5%) (diet N+C). At the end of the experiment (17 d treatment), final survival did not differ among groups, but growth in total length and dry weight in fish oil and *C. cohnii*-fed treatments were higher ($p<0.05$) than in the treatment containing *N. gaditana*. Fatty acid analysis confirmed that both *C. cohnii* and *N. gaditana* were used as essential fatty acid sources in microdiets of gilthead sea bream larvae. These results show that the eicosapentaenoic acid (EPA; 20:5n-3) contained in *N. gaditana* supported larval survival but the availability of docosahexaenoic acid (DHA; 22:6 n-3) from *C. cohnii* was necessary to match the growth performance in weaning diets for gilthead sea bream.

5.2. INTRODUCTION

Total fish production from capture fisheries and aquaculture is estimated to reach 172 million tons in 2021; aquaculture production is expected to exceed capture fisheries production and to increase around 33% because of higher demand for fish consumption (FAO, 2012). Thus, the establishment of a balance between high production, low cost and quality comes into prominence in aquaculture. Successful production mainly depends on broodstock management, larval husbandry and nutritional quality of feed in larval and juvenile feeding period (Izquierdo *et al.,* 2001; Infante and Cahu, 2010).

Fishmeal and fish oil are the main two components of marine fish feeds, from which protein and lipids are obtained respectively. Fish oil is the major source of n-3 highly unsaturated fatty acids (n-3 HUFA) such as docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3) that are crucial for cultured fish and for the aqua feed industry (Tacon and Metian, 2008; Turchini *et al.,* 2009). However, the price of fish oil-based products is increasing due to the increased demand and limited

production. Therefore, it is necessary to obtain alternative sources in order to assure sustainable aquaculture production (Izquierdo, 2005). Terrestrial plant oils such as sunflower, soybean, canola, rapeseed, palm, cottonsed and linseed oils have been evaluated as replacement of fish oil for several fish species (Bell *et al.,* 2003; Bransden *et al.,* 2003; Huang *et al.,* 2007; Piedecausa *et al.,* 2007; Fountoulaki *et al.,* 2009; Bell *et al.,* 2010; Eroldoğan *et al.,* 2012). Except salmonids, most marine fish are not capable of synthesizing n-3 HUFA because of their limited delta 5 and delta 6-desaturase enzymes expression by their genes (Sargent *et al.,* 2002). Since complete or partial replacement of fish oil by vegetable oils can reduce the essential n-3 HUFA level of fish fed with these diets (Bell *et al.,* 2003; Bell *et al.,* 2004; Torstensen *et al.,* 2005; Mourente and Bell, 2006) and could negatively affect health and flesh quality of gilthead sea bream (Montero *et al*., 2003; Izquierdo, 2005; Cruz-Garcia *et al.,* 2011), n-3 HUFA should be present in marine fish diets in order to support high levels of n-3 HUFA in fish.

Marine microalgae are the primary producers of n-3 HUFA in the aquatic food chain, and are commonly used in aquaculture as a source of essential nutrients such as n-3 HUFA, vitamins and pigments to feed molluscs, crustaceans, rotifers and *Artemia* (Muller-Feuga, 2000). More recently, much interest has been paid to the industrial production of microalgae for biofuel and for the nutraceutical market. Many studies have been focused on the production of alternative sources of n-3 HUFA from microalgae (Ratledge, 2001; de Swaaf, 2003; Ganuza and Izquierdo, 2007) and single cell biomass of some species is now commercially available as a free flowing powder that could easily be used as aquafeed ingredient.

Some studies succeeded to use those microalgae based n-3 HUFA sources as an alternative to fish oil (Atalah *et al.,* 2007; Miller *et al.,* 2007; Ganuza *et al.,* 2008; Eryalçın *et al.,* 2013). While production costs for single cell HUFA is still not competitive with current fish oil prices (Pulz and Gross, 2004; Spolaore *et al.,* 2006), development of microalgae for biodiesel and food industry could bring their cost to a level that might eventually compete with the raising fish oil prices. *Nannochloropsis gaditana* L. M. Lubián 1982 is a phototrophic eustigmatophyte widely used in larval rearing, especially for rotifer production and for larval rearing with the greenwater technique. *N. gaditana* is rich in eicosapentaenoic acid (EPA; 20:5n-3) and during

rotifer enrichment provides high levels of EPA and protein (Sukenik, 1991; Rocha *et al.*, 2008; Ferreira *et al*., 2009). *Crypthecodinium cohnii* (Seligo) Javornicky 1962 is a heterotrophic dinoflagellate rich in docosahexaenoic acid (DHA; 22:6n-3) and it is used as a source of this fatty acid in several aquaculture applications (Barclay *et al.,* 1994; Kyle, 1996; Apt and Behrens, 1999; Ganuza and Izquierdo 2007; Ganuza *et al.,* 2008; Eryalçın *et al.,* 2013). It can produce high amounts of DHA (close to 10% dry weight or 40% total fatty acids of its biomass) and is low in other short chain fatty acids (Kyle and Gladue, 1991; Kyle *et al.,* 1992; Sijtsma and de Swaaf, 2004; Ganuza and Izquierdo, 2007).

It was shown that microalgae biomass could be used as DHA sources alternative to fish oil in microdiets for gilthead seabream larvae (Vazhappilly and Chen, 1998; de Swaaf *et al.,* 1999; Ganuza and Izquierdo, 2007; Eryalçın *et al.,* 2013), and that their inclusion of commercial EPA sources in gilthead seabream weaning diets resulted in high total length and in survival rates similar to those obtained with diets containing different microalgae products (Eryalçın *et al.,* 2013). *C. cohnii* was successfully used to substitute fish oil DHA in gilthead seabream weaning diets (Ganuza *et al.*, 2008), but the necessity for EPA sources was highlighted by the authors. When DHA requirements are fulfilled by the diet, EPA unbalance may affect growth, survival and resistance to stress (Liu *et al.,* 2002; Izquierdo and Koven, 2011; Eryalçın *et al.,* 2013). *Nannochloropsis* species are commonly used in greenwater for larvae cultures as a source of EPA and their beneficial effects are well known for Sparidae species, but they are rarely used as a feed ingredient.

The aim of this study was to investigate whether DHA and EPA from fish oil sources could be replaced by freshly cultured lyophilized microalgae *C. cohnii* and *N. gaditana,* respectively. In order to evaluate the effects of the microalgae oils, growth performance, survival, digestive morphology and fatty acid compositions of the gilthead sea bream larvae were studied.

5.3. MATERIAL AND METHODS

5.3.1. Microalgae culture

N. gaditana was cultured phototrophically in 300-L plastic bags using f medium (Guillard and Ryther, 1962). The bags were operated in a batch mode at 25 °C, 5 vol.

air/vol culture min and 400 μM photon/m^2/s. *C. cohnii* ATCC 50060 was produced heterotrophically in a 10-L fermenter (Bioflo 3000, New Brunswick) using a seawater medium with ethanol and yeast extract as sole ingredients. The fermenters were operated on a fed batch mode according to the protocol described by de Swaaf *et al*. (2003). Both algal biomasses were harvested at the stationary phase of the culture by centrifugation (3000 *g*, 5 min, 10 °C). The biomass was washed twice with demineralized water at 3 °C in order to eliminate the residual salts. Fatty acid profiles of both microalgae are shown in Table 5.2.

5.3.2. Fish husbandry

Gilthead seabream (*Sparus aurata*) larvae were obtained from natural spawnings from Instituto Canario de Ciencias Marinas [Grupo de Investigación en Acuicultura (GIA), Las Palmas de Gran Canaria, Spain]. Larvae (5.97±0.4 mm total length, mean±SD; 0.012±0.001 mg dry body weight) previously fed rotifers (*Brachionus plicatilis*) enriched with DHA Protein Selco® (INVE, Dendermond, Belgium) until 20 dph were randomly distributed in 12 light grey fiberglass cylinder tanks (200-L) at a density of 2100 larvae $tank^{-1}$ and fed one of the diets tested in triplicate. All tanks (were supplied with filtered seawater (37 g L^{-1} salinity) at a rate of 0.4 L min^{-1} during the first week, which was increased to 1.0 L min^{-1} during the second week of the experiment. Water entered from the tank bottom and exited from the top to ensure water renewal and maintain high water quality. Ammonia and nitrites were measured twice a week (Marine and Fresh- water Test Lab, Red Sea Europe, Verneuil sur Avre, France) during the experiment. Values were always below detectable levels for ammonia (NH_4^+ <0.2 mg/L) and nitrites (NO_2^- <0.02 mg/L). Water was continuously aerated (125 mL min^{-1}) attaining 6.2±0.5 mg L^{-1} dissolved O_2. Mean water temperature and pH throughout the trial were 18.3±0.2 °C and 7.75, respectively. Photoperiod was kept at 12 h light/12 h dark, by white daylight fluorescent tubes, and light intensity was kept at 1700 lux (digital Lux Tester YF-1065, Powertech Rentals, Osborne Park City, WA, Australia).

5.3.3. Experimental diets

Four experimental microdiets (pellet size <250 μm) with different sources of EPA and DHA were prepared (Table 5.1): a control diet (diet Control) based on fish oil

(Capelin oil, Norsildmel, Bergen, Norway) and squid powder (Rieber and Son, Bergen, Norway) as lipid and protein sources; a diet containing 11 % *Nannochloropsis gaditana* (diet N) to substitute completely fish oil and replace squid meal partially; a diet containing 8% *Crypthecodinium cohnii* (diet C) to substitute completely capelin oil and replace partially squid meal; a diet containing equal amounts of 5.5 % *Nannochloropsis gaditana* and *Crypthecodinium cohnii* (diet N+C) substituting squid powder and capelin oil. The squid powder in diet N+C was lowered in order to balance the protein added as microalgae. Consequently, the lowered EFA content was balanced with 5.5% fish oil. The desired lipid content of experimental diets was adjusted with a non-essential fatty acid source, oleic acid (1.5 %). The proximate and fatty acid composition of the experimental diets are given in Table 5.1 and Table 5.3.

Table 5.1 Formulation and proximate compositions of experimental diets

	Control	N	N+C	C
Ingredients (% of diet)				
Squid Powder [1]	75.6	69.5	65	72
Gelatin[2]	3	3	3	3
Nannochloropsis sp.[3]	-	11	5.5	-
Crypthecodinium cohnii [4]	-	-	5.5	8
Soy lecithin	2	2	2	2
Fish oil[5]	5.9	-	5.5	-
Oleic acid[6]	-	1	-	1.5
Vitamin premix	4.5	4.5	4.5	4.5
Mineral premix[7]	6	6	6	6
Attractant	3	3	3	3
Proximate analysis of experimental diets (% dry weight)				
Crude lipid	16.78	12.9	13.79	15.11
Crude protein	65.79	67.61	69.98	67.14
Ash	6.18	7.21	6.67	6.09
Moisture	7.81	9.56	9.59	6.91
Gross Energy (KJ/kg)[8]	24.02	23.09	23.03	23.75

[1] Squid meal (Riber & Son. Bergen, Norway)
[2] Gelatine 80-100 Blooms, Pancreac, Espana.
[3] Lyophilized *N.gaditana* biomass.
[4] Lyophilized *Crypthecodinium* biomass.
[5] Fish oil (Capelin oil, Denofa, Fredrikstad, Norway)
[6] Oleic acid Vegetable, Merch, Darmstadt, Germany.
[7] Teshima, Kanazawa and Sakamoto (1982).
[8] Microdiet gross energy content was estimated as:
total carbonhydrate x 17.2 J/kg; fat x 39.5 J/kg; protein x 23.5 J/kg
* Mean values (n=3) are quoted in order to include the analytical error.

Table 5.2 Fatty acid composition of microalgae (% of total lipid)

	Nannochloropsis gaditana	*Crypthecodinium cohnii*
12:0	-	2.20
14:0	5.30	16
14:1n-9	0.40	-
15:0	0.20	0.1
16:0	27.60	25
16:4n-3	0.80	-
16:1n-7	35.80	0.4
16:2n-6	-	-
16:2n-4	-	-
17:0	-	0.1
18:0	1.70	-
18:1	-	16
18:1n-9	6.20	-
18:2n-6	0.80	0.5
18:3n-3	4.50	0.4
18:3n-6	2.1	-
20:3n-3	0.40	-
20:5n-3	12.6	0.1
22:4n-6	2.10	-
22:5n-6	-	-
22:6n-3	-	39

Table 5.3 Fatty acid compositions of the experimental diets (% dry weight, n=3)

Fatty acid (%)	Control	N	N+C	C
14:0	0.28	0.20	1.20	1.01
15:0	0.05	0.05	0.04	0.11
16:0	3.01	2.94	3.82	3.52
16:0ISO	0.01	0.00	0.00	0.03

Me16:0	0.02	0.02	0.01	0.02
17:0	0.03	0.02	0.01	0.01
18:0	0.58	0.46	0.49	0.04
20:0	0.03	0.01	0.02	0.02
Σ Saturates	4.01	3.70	5.61	4.76
14:1n-5	0.01	0.01	0.00	0.03
14:1n-7	0.01	0.01	0.00	0.03
15:1n-5	0.00	0.00	0.00	0.04
16:1n-7	0.28	0.62	0.33	0.08
18:1n-9	2.38	1.94	1.05	2.56
18:1n-7	0.30	0.16	0.13	0.13
18:1n-5	0.03	0.16	0.13	0.13
20:1n-9+n7	0.60	0.38	0.31	0.35
22:1n-11	0.23	0.00	0.00	0.01
22:1n-9	0.06	0.03	0.03	0.04
Σ Monounsaturated	3.59	2.96	1.70	3.07
16:2n-6	0.03	0.01	0.01	0.01
18:2n-6	3.30	1.43	2.55	1.39
18:3n-6	0.01	0.01	0.00	0.00
20:2n-6	0.05	0.03	0.02	0.02
20:4n-6	0.13	0.17	0.12	0.11
22:4n-6	0.03	0.01	0.01	0.01
22:5n-6	0.03	0.03	0.02	0.02
Σ (n-6)	3.58	1.69	2.73	1.57
16:3n-3	0.02	0.02	0.02	0.02
16:4n-3	0.04	0.05	0.04	0.04
18:3n-3	0.45	0.12	0.10	0.12
18:4n-3	0.05	0.01	0.01	0.01
20:3n-3	0.07	0.06	0.05	0.06
20:4n-3	0.06	0.01	0.01	0.01
20:5n-3	1.19	1.27	1.90	0.96
22:5n-3	0.15	0.04	0.04	0.04
22:6n-3	3.12	2.61	3.81	4.16

Σ (n-3)	5.14	4.18	5.97	5.42
EPA:DHA	0.38	0.49	0.50	0.23
ARA:EPA	0.11	0.14	0.06	0.11
Σ n-3 HUFA	4.58	3.99	5.81	5.23

The total lipid content of the different diets ranged between 13 and 17% dry weight. The ingredients were mixed in a mortar and then combined with gelatin dissolved in water at 80 °C. The feed paste obtained was compressed, pelleted and dried in an oven at 40 °C for 24 h. The pellets were ground in a Sample Mill (Braun KSM 2) and sieved to obtain a particle size of 150-250 µm (Atalah *et al.,* 2012; Eryalçın *et al.,* 2013).

No microalgae were added to the experimental tanks during the feeding trial. Larvae were fed with the experimental diets for 17 days by manual feeding every 45 min during light periods. The first 4 days of the experiment, larvae were fed with non-enriched rotifers twice a day at 12:00 and 16:00 hours to maintain a rotifer density of 2 ind. mL^{-1} in rearing tanks. During the first week feeding rate was 2 g per day, which was adjusted to 2.5 g per day during the second week. Larvae were observed under the binocular microscope to determine feed acceptance. The tanks were siphoned daily in order to collect uneaten food and faeces.

5.3.4. Growth performance

Larval growth was determined measuring total length of 30 larvae 20, 27 and 34 dph using profile projector (Mitutoyo Profile Projector PJ-A3000). Survival rate was determined counting all live larvae at the end of the experiment. Air exposure stress resistance test was applied to 25 larvae of each tank. For that purpose, larvae were held out of the water for 1 min. 30 sec. in a scoop net and then subsequently moved into 2-L beaker with aerated water. Survival was determined after 24 hours by counting dead larvae. At the end of the feeding trial, microdiets and all larvae in tanks were collected and stored at -80°C in plastic bags until proximate composition was carried out. Moisture, total protein and ash content were determined drying for 24 hours at 105 °C, as total Kjeldahl nitrogen and after incinerating the dried samples at 600 °C for 24 h, respectively (A.O.A.C. 2005). Total lipid contents of larvae and

microdiets were determined gravimetrically after extraction with chloroform-methanol (2:1) (Folch *et al.,* 1957).

Ingestion of all experimental diets was checked by microphotography of larval digestive system after 7 days of feeding. If apparent feed intake differences were observed, diet acceptance was determined observing the abdominal cavity of 30 larvae of each tank with a stereoscope (Leica Wild M3Z, Optotek, California, USA), and measuring the area of the gut occupied by digested matter on the micrographs, using Image Pro Plus® (Media Cybernetics Inc., Silver Springs, MD, USA) semiautomatic image analysis system.

5.3.5. Fatty acid methyl esters preparation and quantification

Fatty acid methyl esters were obtained by transmethylation with 1% sulphuric acid in methanol (Christie, 1982). Fatty acid methyl esters were separated by GC (GC-14A; Shimadzu, Tokyo, Japan) in a Supercolvax-10-fused silica capillary column (constant pressure with 100KPa, length: 30 m; internal diameter: 0.32 mm; 0,25 i.d (Ref.: 24080-U) Supelco, Bellefonte, PA, USA) using helium as a carrier gas. Column temperature was 180 °C for the first 10 min, increasing to 220 °C at a rate of 2 °C min^{-1} and then held at 220 °C for 15 min. Fatty acid methyl esters were quantified by FID following the conditions described in Izquierdo *et al*. (1990) and identified by comparison with external standards and well characterized fish oils (EPA 28, Nippai, Ltd Tokyo, Japan).

5.3.6. Statistical analysis

All data were normal and homoscedastic (Shapiro-Wilk's test was used for normality and Levene's tests), not requiring any transformation and were treated using one-way analysis of variance (ANOVA). Means were compared by Tukey tests ($p<0.05$) using SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).

5.4. RESULTS

Final survival rates were not statistically different (34%-69%, average) ($p>0.05$) (Figure 15). Similarly, air exposure stress resistance survival results were similar among the groups fed with different experimental diets. After 7 days of experimental feeding (27 dph), larvae fed diet C had a significantly ($p<0.05$) higher total length

than larvae fed diets containing *N. gaditana* (diet N and N+C), but differences in weight were not significant. At the end of the experiment, larvae fed either control or C diet had significantly (p<0.05) higher total length and dry weight than larvae fed N and N+C diets, which were not significantly different (Figure 13 and 14).

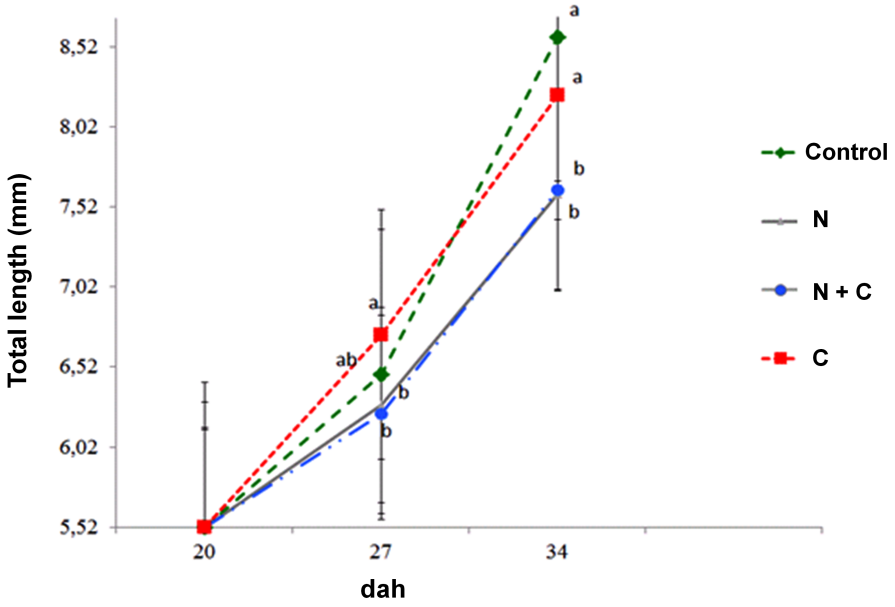

Figure 13. Total length of gilthead sea bream larvae fed experimental diets. Treatments containing different letters in each datapoint were significantly different (p<0.05).

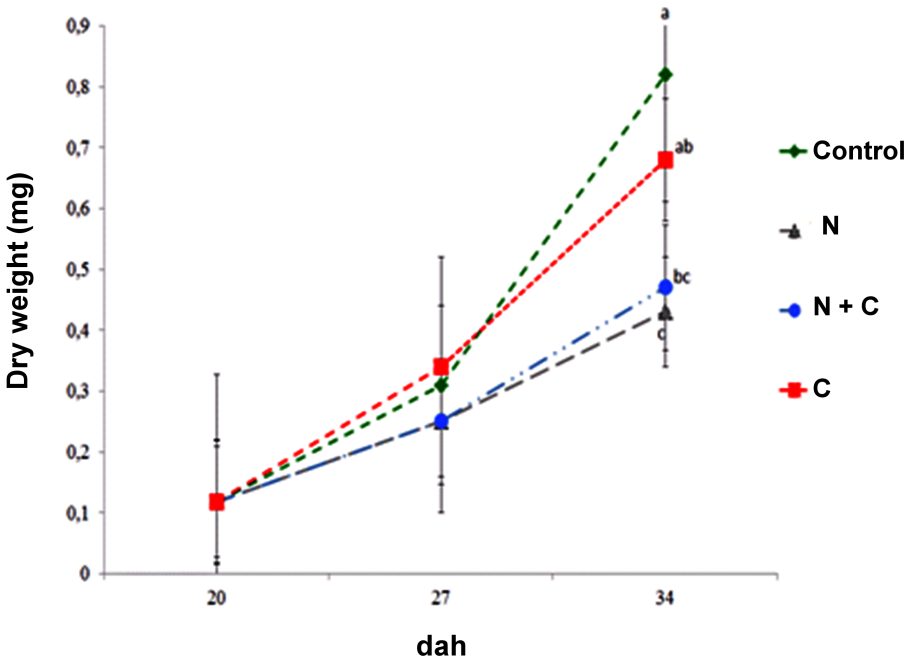

Figure 14. Dry weight of gilthead sea bream larvae fed with experimental diets Treatments containing different letters in each datapoint was significantly different (p<0.05).

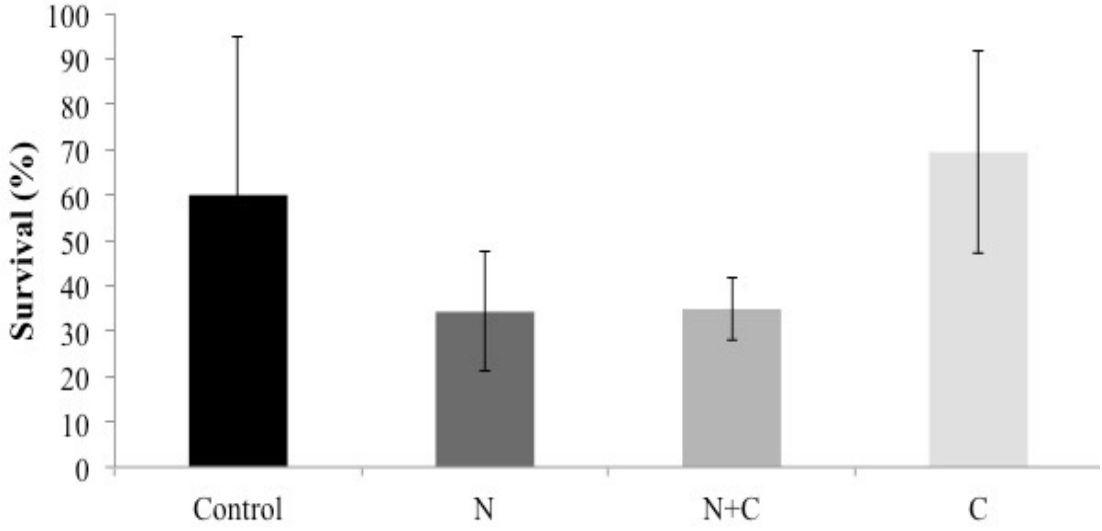

Figure 15. Final survival of larvae fed with different experimental diets.

At the end of the study, larvae fed the Control and C diets showed higher protein level than other groups, and lipids were higher in control group larvae than in C and N groups. The moisture content was higher in the control group in comparison to other groups, while the group with highest ash content was the N group (Table 5.4).

Table 5.4 Proximate compositions of experimental larvae fed diets containing different microalgae for 14 days (% of dry weight)

Proximate Composition *					
(% dw)	**Initial**	**Control**	**N**	**N+C**	**C**
Crude Lipid	2.42±0.15	3.65±0.14^{a}	3.17±0.18 ^{b}	2.6±0.12^{c}	3.2±0.14^{b}
Crude Protein	10.63±0.13	12.47±0.12^{ab}	11.35±0.11^{c}	11.54±0.17^{c}	12.96±0.11^{a}
Ash	1.41±0.02	1.29±0.03^{b}	1.74±0.01^{a}	1.19±0.02^{c}	1.27±0.02^{b}
Moisture	84.77±0.45	86.17±0.35^{a}	85.24±0.21^{ab}	86.25±0.54^{a}	86.09±0.43^{a}

* Mean values (n=3) are quoted in order to include the analytical error.

Fatty acid analysis of experimental fish (% dry body weight) revealed that all the experimental diets caused an increase in saturated fatty acids content, mainly 16:0, in

comparison to larvae fed the control diet. Total monounsaturated fatty acid levels were significantly (p<0.05) lower in larvae fed with N, N+C and C diets compared to control group larvae (Table 5.5). Linolenic (18:3n-3) and linoleic (18:2n-6) acid levels of larvae fed with control diet were significantly (p<0.05) higher than those of the other experimental groups. Total n-3 and n-6 fatty acid content of larvae fed with N, N+C and C diet was lower than in the control group (p<0.05). Additionally, the total n-3 HUFA contents of larvae fed with Control and C diets were higher than those of larvae fed with diet N+C and N diet.

Table 5.5 Fatty acid compositions of experimental larvae (% dry weight, n=3)

Fatty acid (%)	Initial	Control	N	N+C	C
14:0	0.13	0.31	0.18	0.48	0.53
15:0	0.09	0.12	0.10	0.10	0.09
16:0	2.38	6.61[a]	3.98[c]	5.52[b]	5.79[b]
17:0	0.12	0.09	0.10	0.10	0.07
18:0	1.40	2.44[a]	1.76[c]	2.27[a]	2.03[b]
20:0	0.02	0.07	0.05	0.06	0.05
Σ Saturates	4.19	9.68[a]	6.21[c]	8.57[b]	8.59[b]
14:1n-7	0.07	0.03	0.03	0.03	0.02
16:1n-5	0.13	0.08	0.10	0.11	0.07
16:1n-7	0.98	0.49	0.59	0.53	0.31
18:1n-9	2.09	4.20	3.02	2.63	3.77
18:1n-7	0.79	0.73	0.54	0.56	0.43
18:1n-5	0.07	0.08	0.07	0.07	0.19
20:1n-9+n7	0.25	0.73	0.34	0.33	0.44
20:1n-5	0.11	0.05	0.06	0.06	0.05
22:1n-11	0.05	0.21	0.03	0.02	0.01
22:1n-9	0.04	0.18	0.11	0.12	0.13
Σ Monounsaturated	4.34	6.06[a]	4.57[bc]	4.13[c]	4.99[b]
16:2n-6	0.10	0.18	0.12	0.15	0.15
18:2n-6	0.69	4.16[a]	1.48[b]	1.21[c]	1.62[b]
18:3n-6	0.01	0.03	0.03	0.03	0.03

20:2n-6	0.14	0.22	0.13	0.13	0.12
20:3n-6	0.05	0.02	0.04	0.03	0.01
20:4n-6	0.47	0.48^{b}	0.52^{ab}	0.55^{a}	0.39^{c}
22:4n-6	0.02	0.03	0.01	0.01	0.01
22:5n-6	0.11	0.10^{ab}	0.09^{b}	0.11^{a}	0.08^{b}
Σ (n-6)	1.59	5.23^{a}	2.43^{b}	2.22^{c}	2.41^{bc}
16:4n-3	0.04	0.16	0.18	0.22	0.15
18:3n-3	0.05	0.36^{a}	0.10^{b}	0.07^{c}	0.09^{b}
18:4n-3	0.01	0.04	0.01	0.01	0.01
20:3n-3	0.01	0.10	0.05	0.05	0.07
20:4n-3	0.08	0.07	0.02	0.02	0.02
20:5n-3	1.21	1.85^{a}	1.28^{c}	1.21^{d}	1.31^{b}
22:5n-3	0.58	0.44	0.34	0.29	0.22
22:6n-3	2.71	8.28^{ab}	5.54^{c}	8.16^{b}	8.59^{a}
Σ (n-3)	4.70	11.30^{a}	7.52^{c}	10.04^{b}	10.47^{b}
EPA:DHA	0.45	0.22^{a}	0.23^{a}	0.15^{b}	0.15^{b}
ARA:EPA	0.39	0.26^{c}	0.41^{ab}	0.46^{a}	0.30^{b}
Σ n-3 HUFA	4.59	10.74^{a}	7.23^{c}	9.74^{b}	10.21^{a}

Higher DHA (22:6n-3) accumulation was observed in larvae of C and Control groups. Additionally, EPA (20:5n-3) level was significantly higher in larvae fed with theControl diet. On the other hand, ARA (20:4n-6) level was found higher in larvae fed diets N+C and N than in those fed with Control and C diets. This result also reflected the ARA:EPA proportion of larvae. Larvae fed control diet and N diet showed higher EPA:DHA ratio due to the EPA rich in *N. gaditana*. On the other hand, fish fed diets N+C and C also showed very good levels of DHA.

5.5. DISCUSSION

Fatty acid analysis confirmed that microalgae were effectively used as essential fatty acid sources in microdiets. Total n-3 HUFA content of the larvae was improved with the inclusion of *N. gaditana* and *C. cohnii* as substitution to fish oil. Survival and stress resistance of fish are important parameters to ensure good quality fish

production. Survival of larvae depends mainly on nutritional quality of feeds and nursery conditions, and a large range of survival rates were obtained in various dietary experiments (e.g. Robin and Vincent, 2003; Ganuza *et al.*, 2008; Eryalçın *et al.,* 2013). In this study, larval survival rates ranged from 34 to 69% from 20-34 dph larvae, in agreement with these previous studies. The importance of EPA content in larval diet due to its critical effect on growth and survival was previously suggested by Ganuza *et al.* (2008) who concluded that DHA rich single cell microorganisms in microdiets cannot replace completely fish based products. This requirement of EPA sources in microdiets for gilthead seabream larvae was also shown in other studies (Atalah *et al.*, 2007; Eryalçın *et al.,* 2013). It was suggested that the EPA:DHA ratio in marine fish larvae should be in the range 0.67 to 2.0 (Rodriguez *et al.*, 1997; Atalah *et al.*, 2007). In our diets with single cell sources, the EPA:DHA ratio varied from 0.23 to 0.50, which seems adequate for early development. In this study, DHA rich *C. cohnii* diet resulted in high growth rates, while the EPA rich *N. gaditana* diet led to mediocre growth. On the other hand *N. gaditana* and N+C diets gave higher ARA amount which is important for the immune system. Therefore, high growth rates and good survival could be expected from the combined diet containing both microalgae (N+C) but the obtained growth rates were not satisfactory. The combination of microalgae strains that can provide a wider range of fatty acids to obtain better dietary quality than single algae was previously suggested by Harel *et al.* (2002). According to our results, the combined use of *C. cohnii* and *N. gaditana* sustained survival rates, but was not sufficient to support high growth rates.

Dietary energy content is also important for the microdiet nutritional value. Lower amounts of accessible energy to larvae were previously suggested as a reason for total length differences observed at day 14 (Ronnestad *et al.,* 1994). In the present study, *N. gaditana* and N+C diet had lower gross energy content compared to the fish oil diet or *C. cohnii* diet, which could be insufficient to cover the larvae's energy requirements during this fast growing phase. The N and C diets included oleic acid, which is regarded as an important source of energy, during larval development of marine fish larvae (Van der Mereen *et al.*, 1991). The oleic acid content might have supported the growth rates obtained from C diet, but does not seem sufficient for the low DHA containing N diet. The absence of oleic acid in the N+C diet on the other hand, might have led to low growth rates due to less available energy in diets.

Some microalgae species as *N. gaditana* are not only a good source of essential fatty acids, but also of pigments and vitamin E (Rebolloso-Fuentes *et al.*, 2001; Durmaz, 2007; Hemaiswarya *et al.*, 2011), which could be related to the good survival rates of larvae fed *N. gaditana* despite the lower DHA content in their body tissue. Vitamin E is an essential antioxidant synthesized by photosynthetic organisms (Durmaz, 2007) and its effect on growth, survival and resistance to stress is important along the whole life cycle in fish (Izquierdo *et al.*, 2001; Montero *et al.,* 2001; Vismara *et al.,* 2003; Atalah *et al.,* 2012).

In our study, single cell sources such as the dinoflagellate *C. cohnii* and eustigmatophyceae *N. gaditana* were effectively used as essential fatty acid sources in gilthead seabream larvae in comparison to the control group. Particularly fish fed *C. cohnii* containing diet performed similar to fish fed the control diet containing fish oil. Diets with *N. gaditana* alone on the other hand could not effectively replace fish oil. *N. gaditana* could be used to complement possible EPA deficiencies for gilthead sea bream larvae but the combined use of the two microalgae *C. cohnii* and *N. gaditana* seem to necessitate additional energy source such as oleic acid. Future work should focus on longer experimental periods with weaning diets including different levels of these microorganisms in order to address the long-term effects on immune system and survival of gilthead sea bream larvae.

Acknowledgements

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CHAPTER 6

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Effects of dietary mannan oligosaccharides in early weaning diets on growth, survival, fatty acid composition and gut morphology of gilthead sea bream (*Sparus aurata*, L.) larvae

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6.1. ABSTRACT

Early weaning of marine fish larvae with dry diets delays gut maturation and reduces growth rates. In juvenile and adult forms of several marine fish species, inclusion of dietary mannan oligosaccharides (MOS) improves gut integrity and functionality, but the effects of MOS inclusion in gilthead sea bream (*Sparus aurata*, L.) larval diets have not been addressed yet. Thus, this study assesses the effects of dietary MOS inclusion on survival, growth performance, gut morphology, feed acceptance and quality of gilthead sea bream larvae. For that purpose, 16 days post-hatched gilthead sea bream larvae were fed four graded levels of MOS (Biomos, Alltech, Nicholasville, KY, USA) in weaning diets as follows: 0 g kg^{-1} MOS, 0.5 g kg^{-1} MOS, 1.5 g kg^{-1} MOS and 2 g kg^{-1} MOS. Dietary MOS did not affect feed acceptance in gilthead sea bream larvae (p>0.05). MOS supplementation was correlated in a dose-dependent way with higher larval survival (P = 0.026). After 15 days of feeding, dietary MOS increased whole larvae (p<0.01) arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid. Gilthead sea bream larvae fed 2 g kg^{-1} MOS presented higher gut occupation with goblet cells after feeding compared with larvae fed the other dietary treatments. Overall, the results suggest that inclusion of MOS in early weaning diets for gilthead sea bream improves essential fatty acid utilization and may promote growth and final survival.

6.2. INTRODUCTION

Nowadays, production of marine fish larvae is mainly limited by first feeding dependence on live prey together with low hatching rates and reduction of larval gut development produced by first feeding on dry diets (Sargent *et al.*, 1999; Hamre *et al.*, 2013). Due to the incomplete development of the digestive tract, immune and sensory systems of fish larvae after hatch, first feeding on easily digestible, highly appetizing and immune promoter feeds is a priority for the aquaculture larvae feed producer industry (Dimitroglou *et al.*, 2011). Prebiotics are non-digestible food ingredients that beneficially affect the host by stimulating growth and/or health and promoting beneficial bacteria population in the gastrointestinal tract (GI) microbiota of poultry (Patterson and Burkholder, 2003), terrestrial livestock (Flickinger *et al.*, 2003) and humans (Gibson and Roberfroid, 1995). Among them, dietary mannan oligosaccharides (MOS) have been successfully included in adult and juvenile fish

diets showing beneficial effects on growth performance and health, mainly by modulating intestinal microbiota and promoting gut integrity (Torrecillas *et al.*, 2007; Torrecillas *et al.*, 2011a; Torrecillas *et al.*, 2011b; Torrecillas *et al.*, 2013). However, the potential rather than beneficial effects of MOS are clearly dependent on several factors such as dietary level, fish species, fish age, culture conditions and duration of supplementation (Sweetman *et al.*, 2010; Torrecillas *et al.*, 2014). For instance, rainbow trout Oncorhynchus *mykiss* (Walbaum) fed dietary 2 g kg^{-1} MOS (Bio-Mos, Allthech, USA) for 42 days presented better growth performance and higher survival rates (Staykov *et al.*, 2007; Torrecillas *et al.*, 2014). Similarly, European sea bass (*Dicentrarchus labrax*, L.) juveniles fed dietary 0.4% MOS (Bio-Mos) for 67 days presented better SGR and promoted immune response and higher resistance to intestinal bacterial infection (Torrecillas *et al.*, 2014). For the same fish species, MOS supplementation at 4 and 6 g kg^{-1} for three months resulted in better FCR, enhanced immune system response and increased gut mucus production (Torrecillas *et al.*, 2011; Torrecillas *et al.*, 2011, 2014). Likewise, gilthead sea bream (*Sparus aurata*) juveniles fed 2 g kg^{-1} MOS for 12 weeks presented increased growth performance, protein and carbohydrate digestibility (Gültepe *et al.*, 2011). On the other hand, MOS (AQUAMYCES, Vito-mix, Colombia) inclusion at 0, 1.5, 3 and 4.5 g kg^{-1} in hybrid Tilapia (*Oreochromis niloticus* x *O. aureus*) diets did not positively affect growth performance after 80 days of feeding (Genc *et al.*, 2007). Also, dietary partial substitution of fish meal (FM) by soya bean meal (SBM) together with MOS inclusion did not increase growth performance and nutrient digestibility but affected the fatty acid profile of sharpsnout sea bream (*Diplodus puntazzo*, Cetti 1777) (Piccolo *et al.*, 2011; Piccolo *et al.*, 2013). Unfortunately, the effects of dietary MOS in larvae rearing and metabolism have been poorly studied until today. Thus, the aim of this study was to determine the effect of four graded levels of dietary MOS inclusion on growth performance, fatty acid composition, feed acceptance percentages, stress resistance and intestinal morphology in gilthead sea bream larvae.

6.3. MATERIAL AND METHODS

6.3.1. Diets

Four experimental microdiets (pellet size <250, 250–500 and >500 μm) were formulated with increasing levels of MOS (Biomos, Alltech, Nicholasville, KY, USA) (BM0: 0 g kg^{-1}, BM5: 5 g kg^{-1}, BM15: 15 g kg^{-1} and BM20: 20 g kg^{-1}). Their

formulation and proximate analysis are shown in Table 6.1. Diets fatty acid compositions of the experimental diets are shown in Table 6.2. The desired lipid content (about 125 g kg^{-1} DW) was obtained from krill phospholipids and protein content was obtained from squid powder (690 g kg^{-1} DW). The microdiets were prepared by mixing squid meal and water-soluble components, followed by the lipids and fat-soluble vitamins and, finally, the gelatin dissolved in warm water. This paste was compressed pelleted (Severin, Suderm, Germany) and dried in an oven at 38°C for 24 h (Ako, Barcelona, Spain). Pellets were ground (Braun, Kronberg, Germany) and sieved (Filtra, Barcelona, Spain) to obtain a particle size below 250 μm following Grupo de Investigación en Acuicultura (GIA, ULPGC, Las Palmas, Spain) standard procedures. Diets were manually supplied fourteen times per day each 45 min from 9:00 to 19:00 for 30 days. Non-enriched rotifers were added to the tanks only during the first 2 days of feeding (1 rotifer mL^{-1}). To assure feed availability, daily feed supply was maintained at 1.5 (pellet size <250 μm) and 2.5 (250-500 μm) g L $tank^{-1}$ during the first and second weeks of feeding, respectively. Following feeding, larvae were observed under a binocular microscope to determine feed acceptance. If apparent feed intake differences were observed among different experimental diets, diet acceptance was determined by calculating the percentage of gut occupation by the microdiet, using image analysis of larvae pictures. For this study, 30 larvae per tank were taken at the beginning and middle of the experiment, their abdominal cavity was observed in a stereoscope (Leica Wild M3Z, Optotek, CA, USA) and the area of the gut occupied by digested matter was measured on the optic micrographs taken at a magnification of 25 μm, using Image ProPlus (Media Cybernetics, Silver Springs, MD, USA) semiautomatic image analysis system. Data are represented as mean of occupied gut area % in each individual.

Table 6.1. Ingredients and proximate composition of experimental diets for gilthead sea bream (*Sparus aurata*) larvae containing graded levels of MOS

Ingredients (g kg^{-1} dry weight)	**BM0**	**BM5**	**BM15**	**BM20**
Squid powder*	690	690	690	690
Gelatine†	30	30	30	30
Krill phospholipids‡	125	125	125	125
MOS§	-	5	15	20
CMC¶	20	15	5	-
Fish oil**	-	-	-	-
Minerals††	45	45	45	45
Vitamins‡‡	60	60	60	60
Attractants§§	30	30	30	30
Lipid	15.97±0.10	15.86±0.77	15.72±1.36	16.02±0.36
Protein	61.96±1.31	63.26±0.70	64.68±0.45	65.38±0.48
Ash	6.29±0.04	5.95±0.04	5.50±0.06	5.67±0.06
Moisture	9.79±0.25	10.17±0.18	8.90±0.19	9.11±0.09

Control = 0 g kg^{-1} MOS; BM5 = 5 g kg^{-1} MOS; BM15 = 15 g kg^{-1} MOS; BM20 = 20 g kg^{-1} MOS.
*Rieber & Son, Bergen, Norway.
†Panreac, Barcelona, Spain.
‡Qrill, high PL, Aker BioMarine, Fjordalleen, Norway.
§Bio-Mos, Alltech.
¶Carboxymethyl cellulose (sodium salt, Sigma-Aldrich, Munich, Germany).
**Peruvian fish oil.
††Teshima, Kanazawa, Horinouchi,Yamasali and Hirata (1987).
‡‡Eryalcin, Roo, Saleh, Atalah, Benitez, Betancor, Hernandez-Cruz and Izquierdo (2013).
§§Betancor, Nordrum, Atalah, Caballero, Benitez-Santana, Roo, Robaina and Izquierdo (2012a) and Betancor, Caballero, Terova, Cora, Saleh, Benitez-Santana, Bell, Hern andez-Cruz and Izquierdo (2012b).

6.3.2. Experimental conditions

Gilthead sea bream larvae were obtained from a natural spawn from GIA. Larvae (initial total length 5.09±0.5 mm; dry body weight 0.36±0.001 mg) were previously fed rotifers (*Brachinus plicatilis*) enriched with DHA Protein Selco (INVE,

Dendermonde, Belgium) until they reached 15 days post hatch (dph), and afterwards, they were randomly distributed in 15 experimental tanks at a density of 2100 larvae $tank^{-1}$ and experimental diets tested in triplicate for 30 days. All tanks (200-L fibreglass cylinder tanks with conical bottom and painted a light grey colour) were supplied with filtered seawater (37 mg L^{-1} salinity) at an increasing rate of 0.4-1.0 L min^{-1} to assure good water quality during the entire trial. Water entered from the tank bottom and exited from the top to ensure water renewal and maintain high water quality, which was tested daily and no deterioration was observed. Water was continuously aerated (125 mL min^{-1}) attaining 6.0±1 ppm dissolved O_2. Average water temperature and pH along the trial were 21.0±1°C and 7.85±0.1, respectively. Photoperiod was kept at 12-h light: 12-h dark, by fluorescent daylights and the light intensity was 1700 lux (digital Lux Tester YF-1065; Powertech Rentals, Osborne Park City, Australia) at the water surface. Final larval survival was determined at the end of the feeding trial by counting all the remaining alive larvae. Before that, at 45 dph, 20 larvae per tank were submitted to a stress resistance test conducted by handling them out of the water for 90 s and returning them in a tank with seawater and aeration to determine their survival rate 24 h later. Larval growth was determined by measuring the total length and dry weight of 30 larvae at 15-30-45 dph using a profile projector (Mitutoyo Profile Projector PJ-A3000).

6.3.3. Biochemical analysis

At the end of the feeding trial, all larvae in the tanks and microdiets were kept in -80°C until analysed. Moisture (A.O.A.C., 1995), crude protein (A.O.A.C., 1995) and crude lipid (Folch *et al.*, 1957) contents of larvae and diets were analysed. Fatty acid methyl esters were obtained by transmethylation with 1% sulphuric acid in methanol (Christie, 1982). Fatty acid methyl esters were separated by GC (GC-14A; Shimadzu, Tokyo, Japan) using helium as a carrier gas. Fatty acid methyl esters were quantified by FID following the conditions (Izquierdo *et al.*, 1990) and identified by comparison with external standards and well-characterized fish oils (EPA 28, Nippai, Tokyo, Japan).

6.3.4. Histological studies

At the end of the feeding trial, ten larvae from each tank were fixed in 10% neutral-buffered formalin and embedded in paraffin. Whole larvae 4-µm sections were

stained with H&E for morphological studies and with Alcian blue/periodic acid–Schiff (PAS) for mucus production evaluation (Martoja and Martoja, 1970; Vandewalle *et al.*, 1998). Sections were evaluated under light microscopy with 50x magnification (Olympus CX41 microscope). Micrographs were taken at a final magnification of 400x using an Olympus DP50 (Olympus Optical, Shinjuku-ku, Tokyo, Japan) camera. The number of goblet cells stained for specific acid mucin staining by unit of fold area was determined using an ANALYSIS® (Image Pro Plus®, Media Cybernetics, Silver Spring, MD, USA) (Torrecillas *et al.*, 2013).

6.3.5. Statistical analysis

All data were statistically analysed using a SPSS Statistical Software System 15.0 (SPSS Inc., Chicago, IL, USA). The significant level for all the analysis was set 5%, and results are given as mean values and standard deviation. All values presented as percentage were arcsine transformed. Also, all variables were checked for normality and homogeneity of variance, using the Kolmogorov-Smirnoff and the Levene's test respectively. Means were compared using one-way ANOVA or a Kruskal-Wallis test.

Table 6.2 Fatty acids composition of experimental diets (%dry weight, n=3)

	BM0	**BM5**	**BM15**	**BM20**
14:0	1.196	1.416	1.385	1.419
14:1n-7	0.019	0.023	0.023	0.023
14:1n-5	0.024	0.027	0.026	0.026
15:0	0.050	0.057	0.056	0.058
15:1n-5	0.009	0.010	0.010	0.010
16:0ISO	0.009	0.012	0.011	0.011
16:0	3.304	3.629	3.583	3.686
16:1n-7	0.790	0.890	0.900	0.949
16:1n-5	0.035	0.035	0.035	0.036
16:2n-4	0.068	0.075	0.074	0.074
17:0	0.024	0.026	0.026	0.027
16:3n-4	0.022	0.024	0.023	0.024
16:3n-3	0.045	0.044	0.045	0.045
16:3n-1	0.042	0.043	0.043	0.044

16:4n-3	0.090	0.099	0.097	0.098
16:4n-1	0.004	0.005	0.005	0.004
18:0	0.413	0.406	0.418	0.429
18:1n-9	1.535	1.540	1.535	1.597
18:1n-7	0.917	0.909	0.894	0.911
18:1n-5	0.042	0.042	0.041	0.042
18:2n-9	0.007	0.006	0.006	0.007
18:2n-6	0.220	0.227	0.231	0.243
18:2n-4	0.009	0.009	0.009	0.009
18:3n-6	0.033	0.033	0.032	0.033
18:3n-4	0.007	0.011	0.010	0.011
18:3n-3	0.165	0.163	0.160	0.162
18:3n-1	0.001	0.001	0.001	0.001
18:4n-3	0.451	0.450	0.446	0.444
18:4n-1	0.007	0.007	0.009	0.006
20:0	0.020	0.018	0.018	0.019
20:1n-9	0.017	0.016	0.021	0.016
20:1n-7	0.439	0.391	0.396	0.399
20:1n-5	0.055	0.048	0.048	0.048
20:2n-9	0.002	0.001	0.001	0.001
20:2n-6	0.020	0.018	0.018	0.018
20:3n-9	0.006	0.006	0.006	0.006
20:3n-6	0.008	0.007	0.007	0.007
20:4n-6	0.117	0.106	0.111	0.110
20:3n-3	0.043	0.038	0.040	0.039
20:4n-3	0.073	0.051	0.050	0.050
20:5n-3	2.742	2.508	2.470	2.477
22:1n-11	0.169	0.158	0.138	0.140
22:1n-9	0.032	0.026	0.025	0.026
22:4n-6	0.021	0.017	0.017	0.016
22:5n-6	0.026	0.021	0.022	0.022
22:5n-3	0.081	0.068	0.067	0.068
22:6n-3	2.548	2.135	2.133	2.129
ΣSaturated	5.016	5.564	5.497	5.649

ΣMonoenoics	4.084	4.114	4.092	4.223
Σn-3	6.386	5.658	5.607	5.614
Σn-6	0.445	0.428	0.437	0.449
Σn-9	1.598	1.595	1.594	1.652
Σn-3 HUFA	5.488	4.799	4.759	4.763
EPA/ARA	23.356	23.704	22.274	22.433
ARA/EPA	0.043	0.042	0.045	0.045
DHA/EPA	0.929	0.851	0.864	0.860
DHA/ARA	21.702	20.179	19.235	19.283
Σ(n--3)/ Σ(n-6)	14.335	13.206	12.821	12.493

6.4. RESULTS

There was a good feed acceptance from the beginning of the trial (15 dph) for all the dietary treatments. Increase in dietary MOS was positively correlated to feed content in gut ($y = 6.1811x + 52.322$, $r = 0.9522$, P value=0.0024), with a significantly higher ($p<0.05$) value for larvae fed BM20 (Figure 16). There was also a significant ($y = 7.201x + 13.779$, $r = 0.8155$, P value=0.026) positive correlation between MOS dietary levels and larval survival at the end of the trial (Figure 17a). Increased levels of dietary MOS did not significantly affect survival after handling stress resistance test ($y = -11.482x^2 + 26.964x + 75.722$, $r = 0.8749$, P value = 0.4018); however, larvae fed BM5 diet showed a 14.44% higher survival than larvae fed BM0 (Figure 17b).

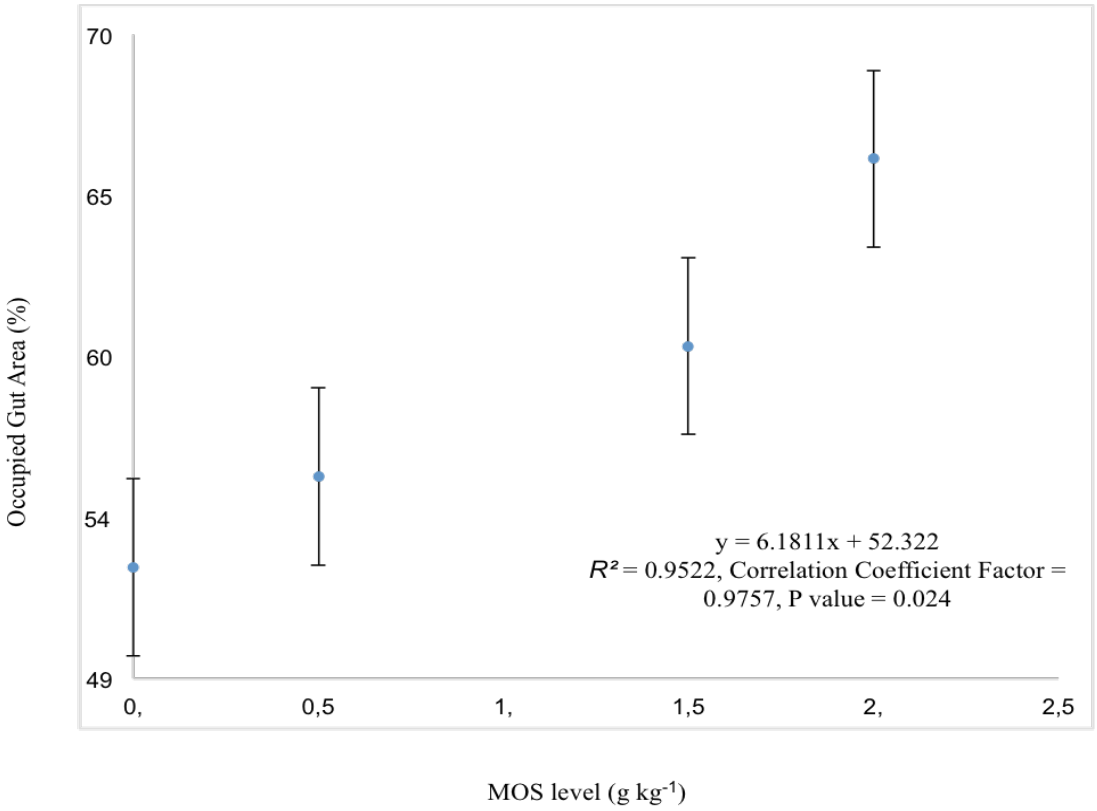

Figure 16. Gut occupation percentage of larvae fed experimental microdiets containing graded levels of MOS from 15-45 dph.

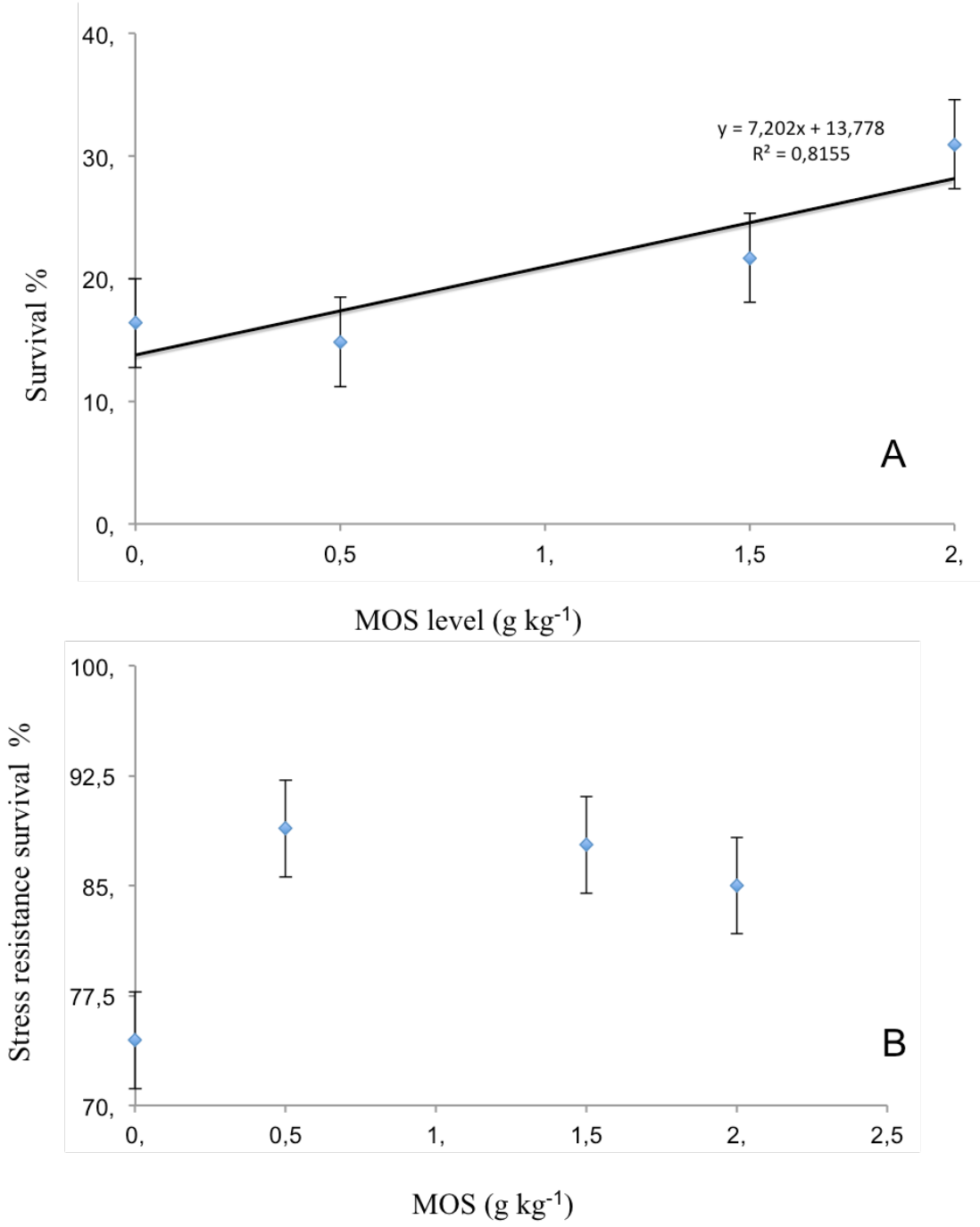

Figure 17. (A) Survival and (B) survival after stress resistance test of gilthead seabream larvae fed microdiets with different levels of MOS from 15 to 45 dph.

Regarding growth, at day 30 dph, larvae fed BM15 showed higher dry body weight (p<0.05) than larvae fed BM5 and BM20. However, at the end of the feeding trial, larvae fed different dietary MOS levels were not different in body weight or total length (p>0.05) (Figures 18a and 18b) that was negatively correlated with final survival (y = -0.0308x + 10.541, r = 0.978, P value = 0.011) (Figure 19).

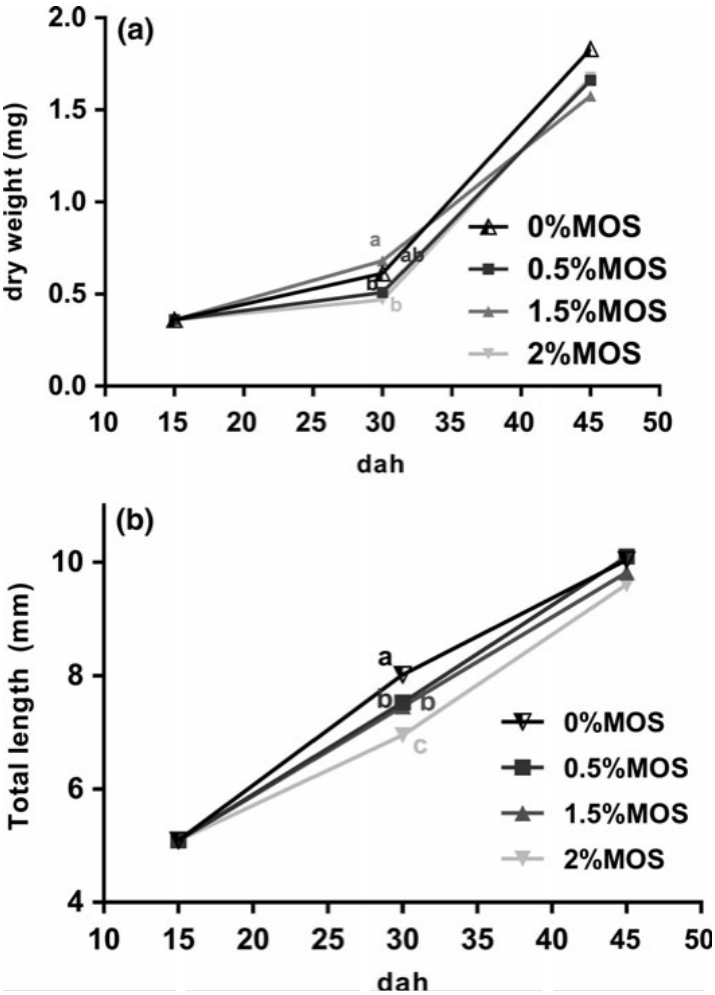

Figure 18. (a) Dry weight and (b) total length of gilthead sea bream larvae fed microdiets with different levels of MOS from 15 to 45 dph.

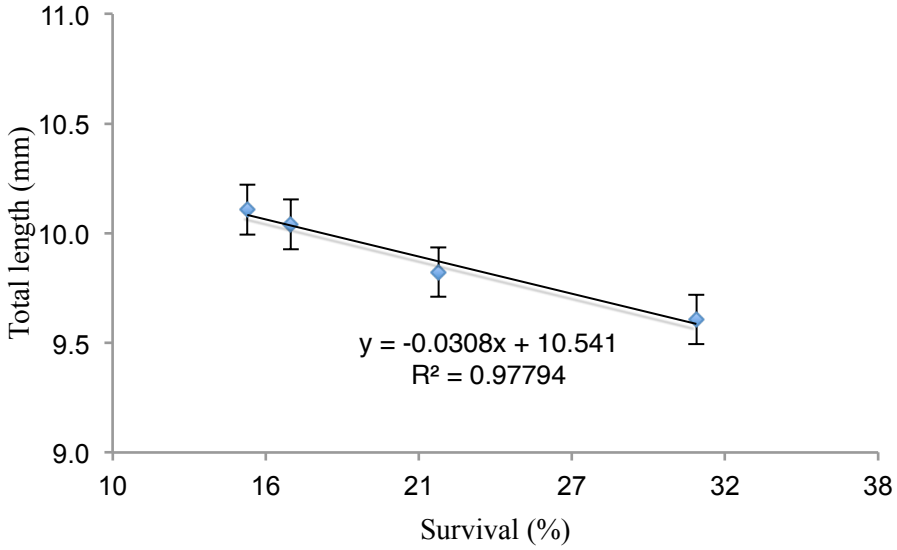

Figure 19. Relation between larval survival and total length in gilthead seabream (*Sparus aurata*) larvae fed microdiets with increased levels of MOS from 15-45 dph.

At the end of the feeding trial, larvae fed BM20 presented a significantly (p<0.05) higher crude lipid content than other larvae (Table 6.3). Increase in dietary MOS up to 1.5% (BM15) significantly (p<0.05) reduced larval content in saturated fatty acids, particularly 16:0 and 18:0, monounsaturated, such as 18:1n-9 and n-9 fatty acids (Table 6.4). Besides, larval total n-3, n-3 HUFA, EPA and DHA were increased by the elevation of MOS dietary levels up to 1.5% (BM15 diet). Consequently, there was a significant correlation between dietary MOS levels and larval n-3 HUFA contents (y =0.4036x + 0.6272, r=0.9968, P value=0.004) (Figure 20).

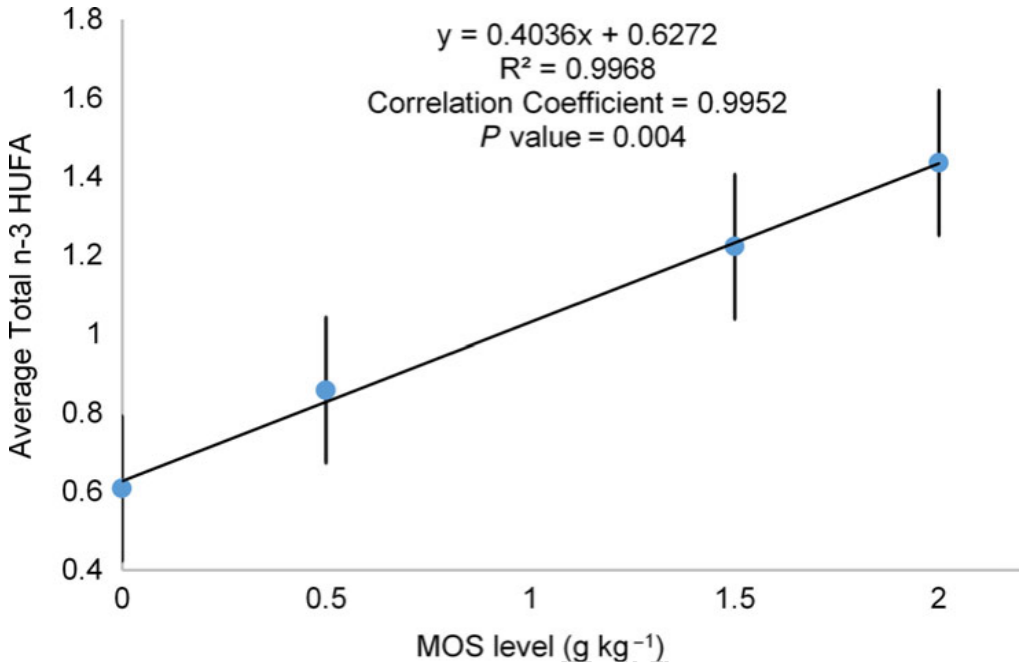

Figure 20. Total n-3 HUFA content in gilthead sea bream larvae (45 dph) fed microdiets with increased levels of MOS from 15 to 45 dph.

The total n-6 fatty acids were not increased, but 18:2n-6 showed a tendency to be reduced by the increase in dietary MOS, as well as 18:3n-6 (p<0.05), whereas 20:4n-6 was significantly increased (p<0.05, Table 6.4). Survival to handling stress seemed to be related to larval contents in ARA (y = -0.44 + 0.36x, r = 0.603, P value=0.223) and DHA (y = -59.82 + 15.3x, r = 0.596, P value=0.228). Liver histology showed that addition of MOS produced large hepatocytes due to the presence of lipid vacuoles in the cytoplasm, being more evident in larvae fed BM20 (Figure 21). Gut morphometric studies revealed similar (p>0.05) number of mucus secreting acid mucins by unit of intestinal area for both anterior and posterior intestine. However, after 30 days of supplementation with MOS, posterior gut presented a tendency to a higher number of

cells secreting acid mucins by unit of area when MOS was included in a dose-dependent way ($y = -0.53x^2 + 1.13x+0.54$, $R^2 = 0.74$, correlation coefficient=0.2156, P value=0.784) (Figure 22).

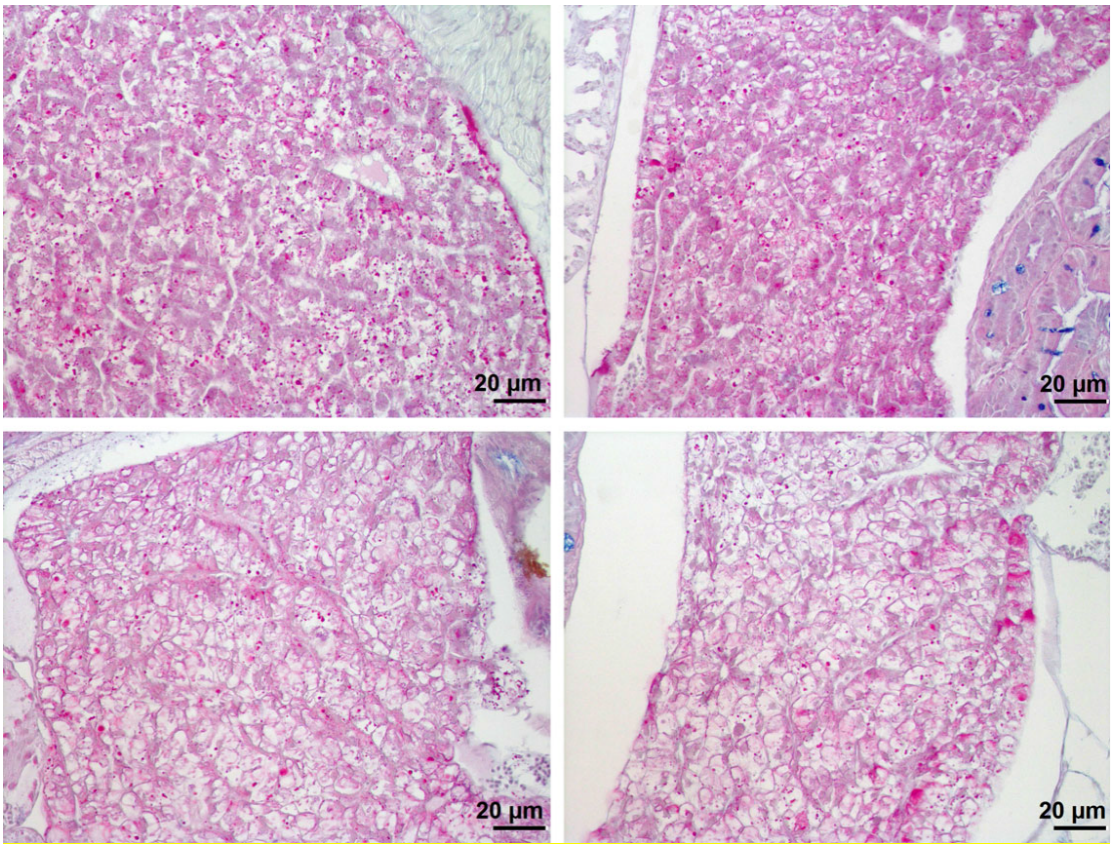

Figure 21. Liver (Alcian/PAS) from 45 dph gilthead sea bream (Sparus aurata) fed for 30-days of experimental microdiets containing (a) BM0, (b) BM5, (c) BM15 and (d) BM20. BM0 = 0 g kg^{-1} MOS; BM5 = 5 g kg^{-1} MOS; BM15 = 15 g kg^{-1} MOS; BM20 = 20 g kg^{-1} MOS.

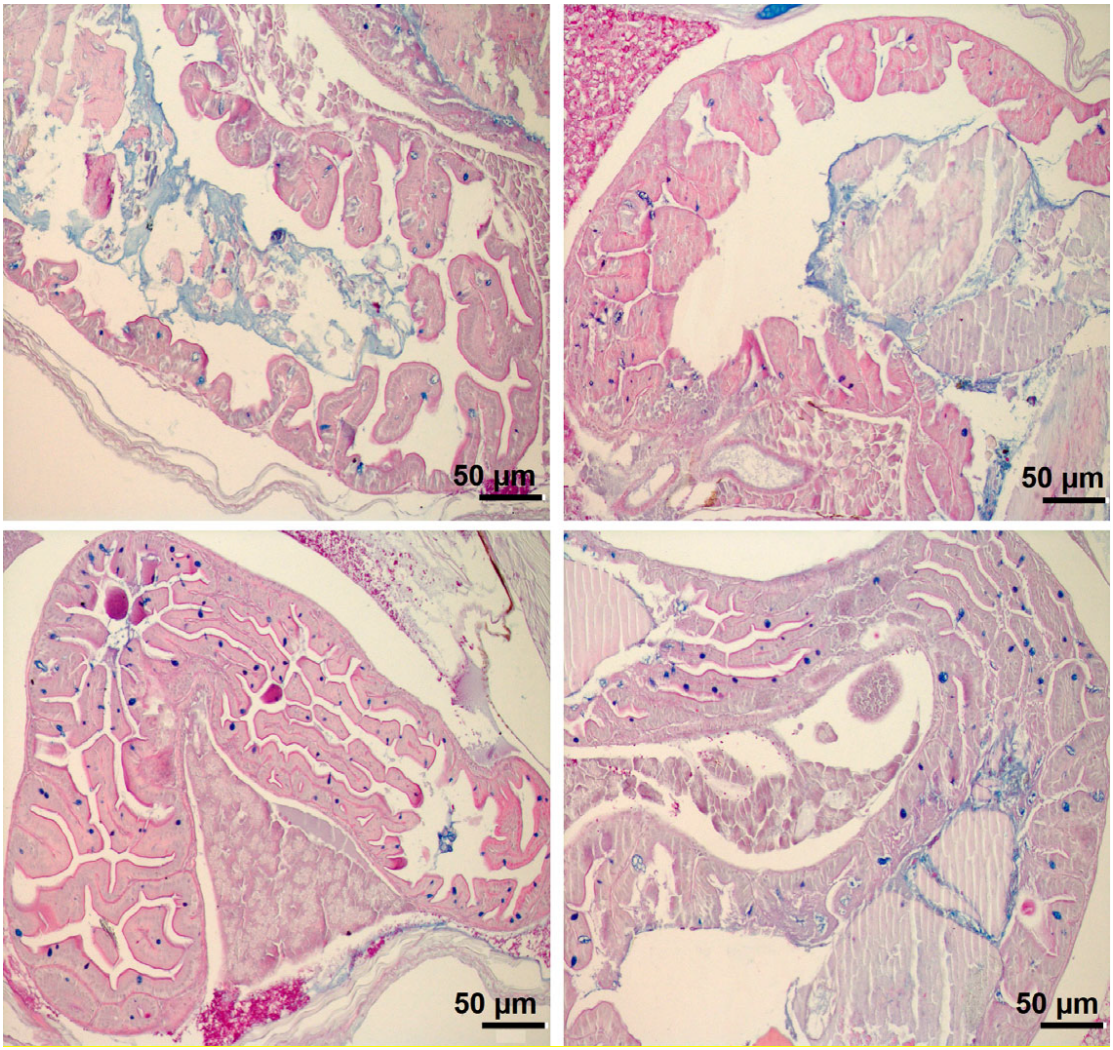

Figure 22. Gut (Alcian/PAS) from 45 dph gilthead sea bream (*Sparus aurata*) fed for 30 days of experimental microdiets containing (a) BM0, (b) BM5, (c) BM15 and (d) BM20. BM0 = 0 g kg^{-1} MOS; BM5 = 5 g kg^{-1} MOS; BM15 = 15 g kg^{-1} MOS; BM20 = 20 g kg^{-1} MOS.

At the end of the experiment, crude lipids levels of larvae were significantly found different in larvae fed 20 g kg^{-1} MOS (Table 6.3). Dietary MOS affected larvae fatty acid profiles (% dry body weight) by increasing saturated fatty acids content (mainly 14:0 and 16:0). Besides, larvae n-3 percentages were increased (p<0.05) in a dose dependent way after feeding MOS (y = 0.4004x + 0.6348 and R^2 = 0.99383; Pearson correlation coefficient = 0.999) (p<0.05), especially ARA (y = 0.0128x + 0.034 and R^2 = 0.9755; Pearson correlation coefficient =0.997) (p<0.05), EPA (y = 0.1061x + 0.1984 and R^2 = 0.9532; Pearson correlation coefficient =0.992) (p<0.05), DHA (y = 0.27x + 0.3945 and R^2 = 0.9936; Pearson correlation coefficient =0.998) (p<0.05), and total n-3 HUFA (y = 0.4004x + 0.6348 and R^2 = 0.99383, Pearson correlation coefficient = 0.987) (p<0.05). Moreover, larvae fed increased dietary MOS levels

presented higher (p<0.05) percentages of n-3, n-6 and DHA/EPA and DHA/ARA ratios (p<0.05) (Table 6.4). Interestingly, survival to handling stress was correlated with the levels of ARA and DHA found in larvae fed the different dietary treatments (ARAy= 10.07x + 4.8 with R^2 =0.370; Pearson correlation coefficient = 0.608; DHAy = 0.53x + 4.9 with R^2 = 0.372; Pearson correlation coefficient =0.610) (p<0.05). Dietary MOS inclusion showed no significant effects on goblet cells number in total interior and posterior intestine area of larvae. After 30 days of supplementation with MOS, morphological studies of the posterior gut showed a tendency ($y = -0.53x^2 + 1.13x + 0.54$, $R^2 = 0.74$) for a higher number of cells secreting acid mucins by unit of area when MOS was included in the diet. However, the analysis for multiple comparison of means denoted no significant differences among larvae fed the different MOS levels (p>0.05) (Figure 23).

Table 6.3 Final proximate composition (g 100 g^{-1} dry weight) of gilthead seabream (*Sparus aurata*) larvae fed MOS diet at the end of the feeding trial (t = 30 days)

	Initial	**BM0**	**BM5**	**BM15**	**BM20**
Crude protein	11.17±0.08	13.72±0.80	13.62±0.39	14.14±1.42	12.85±0.59
Crude lipids	21.28±0.15	14.05±2.14^{b}	19.59±1.68^{b}	18.64±3.94^{b}	28.30±4.97^{a}
Ash	0.80±0.10	1.32±0.42	1.85±0.62	1.63±0.16	1.43±0.22
Moisture	87.51±0.09	84.06±1.17	86.06±1.12	83.13±1.00	84.68±3.00

Different letters within a line denote significant differences (p<0.05). Values expressed in mean ± SD (n =3 tanks/diet).

Table 6.4 Fatty acids composition (% total identified fatty acids) of gilthead seabream larvae fed different levels of MOS from 15 to 45 dph

	Initial	**BM0**	**BM5**	**BM15**	**BM20**
14:0	0.02	0.07±0.01^{ab}	0.07±0.00^{ab}	0.07±0.01^{b}	0.09±0.01^{a}
15:0	0.01	0.09±0.00^{b}	0.09±0.00^{b}	0.09±0.00^{b}	0.012±0.00^{a}
16:0	0.08	0.73±0.07^{b}	0.70±0.03^{b}	0.73±0.06^{b}	0.94±0.04^{a}
16:1n-7	0.01	0.08±0.01^{b}	0.07±0.00^{b}	0.08±0.01^{b}	0.11±0.00^{a}

16:2n-4	0.01	0.06±0.01^{b}	0.06±0.00^{b}	0.06±0.01^{b}	0.08±0.00^{a}
16:3n-1	0.01	0.03±0.00^{b}	0.03±0.00^{b}	0.03±0.00^{b}	0.03±0.00^{a}
18:0	0.28	0.25±0.03^{b}	0.24±0.01^{b}	0.28±0.02^{b}	0.33±0.01^{a}
18:1n-9	0.07	0.32±0.04^{b}	0.30±0.01^{b}	0.36±0.01^{b}	0.45±0.01^{a}
18:1n-7	0.01	0.12±0.01^{b}	0.12±0.00^{b}	0.13±0.01^{b}	0.16±0.01^{a}
18:2n-6	0.00	0.03±0.00^{b}	0.03±0.00^{ab}	0.04±0.01^{ab}	0.04±0.00^{a}
18:3n-3	0.00	0.01±0.00^{b}	0.01±0.00^{ab}	0.02±0.00^{ab}	0.02±0.00^{a}
18:4n-3	0.00	0.01±0.00^{b}	0.01±0.00^{b}	0.02±0.00^{ab}	0.03±0.00^{a}
20:1n-7	0.02	0.04±0.01^{b}	0.04±0.00^{b}	0.04±0.00^{ab}	0.05±0.00^{a}
20:4n-6	0.01	0.03±0.00^{d}	0.04±0.00^{c}	0.05±0.00^{b}	0.06±0.00^{a}
20:3n-3	0.01	0.01±0.00^{b}	0.01±0.00^{b}	0.01±0.00^{ab}	0.01±0.00^{a}
20:4n-3	0.01	0.01±0.00^{b}	0.01±0.00^{ab}	0.01±0.00^{a}	0.01±0.00^{a}
20:5n-3	0.10	0.20±0.02^{c}	0.26±0.06^{bc}	0.33±0.02^{b}	0.43±0.02^{a}
22:1n-9	0.01	0.02±0.00	0.02±0.00	0.03±0.00	0.02±0.00
22:4n-6	0.01	0.00±0.00^{c}	0.00±0.00^{bc}	0.00±0.00^{ab}	0.01±0.00^{a}
22:5n-6	0.01	0.01±0.00^{d}	0.01±0.00^{c}	0.01±0.00^{b}	0.01±0.00^{a}
22:5n-3	0.05	0.02±0.00^{c}	0.02±0.00^{b}	0.04±0.00^{a}	0.05±0.00^{a}
22:6n-3	0.41	0.38±0.05^{c}	0.56±0.15^{b}	0.78±0.05^{a}	0.94±0.03^{a}
ΣSaturated	0.637	1.08±0.11^{b}	1.09±0.13^{b}	1.10±0.07^{b}	1.39±0.06^{a}
ΣMonounsaturated	0.501	0.60±0.07^{ab}	0.50±0.15^{b}	0.68±0.03^{a}	0.56±0.01^{ab}
Σn-3	0.601	0.65±0.09^{d}	0.91±0.21^{c}	1.22±0.05^{b}	1.50±0.04^{a}
Σn-6	0.317	0.08±0.01	0.11±0.01	0.12±0.01	0.14±0.00
Σn-9	0.297	0.49±0.06^{a}	0.37±0.02^{c}	0.40±0.01^{bc}	0.48±0.01^{ab}
Σn-3 HUFA	0.570	0.61±0.08^{c}	0.86±0.21^{b}	1.22±0.05^{a}	1.44±0.05^{a}
EPA/ARA	1.081	5.88±0.25^{b}	6.26±0.79^{ab}	6.46±0.71^{ab}	7.02±0.27^{a}
DHA/EPA	3.845	1.90±0.08^{b}	2.13±0.29^{ab}	2.40±0.27^{ab}	2.18±0.08^{a}
DHA/ARA	4.157	11.15±0.08^{c}	13.29±2.05^{b}	15.40±0.18^{a}	15.32±0.09^{a}
n-3/n-6	1.896	7.93±0.13^{c}	8.53±2.49^{ab}	10.14±0.44^{ab}	10.70±0.08^{a}

Different letters within a line denote significant differences ($p<0.05$). Control = 0 g kg^{-1} MOS; BM5 = 5 g kg^{-1} MOS; BM15 = 15 g kg^{-1} MOS; BM20 = 20 g kg^{-1} MOS. Values expressed in mean ± SD.

6.5. DISCUSSION

Several authors have demonstrated the benefits of dietary MOS supplementation in marine fish species growth performance and food efficiency by mainly reinforcing the functionality and integrity of the intestinal epithelium (Torrecillas *et al.*, 2014). In the present study, dietary MOS inclusion significantly increased larval survival, feed occupancy in gut and larval lipid contents, in relation to a possible positive MOS effect on gut development and integrity during early larval development that may result in a better nutrient absorption. In agreement, fish fed the highest BM dietary content resulted in higher liver lipid vacuolization grade in agreement with the higher larval lipid content and higher feed intake. Nevertheless, the potential beneficial effects of MOS are highly dependent on the type of product used, the dose and time of supplementation, the feed production process, fish species and age (Torrecillas *et al.*, 2014). In agreement, in the present study, a short-term MOS supplementation (15 days of feeding) resulted in improved gilthead sea bream larval growth when fed 15 g kg^{-1} MOS treatment. However, when longer periods of dietary MOS supplementation were evaluated (30 days of feeding), this effect was limited to a positive trend between the level of dietary MOS and larval growth and survival, denoting a dose- and time-dependent effect on gilthead sea bream larvae. Similar results have been reported before in white sea bream *Diplodus sargus* (L.) larvae fed with 0.2% MOS (Bio-Mos, Alltech) enriched Artemia between 17 and 23 dph (Dimitroglou *et al.*, 2010) and cobia *Rachycentron canadum* (L.) larvae fed with 0.2% MOS (Bio-Mos) (Salze *et al.*, 2008). These results suggest that MOS supplementation to gilthead sea bream larvae has a more important effect during the early stages of larval development (15–30 dph) when fish gut is poorly developed, than at 45 dph when gilthead sea bream acid digestion starts to be detected and enterocytes and gut-associated lymphoid tissue (GALT) functionality are better developed (Picchietti *et al.*, 2007). Furthermore, the inverse relation between larval survival and growth performance found in the present study suggests that the mortality of small size larvae in treatments with the lowest survival (BM0 and BM5) rendered a higher average larval size, in view of their lower size dispersion. Besides, the mortality of small larvae would increase feed availability for others, as all tanks were supplied with the

same amount of feed. In terms of stress resistance, larvae fed 5 g kg^{-1} MOS presented a better stress resistance to air exposure, than those fed lower levels of MOS dietary inclusion. Similar results have been reported for white sea bream (*Diplodus sargus*) larvae fed MOS after a salinity stress test (Dimitroglou *et al.*, 2010). This improved stress resistance could be related to a potential beneficial effect of dietary MOS on skin and gills mucous cell areas and density in relation to a better protection on the mucosal epithelium (Torrecillas *et al.*, 2015). Moreover, in the present study a stress resistance was related to a higher whole body content of essential fatty acids such as ARA and DHA, important components of body tissues that promote fish growth, survival stress resistance and metabolism (Samrongpan *et al.*, 2008; Sang and Fotedar, 2010). Besides, dietary inclusion of 1.5% MOS reduced monounsaturated and saturated fatty acid contents in the larvae and increased the relative contents of ARA, EPA and DHA, in agreement with the higher intermediate growth. Dietary concentrated MOS (Actigen®, Alltech, Nicholasville, KY, USA) supplementation to European sea bass juveniles has also been related to higher liver percentages of HUFA and lower levels of long-chain monoenoic fatty acids, which are preferentially catabolized in ß-oxidation in relation to a better fish performance (Torrecillas *et al.*, 2015). Actually, MOS effects on lipid metabolism include decreased lipogenesis by reduction in the activity of enzymes such as glucose-6-phosphate dehydrogenase and malic enzyme compared with fish fed control diet (Santoso *et al.*, 1995; Torrecillas *et al.*, 2011) as well as increased lipolytic activities in liver (Gallaher *et al.*, 2000). In agreement, in the present study, increase in dietary MOS up to 1.5% reduced body content of 16:0. 18:0 and 18:1n-9, terminal fatty acids of lipogenesis in fish and main substrates for lipolytic activities.

In summary, the results of the present study demonstrated the positive effect of MOS inclusion in early weaning diets on gilthead sea bream larvae feed acceptance and survival, together with improved growth and fatty acid utilization when larvae are fed 1.5% MOS from 15 dph. Further studies determining the optimal larva age and dose and time of MOS supplementation while observing larval gut development might help to optimize culture performance of gilthead sea bream.

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CHAPTER 7

EFFECT OF DIETARY MINERALS IN EARLY WEANING DIETS ON GROWTH, SURVIVAL, MINERAL CONTENTS AND GENE EXPRESSION IN GILTHEAD SEA BREAM (*Sparus aurata,* L.) LARVAE

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Keywords: Larval nutrition, copper, zinc, manganese, selenium, gene expression, growth, gilthead sea bream.

7.1. ABSTRACT

Despite the importance of trace minerals for the normal development of marine fish larvae, previous studies have demonstrated that the contents of these nutrients in the live preys used in larviculture are much lower than those of copepods. Moreover, our previous studies have shown the importance of a premix inclusion of Zn, Mn and Se in early weaning diets for larval growth, and suggested a potential toxicity by one of these elements. The objective of the present study was to determine the effect of the single dietary inclusion of Zn, Mn, Se and Cu, their combination (Control+) or their absence (Control-). These 6 diets were fed to 22 day-old gilthead seabream larvae in triplicates for three weeks. At the end of the trial, survival was significantly ($p<0.05$) lowest in fish fed all these minerals (C+ diet, 17.16±7 % mean±SD), followed by that of larvae fed Mn (Mn diet, 21.91±7). The highest survival was obtained by supplementation with Cu (Cu diet, 35.27±15), followed by negative control (C- diet, 34.58±9). Cu and Se significantly improved larval growth, in terms of total length and body weight, in comparison to the C- fish, whereas fish fed Mn and C+ showed the lowest fish growth. Supplementation with Zn or Cu significantly increased *CuZnsod* whereas *gpx* was significantly up-regulated in fish fed Se and C+ diets. Other oxidative stress related enzymes were not affected. Fatty acid composition of the diets was homogeneous as well as that of the larvae, except for a significantly highest ARA/EPA and lowest DHA/EPA in larvae fed Cu diet and the opposite in fish fed C+ diet. The results pointed out the importance of supplementation with Cu, as well as Se and Zn on early weaning diets for gilthead seabream, and the potential toxic effect of Mn. Further studies are being conducted to determine optimum levels of these nutrients in diets for marine fish larvae.

7.2. INTRODUCTION

Improvement of diets for marine fish larvae requires the complete understanding of nutritional requirements of these early life stages in the different species (Kolkovski *et al.*, 2009; Hamre *et al.*, 2013). In spite of the abundance of research regarding lipids and essential fatty acids (Izquierdo and Koven, 2011), proteins and aminoacids (Conceição *et al.*, 2010) or vitamins A, C and E (Moren *et al.*, 2011), studies aiming to determine the essentiality of other micronutrients are more scarce (Hamre et al., 2011). Regarding microminerals, essentiality of iodine (I) and selenium (Se) has been

established for marine fish larvae (Ribeiro *et al.*, 2009; Hamre *et al.*, 2008b; Moren *et al.*, 2006; Saleh *et al.*, 2014), whereas the effects of other dietary microminerals such as zinc (Zn), copper (Cu) or manganese (Mn) on fish larvae have been much less studied (Hamre et al., 2013). Despite these minerals form part of key metalloenzymes that play important roles in fish physiology, it is not yet known if their requirements in larvae are covered by the dietary inputs obtained by live preys.

Zn is an important mineral included in 20 metallo-enyzmes that catalyze different metabolic pathways related to growth, reproduction, tissue development, vision or immunity (Watanabe *et al.,* 1997). Metabolism of carbohydrates, lipids and proteins is directly related to Zn-chained enzymes such as carbonic anhydrase, alkaline phosphatase or carboxypeptidase A. Moreover, Zn plays important roles in oxidative stress and reducing cellular damage through antioxidant enzymes like alcohol dehydrogenases or cytosolic superoxidase dismutase (Watanabe *et al.*, 1997; Halver and Hardy, 2002). Zn intervenes on bone formation and mineralization by activating osteoblastic cells and inhibiting osteoclastic bone resorption, hence promoting ossification, bone development and, subsequently, growth (Yamaguchi, 1998). Studies have demonstrated that Zn can partially substitute calcium (Ca) in the bone due to their similar physic-chemical properties (Do Carmo e Sá *et al.*, 2004). Uptake of Zn during larval development seems to be determinant to control mineral status in the larvae of amberjack (*Seriola dumerilii*) (Yamamoto *et al.*, 2013) and improves growth and survival in red sea bream (*Pagrus major*) (Nguyen *et al.,* 2008).

Cu forms part of several enzymes such as cytochrome oxidase, superoxide dismutase, dopamine hydroxylase or tyrosinase and regulates lipid and blood metabolism and counteracts oxidative stress. Therefore, Cu plays important functions in vital organs such as heart, eye, brain, liver or bone (Watanabe *et al.,* 1997; Lall and Lewis-McCrea, 2007), whereas excessive levels lead to reduced growth (Lin *et al.*, 2010) or toxicity that may induce necrosis in gills or liver (Murai *et al.,* 1981; Woody and O'Neal, 2012). Deficient Cu levels in diets for juveniles may lead to Cu reduction in tissues and low growth (Tang *et al.*, 2013). However, its importance in larval diets has not been sufficiently addressed. For instance, Cu and Zn contents in rotifers are markedly reduced when they are added to fish larval tanks, even if microalgae is supplied to the larval rearing tanks, whereas availability of both elements increase in rotifers with digestive replete in microalgae (Wang *et al.*, 2019).

Mn is known to be an essential mineral for growth, reproduction and prevention of skeletal abnormalities in terrestrial animals and fish. It is a cofactor in many enzymatic processes, being a component of metalloenzymes such as arginase, pyruvate carboxylase and Mn superoxidase dismutase (Leach, 1976). Mn is important for mitochondria functioning, affecting lipid, protein and carbohydrate metabolism (Lall, 2002). Thus, the lack of dietary Mn leads to low growth in rainbow trout or carp (Ogino and Yang, 1980), whereas its presence promotes protein synthesis and growth (Romanenko, 1984). Despite fish can directly absorb Mn from water, Ca and Phosphorus (P) presence reduces Mn absorption that, therefore, must be included in the diet (Watanabe *et al.*, 1997). Mn deficiency in fish reduces growth (Satoh *et al.,* 1987; Tan *et al.,* 2012) and affects manganese superoxide dismutase (Mnsod) and copper/zinc superoxide dismutase (Cu/Znsod) activities (Lin *et al.,* 2008a,b), as well as tissue mineral composition. Artemia seems to be deficient in Mn and enrichment with this mineral improves growth and reduces skeletal anomalies in red sea bream larvae (Nguyen *et al.*, 2008).

Se forms part of a considerable number of selenoproteins including glutation peroxidase (*gpx*) (Penglase *et al.*, 2014) and plays an important antioxidant role (Pacitti *et al.*, 2013), being essential for reproduction and correct larval development (Hamre *et al.*, 2008). Se deficiency may reduce growth, *gpx* activity and survival (Hardy *et al.*, 2010; Moren *et al.*, 2011; Le and Fotedar, 2013; Saleh *et al.*, 2014). Absorption of organic Se has been found to be higher than that of mineral salts (Schrauzer, 2003). Se content in the different live preys used in larviculture markedly differs and has been related to growth improvements (Penglase *et al.*, 2010; Ribeiro *et al.*, 2012). Increase of organic (yeast) Se in larval diets for European seabass (*Dicentrarchus labrax*) reduces oxidative damage (Betancor *et al.*, 2012) and for gilthead seabream (*Sparus aurata*) improves survival and stress resistance (Saleh *et al.*, 2014).

Supplementation of these minerals seems to be necessary in microdiets for larval gilthead seabream, since non-supplementation reduces growth and bone mineralization, leading to a high incidence of bone anomalies (Izquierdo *et al*., 2017). However, supplementation with three of these minerals (Zn, Mn and Se) at the same time in inorganic forms in levels similar to those found in copepods tend to reduce larval survival after a stress test, suggesting the deleterious effect of one or more of these minerals (Izquierdo *et al*., 2017). Thus, the aim of the present study was to

determine the effect of single inclusion of Zn, Cu, Mn and Se in microdiets for gilthead seabream on larval performance, whole body composition, and expression of selected genes.

7.3. MATERIALS AND METHODS

7.3.1 Feeding trial and larval performance

Larvae were obtained from natural spawns from the gilthead sea bream (*Sparus aurata*) broodstock of Grupo de Investigación en Acuicultura (GIA) (EcoAqua Institute, Las Palmas de Gran Canaria, Spain). Larvae (initial total length 6.79±0.42 mm, dry body weight 0.22±0.02 mg, mean±SD) previously fed rotifers (*Brachionus plicatilis*) enriched with DHA Protein Selco® (INVE, Dendermond, Belgium) until 20 days post hatching (dph) were randomly distributed in 18 experimental tanks at a density of 2100 larvae each tank (0.9 larvae l^{-1}) and were fed one of the experimental diets tested in triplicate for 22 days, at a water temperature of 19.2-21.1ºC. All tanks (200 L light grey colour cylinder fiberglass tanks) were supplied with filtered seawater (37 g/L salinity) at an increasing rate of 0.4-1 L/min along the feeding trials. Water entered the tank from the bottom and was let out from the top; water quality was tested daily, and no deterioration was observed. Water was continuously aerated (125 mL/ min), attaining 6.0-6.2 g/L dissolved O_2.

Six isonitrogenous and isolipidic experimental microdiets (pellet size <250 µm) based on squid meal and krill oil with gelatin as a binder, attractants (Kanazawa *et al*., 1989), and vitamin and mineral premixes (Teshima *et al*., 1982) lacking Zn, Cu, Mn and Se were formulated and produced according to Saleh *et al.* (2014). The negative control diet (Diet C-) was not supplemented with the target minerals and their levels were low (83 mg kg^{-1} Zn, 17 mg kg^{-1} Cu, 4 mg kg^{-1} Mn, and 1.9 mg kg^{-1} Se), being lower than their levels in copepods (120 mg kg^{-1} Zn (Fujita, 1972); 3 mg kg^{-1} Se and 8 mg kg^{-1} Mn (Hamre *et al.*, 2008a,b). Another four diets were respectively supplemented with either $ZnSO_4$, $CuSO_4$, $MnSO_4$ or organic Se (yeast Se, Alltech, Lexington, KY, USA) at similar levels to those found in copepods and, finally, a fifth positive control diet was supplemented with the four minerals (Table 7.1). Sulphate forms were preferred over oxide forms for the better absorption (Lorentzen and Maage, 1999; Maage *et al.*, 2000), whereas organic Se was very effectively used by gilthead seabream larvae in our previous studies (Betancor *et al.*, 2012; Izquierdo *et*

al., 2017). Diets were supplied manually every 45 min from 9:00 to 19:00 at a rate of 2.5-3.5 g tank^{-1}. Larvae were observed under the binocular microscope to determine feed acceptance. If apparent feed intake differences were observed along different experimental diets, diet acceptance was determined calculating the percentage of gut occupation of the microdiet by image analysis. For such studies, pictures were taken of the abdominal cavity of 30 larvae per tank (Leica Wild M3Z, Optotek, California, USA).

Table 7.1 Ingredients and analyzed proximate composition and mineral contents in the experimental microdiets supplemented with minerals to feed gilthead seabream larvae from 20 to 24 dph

Ingredients (% dry diet)	**C-**	**Zn**	**Cu**	**Mn**	**Se**	**C+**
Squid Powder[a]	71	71	71	71	71	71
Gelatine[b]	3	3	3	3	3	3
Krill Phospholipids[c]	12.5	12.5	12.5	12.5	12.5	12.5
Mineral Premix (no Zn, Mn, Cu or Se)	4.48	4.48	4.48	4.48	4.48	4.48
$ZnSO_4.7H_2O$[d]	-	0.015	-	-	-	0.015
$CuSO_4.5H_2O$[e]	-	-	0.0013	-	-	0.0013
$MnSO_4.H_2O$[f]	-	-	-	0.003	-	0.003
Organic Se[g]	-	-	-	-	0.000003	0.000003
Vitamins[h]	6	6	6	6	6	6
Attractants[k]	3	3	3	3	3	3
Lipid (% dw)	16.80±0.12	17.73±0.18	17.50±0.19	17.58±0.11	17.55±0.28	15.69±0.13
Protein (% dw)	65.40±0.16	65.25±0.11	65.06±0.12	65.65±0.08	65.47±0.13	67.51±0.16
Ash (% dw)	6.78±0.02	6.52±0.06	6.75±0.03	6.72±0.05	6.89±0.01	6.34±0.06
Moisture (% dw)	9.78±0.13	10±0.09	9.73±0.11	8.72±0.07	9.45±0.12	10.19±0.08
Zn (mg.kg-^{1})	83	119	88	85	83	112
Cu (mg.kg-^{1})	17	17	21	17	17	20
Mn (mg.kg-^{1})	4	4	4	13	4	11

Se (mg.kg-[1])	1.9	1.8	1.9	1.7	2	1.8

[a] Rieber & Son, Bergen, Norway.
[b] Panreac, Barcelona, Spain.
[c] Qrill (high in PL) Aker BioMarine, Fjordalle´en, Norway.
[d] $ZnSO_4.7H_2O$
[e] $CuSO_4.5H_2O$
[f] $MnSO_4.H_2O$
[g] Organic Se (yeast Se, Alltech, Lexington, KY, USA).
[h] Eryalçın, Roo, Saleh, Atalah, Benitez, Betancor, Hernandez-Cruz and Izquierdo (2013).
[k] Betancor, Nordrum, Atalah, Caballero, Benitez-Santana, Roo, Robaina and Izquierdo (2012a) and Betancor, Caballero, Terova, Cora, Saleh, Benitez-Santana, Bell, Hern andez-Cruz and Izquierdo (2012b).

Growth was determined by measuring dry body weight (105ºC until constant weight) and total length (Profile Projector V-12A; Nikon, Tokyo, Japan) of 30 fish/tank at the beginning, in the middle, and at the end of the trial. Final survival was calculated by individually counting all the live larvae at the beginning and at the end of the experiment. Before the end of the experiment, an activity test was conducted by handling 20 larvae/tank out of the water in a scoop net for 1 min and, subsequently allocating them to another tank supplied with clean seawater and aeration to determine survival after 24 h. The remaining larvae in each tank were starved for 16 hours, washed with distilled water, sampled, and kept at 80ºC to analyze biochemical composition.

7.3.2. Proximate composition and fatty acids and mineral profiles

Moisture (A.O.A.C., 1995), protein (A.O.A.C., 1995) and crude lipid (Folch *et al*., 1957) contents of larvae and diets were analyzed. Fatty acid methyl esters were obtained by transmethylation of crude lipids as described by Christie (1982), separated by gas-liquid chromatography (GLC), quantified by FID (GC-14A; Shimadzu, Tokyo, Japan) under the conditions described by Izquierdo *et al.* (1992), and identified by comparison with previously characterized standards and GLC-MS. Mineral analysis was conducted according to the method of Julshamn *et al.* (2004). Samples were acidified in a microwave digester (MarsXpress, CEM, Kamp-Lintfort, Germany) with 5 mL of 69% pure nitric acid, then poured into a 10-mL volumetric flask, and made up to volume with distilled water. A total of 0.4 mL of this solution was then added to a 10-mL sample tube; 10 µL of the internal standard (Ga and Sc, 10 ppm) was included and 0.3 mL of methanol added. The tubes were made up to volume with distilled water and total selenium was measured by collision/reaction

ICP-MS (Thermo Scientific, Cheshire, UK) using argon and hydrogen as carrier gasses.

7.3.3. Gene expression

Molecular biology analysis was carried out at GIA laboratories. Total RNA from larvae samples (average weight per sample 60 mg) was extracted using the RNeasy Mini Kit (Qiagen). Total body tissue was homogenized using the Tissue Lyzer-II (Qiagen, Hilden, Germany) with QIAzol lysis reagent (Qiagen). Samples were centrifuged with chloroform for phase separation (12000 g, 15 min, 4°C). The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into an RNeasy spin column where total RNA bonded to a membrane and contaminants were washed away by RW1 and RPE buffers (Qiagen). Purified RNA was eluted with 30 lL of RNase-free water. The quality and quantity of RNA were analyzed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Synthesis of cDNA was conducted using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Primer efficiency was tested with serial dilutions of a cDNA pool (1, 1:5, 1:10, 1:15, 1:20 and 1:25). The product size of the real-time qPCR amplification was checked by electrophoresis analyses using PB322 cut with HAEIII as a standard. Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) using b-actin as the house-keeping gene in a final volume of 20 lL per reaction well, and 100 ng of total RNA reverse-transcribed to complementary cDNA. Each gene sample was analyzed once per gene. The PCR conditions were the following: 95 °C for 3 min 30s followed by 40 cycles of 95°C for15s, 61°C for 30s, and 72°C for 30s; 95°C for 1 min; and a final denaturing step from 61 °C to 95 °C for 10 s. Data obtained were normalized and the Livak method (2-‴Ct) used to determine relative mRNA expression levels. Gilthead sea bream specific gene primers were designed after searching the NCBI nucleotide database and using the Oligo 7 Primer Analysis Software (Molecular Biology Insights, Cascade, CO, USA). Detailed information on primer sequences and accession numbers is presented in Table 7.2.

Table 7.2. Sequences of forward and reverse primers (5'-3') for real-time quantitative PCR of gilthead sea bream

Gene	Primers	Accesion no
Catalase (*cat*)	Forward primer: 5′-ATGGTGTGGGACTTCTGGAG-3′ Reverse primer: 3′-AGTGGAACTTGCAGTAGAAAC-5′	FJ860003
Superoxide dismutase (*CuZnsod*)	Forward primer: 5′-AAGAATCATGGCGGTCCTACTGA-3′ Reverse primer: 3′-TGAGCATCTTGTCCGTGATGTCT -5′	AJ937872
Manganese superoxide (Mn*sod*)	Forward primer: 5'-AGTGCCTCCTGATATTTCTCCTCTG-3' Reverse primer: 5'-CCTGACCTGACCTACGACTATGG-3'	JQ3088331
Glutathione peroxidase (*gpx*)	Forward primer: 5′-TCCATTCCCCAGCGATGATGCC-3′ Reverse primer: 3′-TCGCCATCAGGACCAACAAGGA-5′	DQ524992
osteocalcin (*oc*)	Forward primer: 5′-AGCCCAAAGCACGTAAGCAAGCTA-3′ Reverse primer: 3′-TTTCATCACGCTACTCTACGGGTT-5′	AF048703

7.3.4. Statistical analysis

All data were tested for normality and homogeneity of variances with Levene´s test, and treated using one-way ANOVA. Means were compared by Duncan's test ($p<0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA). All values presented as percentage were arc cosine transformed before performing any statistical test.

7.4. RESULTS

7.4.1. Feeding trial and larval performance

Microdiets were well accepted by the larvae and no differences were found in ingestion of the various experimental diets. After 21 days of feeding seabream larvae had increased 4 times their initial body weight and significant differences were found in survival and growth (Table 7.2). The highest survival was found in fish fed the Cu supplemented diets, together with those fed non-supplemented target minerals (C-) ($p<0.05$, Table 7.2). On the contrary, the lowest survival rate was found in larvae fed the combination of the four minerals (C+), followed by those fed diets supplemented with Zn, Se and, particularly, Mn ($p<0.05$, Table 7.2). Besides, Cu supplementation significantly improved total length in comparison to larvae fed a non-supplemented diet (C-) ($p<0.01$, Table 7.2), whereas the lowest length was obtained in larvae fed Mn-supplemented and C+ diets. Zn and Se supplementation did not negatively

affected larval total length. Similarly, lowest larval body weight was found in C+ larvae, followed by C- ($p<0.01$, Table 7.2), whereas Cu or Se supplementation significantly increased larval body weight, and Zn and Mn supplementation did not negatively affected larval weight ($p<0.01$, Table 7.2). Regarding condition index, Cu supplementation produced heavier larvae, whereas larvae fed with either C- or C+ diets were 33% thinner (Table 7.2). No significant differences were found in survival after air exposure stress test (92-98%).

7.4.2. Proximate composition and fatty acids and mineral profiles

At the end of the feeding trial there were no significant differences in lipid or protein contents in larval whole body (Table 7.3). There were neither significant differences in the fatty acid profiles of whole body among larvae fed the different microdiets (Table 7.3).

Table 7.3 Survival and growth in gilthead seabream larvae fed for 21 days with diets containing different mineral supplementation (mean ± SD)

	Age (dph)	**C-**	**Zn**	**Cu**	**Mn**	**Se**	**C+**
Survival* (%)	42	34.58±9^{a}	26.31±5^{ab}	35.27±15^{a}	21.91±7^{ab}	22.29±4^{ab}	17.16±7^{b}
Total length** (mm)	31	7.63±0.68	7.66±0.68	7.83±0.76	7.68±0.77	7.87±0.81	7.83±0.82
	42	9.42±1.20^{bc}	9.51±1.02^{abc}	9.76±0.99^{a}	9.30±0.95^{c}	9.65±0.96^{ab}	9.30±0.78^{c}
Dry body weight*** (mg)	31	0.39±0.04	0.43±0.02	0.58±0.17	0.43±0.08	0.44±0.03	0.40±0.08
	42	0.89±0.07^{cd}	1.05±0.19^{bc}	1.34±0.07^{a}	1.00±0.18^{bcd}	1.20±0.07^{ab}	0.79±0.08^{d}
CI (L/W)*	31	20	18	14	18	18	20
	42	11	9	7	9	8	12

*n=3, $p<0.05$. **n=90, $p<0.01$. ***n=9, $p<0.01$. Different low-case letters at a given day denote significant differences.

However, ARA content was slightly lowest in larvae fed the Cu supplemented diet and highest in those fed the C+ diet. Subsequently, EPA/ARA ratios were significantly ($p<0.05$) highest in larvae fed Cu or Zn supplemented diet, as well as in those fed C- diet (Table 7.4), and lowest in fish fed C+. Moreover, there was a significant negative relation between DHA/EPA contents in larvae and survival rate ($R^2 = 0.70165$; P value: 0.156).

Table 7.4 Lipid and protein contents (% dry weight) and main fatty acids profiles (% total identified fatty acids) of gilthead seabream larvae fed microdiets with different minerals from 20 to 42 dph

	Initial	**C-**	**Zn**	**Cu**	**Mn**	**Se**	**C+**
Crude lipid	16.46	18.44±2.33	19.29±1.28	17.32±1.71	19.90±1.54	19.82±1.22	17.88±4.43
Crude protein	69.81	66.06±1.32	65.23±1.08	66.53±0.45	65.62±1.25	66.52±2.74	67.75±4.70
14:0	0.02	0.08±0.01	0.09±0.01	0.08±0.01	0.09±0.02	0.083±0.0	0.08±0.04
15:0	0.01	0.01±0.00	0.01±0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
16:0ISO	0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
16:0	0.33	0.69±0.19	0.77±0.05	0.61±0.04	0.69±0.08	0.82±0.19	0.81±0.23
16:1n-7	0.11	0.08±0.02	0.09±0.00	0.07±0.00	0.08±0.01	0.09±0.02	0.09±0.03
16:1n-5	0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
16:3n-4	0.01	0.01±0.00	0.01±0.00	0.00±0.00	0.01±0.00	0.01±0.00	0.01±0.00
18:0	0.22	0.22±0.06	0.25±0.02	0.20±0.01	0.23±0.03	0.27±0.05	0.28±0.07
18:1n-9	0.33	0.27±0.07	0.29±0.02	0.24±0.02	0.27±0.03	0.32±0.07	0.31±0.08
18:1n-7	0.11	0.13±0.04	0.15±0.01	0.12±0.01	0.13±0.01	0.16±0.04	0.15±0.04
18:1n-5	0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.01	0.01±0.00	0.01±0.00

18:2n-6	0.21	0.04±0.01	0.04±0.00	0.03±0.00	0.04±0.01	0.04±0.01	0.04±0.01
18:3n-3	0.09	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
18:4n-3	0.01	0.02±0.00	0.03±0.00	0.03±0.02	0.02±0.00	0.03±0.00	0.02±00
20:0	0.00	0.01±0.00	0.01±0.01	0.02±0.02	0.01±0.00	0.01±0.00	0.01±0.00
20:1n-9	0.03	0.05±0.01	0.05±0.00	0.06±0.03	0.05±0.00	0.05±0.01	0.06±0.02
20:4n-6	0.07	0.04±0.01	0.05±0.00	0.04±0.01	0.04±0.01	0.05±0.00	0.05±0.00
20:3n-3	0.01	0.01±0.00	0.01±0.00	0.01±0.01	0.01±0.00	0.01±0.00	0.01±0.00
20:4n-3	0.02	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
20:5n-3	0.11	0.34±0.04	0.37±0.08	0.31±0.07	0.32±0.08	0.35±0.02	0.30±0.02
22:5n-6	0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
22:5n-3	0.04	0.04±0.00	0.04±0.01	0.03±0.01	0.04±0.00	0.04±0.00	0.04±0.01
22:6n-3	0.29	0.58±0.05	0.62±0.16	0.45±0.12	0.54±0.18	0.59±0.10	0.58±0.13
Saturated	0.59	0.93±0.26	1.13±0.07	0.92±0.04	1.03±0.10	1.19±0.28	1.19±0.34
Mono-unsaturated	0.64	0.57±0.15	0.64±0.04	0.56±0.05	0.60±0.05	0.70±0.16	0.68±0.18
Σ n-3	0.59	1.03±0.10	1.10±0.25	0.93±0.22	0.96±0.28	1.06±0.11	0.99±0.15
Σ n-6	0.33	0.11±0.02	0.12±0.01	0.10±0.01	0.12±0.02	0.13±0.01	0.13±0.01
Σ n-9	0.36	0.28±0.07	0.31±0.01	0.26±0.03	0.29±0.03	0.34±0.07	0.33±0.08
Σ n-3 HUFA	0.48	0.98±0.10	1.05±0.25	0.86±0.20	0.91±0.27	1.00±0.11	0.94±0.16
EPA/ARA	1.65	7.64±0.69[a]	7.87±1.09[a]	7.97±0.71[a]	7.25±0.32[ab]	6.01±0.50[ab]	5.73±0.36[b]
DHA/EPA	2.56	1.68±0.09	1.67±0.10	1.60±0.14	1.69±0.16	1.68±0.21	1.90±0.33
DHA/ARA	4.21	12.84±1.82	13.17±2.55	12.77±2.02	12.29±1.63	11.76±2.28	10.92±2.55

n-3/n-6	1.77	9.132±1.13	9.057±1.53	9.26±1.21	8.32±1.08	8.26±1.52	7.56±1.62

Different letters within a line denote significant differences ($p<0.05$). Values expressed in mean ± SD.

Increase in dietary Zn levels in diets Zn and C+ did not significantly increased whole body Zn content in gilthead seabream larvae (Table 7.5, $p>0.05$). However, whole body Zn contents increased in larvae fed diets supplemented with Cu (Table 7.5, $p<0.05$). Cu whole body contents were not significantly different, despite a tendency to be slightly higher in larvae fed increased dietary Cu levels (diets Cu and C+) (Table 7.5, $p>0.05$). Elevation of dietary Mn levels (diets Mn and C+) significantly raised larval Mn contents ($p<0.05$). Whole body Se contents were not affected by dietary Se contents. Finally, Fe body contents were significantly ($p>0.05$) higher in larvae fed Mn, followed by those fed Zn or Cu.

Table 7.5 Mineral content of gilthead seabream larvae fed for 21 days with diets containing different mineral supplementation

DIET	C-	Zn	Cu	Mn	Se	C+
Zn (mg kg^{-1})	16.21 ± 2.42^{b}	15.94 ± 0.39^{c}	23.17 ± 6.32^{a}	18.40 ± 4.10^{ab}	14.56 ± 0.83^{c}	16.33 ± 2.07^{b}
Cu (mg kg^{-1})	0.61 ± 0.04^{b}	0.59 ± 0.03^{b}	0.75 ± 0.17^{a}	0.64 ± 0.07^{b}	0.63 ± 0.03^{b}	0.70 ± 0.08^{ab}
Mn (mg kg^{-1})	0.19 ± 0.04^{b}	0.19 ± 0.02^{b}	0.19 ± 0.07^{b}	0.38 ± 0.11^{a}	0.18 ± 0.01^{b}	0.34 ± 0.07^{a}
Se (mg kg^{-1})	0.27±0.04	0.26±0.01	0.29±0.03	0.27±0.01	0.24±0.03	0.29±0.03
Fe (mg kg^{-1})	5.4 ± 0.4^{d}	10.8 ± 4.6^{b}	14.9 ± 7.4^{a}	7.8 ± 1.2^{c}	5.5 ± 1.1^{d}	8.7 ± 2.7^{c}

7.4.3. Gene expression

Molecular studies for antioxidant enzymes genes showed that *cat* expression was highest in larvae fed C- diet and tend to be down-regulated by dietary mineral supplementation being double than that of larvae fed C+, but not significantly different (Table 7.6, $p>0.05$). Expression of *CuZnsod* was highest in larvae fed Zn

supplemented diets, being significantly (p<0.05) different from that in larvae fed C- diet. *CuZnsod* expressions tend to be also higher in larvae fed Cu and Mn supplemented diets, followed by those fed C+ and Se diets. No significant differences were found in expression of *Mnsod* (Table 7.6). Expression of *gpx* was significantly (p<0.05) highest in larvae fed C+ diet, followed by those fed Se supplementation, and lowest in C- fed larvae (Table 7.6). There were neither differences in the *oc* expression.

Table 7.6 Expression of antioxidant enzymes and osteocalcin genes in gilthead seabream larvae fed for 21 days with diets containing different mineral supplementation

DIET	C-	Zn	Cu	Mn	Se	C+
cat	1.24±0.19^{a}	1.15±0.25^{b}	1.16±0.24^{b}	0.94±0.26^{c}	0.76±0.37^{d}	0.62±0.32^{d}
CuZnsod	0.81±0.26^{c}	1.38.0.19^{a}	1.26±0.26^{ab}	1.23±0.22^{ab}	0.99±0.14^{b}	1.01±0.26^{b}
Mnsod	1.20±0.38^{a}	1.02±0.05^{b}	1.01±0.47^{b}	0.98±0.16^{b}	1.04±0.10^{b}	0.97±0.51^{b}
gpx	0.78±0.34^{c}	0.95±0.23^{c}	0.91±0.15^{c}	0.89±0.21^{c}	1.25±0.31^{b}	2.03±0.73^{a}
oc	0.84±0.57^{b}	0.98±0.24^{b}	1.05±0.49^{b}	1.05±0.36^{b}	1.11±0.30^{b}	1.58±0.79^{a}

*Values (mean ± SD, n=3) with same letters are not significantly different (p>0.05).

7.5. DISCUSSION

Even though microminerals are present in rotifers and Artemia in lower amounts than in copepods (Hamre *et al.*, 2007, 2008b), a natural prey for marine fish larvae, studies on the importance of supplementation of these minerals in larval diets are scarce (Hamre *et al.*, 2013). In the present study, combined supplementation of Zn, Cu, Mn and Se (112, 20, 11 and 1.8 mg.kg^{-1}, respectively) at dietary levels similar to those found in copepods (Fujita, 1972; Hamre *et al.*, 2008), negatively affected survival. The results are in agreement with previous studies (Izquierdo *et al.*, 2017) and suggest the harmful effect of at least one of those minerals.

Analysis of the mineral contents in larvae fed the combined supplementation of these four target minerals showed that only Mn contents were significantly raised in comparison to larvae fed the non-supplemented diets. Moreover, Mn contents in

larvae fed the Mn supplemented diet were also increased, and these larvae showed as well a low survival rate, not significantly different from that of larvae fed Mn in combination with the other minerals. In agreement, in juveniles of other fish species Mn contents are also increased in several tissues, particularly bone, by the elevation of dietary Mn levels (Ogino *et al.,* 1980; Gatlin *et al.*, 1986; Knox *et al.*, 1981; Lorentzen and Maage, 1999; Lorentzen *et al.*, 1996; Maage *et al.*, 2000; Pan *et al.,* 2008). These results suggest that increase in dietary Mn supplementation from 4 to 11-13 mg kg^{-1} as $MnSO_4$ in microdiets for gilthead seabream larvae was harmful and reduced survival in a 40-50%. This is in agreement with the negative correlation found between larval survival and dietary Mn levels previously found in gilthead sea bream (Izquierdo *et al.*, 2017), when Mn was supplemented together with Zn and Se. Thus, the present study confirms the negative effect of excess dietary $MnSO_4$ levels on sea bream growth. However, increase in dietary Mn (10–43 mg kg^{-1}) improves growth in larvae of another sparid of similar age (Nguyen *et al.*, 2008). Besides, the Mn levels tested in the present study (11-13 mg kg^{-1}) were in the range of those found in live preys for marine fish larvae. Therefore, it could be possible that, rather than the dietary Mn levels themselves, the supplementation with an inorganic form of Mn would be responsible for the reduced survival. In a recent study in gilthead sea bream juveniles, in comparison to amino acids chelated Mn, supplementation with another inorganic form of Mn (Mn oxide) had a pro-oxidant effect, raising the presence of thiobarbituric acid reactive substances, without affecting *Mnsod* expression, and reduced Zn deposition in whole body, affecting also other elements such as Ca, Mg and P (Dominguez *et al.*, 2019). In agreement, in the present study, *Mnsod* expression was not affected by dietary Mn levels, but neither were affected the whole body Zn contents. In mammals, prolonged or excess exposure to Mn has been associated to several behavioral and neuronal pathologies including atypical parkinsonian syndrome (manganism) and causes neuronal apoptosis and mitochondrial dysfunction, in relation to increased mitochondrial H_2O_2 production and SOD2 activity (Zolkipli-Cunningham and Falk, 2017). In fish juveniles, MnSOD activity in liver is reduced either by insufficient or excess dietary levels of Mn (Satoh *et al.*, 1991; Lin *et al.*, 2008; Tan *et al.*, 2012; Prabhu *et al.*, 2016). In the present study, elevation of dietary and body Mn contents did not up-regulated *Mnsod* or *cat* gene expression in whole body. This fact suggests that even though the Mn contents in whole body doubled those of the fish fed diets non-supplemented with minerals, their levels were not

causing a pro-oxidant effect of excess Mn as described in zebrafish (Arndt *et al.*, 2014). Indeed, larval fatty acid composition did not reflected a potential pro-oxidant effect with increased dietary Mn levels. Further studies are being conducted to determine the mechanisms implied on the negative effect of dietary Mn levels on gilthead sea bream survival and the consequences on larval behavior or neural development.

On the contrary, Cu supplementation markedly improved growth in terms of total length and body weight, raised Zn contents in whole body and led to the highest Cu and Fe larval contents. Cu^{2+} shares with Zn^{2+} and Fe^{2+} and other elements, the same metal ion transporter protein, the solute carrier transporter SLC11A2β, whose gene expression may be regulated by dietary minerals and maintains their homeostasis (Terova *et al.*, 2018). Besides, dietary Cu up-regulated *CuZnsod* expression in whole body, without affecting *cat* expression, increasing the protection against peroxidation risk. In agreement, in juveniles of different fish species, the low growth and low superoxide dismutase activity in fish fed deficient dietary Cu levels is increased by the elevation of dietary Cu (Shiau and Ning, 2003; Tan *et al.*, 2011; Tang *et al.*, 2013; Wang *et al.*, 2015, 2018; Abdel-Hameid *et al.*, 2017). Apart from its important role in defense against oxidation, Cu is an essential element involved as well in energy production through the C cytochrome oxidase, neurotransmission, collagen synthesis and melanin production (Lall, 2002). The larvae of the present study were previously fed on rotifers, which in comparison to copepods, have a low content in Cu (around 6 mg kg^{-1} dry matter) (Watanabe *et al.*, 1997) that could be insufficient to cover the requirements of the fast growing gilthead sea bream larvae. This would explain the elevation of Cu content in larvae fed increased dietary Cu levels. Whereas dietary Cu requirements in juveniles range between 3-13 mg kg^{-1}, these requirements may be increased during fast growing larval development (Prabhu *et al.*, 2014).

Supplementation with Zn raised dietary Zn levels from 86 to 110 mg kg^{-1}, increasing in a 6% the whole body Zn contents and up-regulating *CuZnsod.* These results agree well with the increase in superoxide dismutase activity found in Atlantic cod when rotifer contents in Zn where increased up to 90 mg kg^{-1} (Penglase *et al.*, 2011b). However, the present levels of Zn supplementation did not affected sea bream growth, in agreement with the results found in red sea bream larvae fed rotifers with increased

Zn supplementation (119–306 mg kg^{-1}) (Nguyen *et al.*, 2008). Moreover, increased dietary Zn did neither negatively affected gilthead sea bream larval survival in agreement with studies in larvae of other species fed enriched rotifers (33–245 mg kg^{-1}), Artemia (119–306 mg kg^{-1}) or copepods (340–570 mg kg) (Nguyen *et al.*, 2008; Yamamoto *et al.*, 2013).

Supplementation with organic Se raised dietary Se contents, increased final larval body weight and condition index, up-regulating *gpx*. These results agree well with the increased *gpx* activity found in other species fed increased dietary Se levels (Hamre et al. 2008b; Pacitti *et al.*, 2013; Penglase *et al.*, 2014; Ramesh *et al.*, 2014; Kim *et al.*, 2014). Optimum organic Se (yeast Se) contents in microdiets for gilthead sea bream have been recently determined (Saleh *et al.*, 2014) and show that dietary Se increase from 1.73 up to 11.65 mg kg^{-1} reduces oxidative risk and improves survival and stress resistance, but did not affect larval growth in terms of total length in agreement with the present study.

In summary, the results pointed out the importance of supplementation with Cu, as well as Se and Zn on early weaning diets for gilthead seabream, and the potential toxic effect of Mn. Further studies are being conducted to determine optimum levels of these nutrients in diets for marine fish larvae.

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8. CONCLUSIONS

1) Hatchery produced spray-dried *Nannochloropsis oculata* is a preferred feed over fresh *Nannochloropsis oculata,* Protein Plus, inactive baker's yeast or Algamac, for cultivation of rotifers, but must be followed by enrichment to optimize its nutritional value for marine fish larvae.

2) Single cell oils high in DHA obtained from the marine dinoflagellate *Crypthecodinium cohnii* are a good replacement for fish oil in weaning diets for gilthead seabream, without a complementary addition of ARA, but must be supplemented with EPA to further promote larval performance.

3) Single cell oils from the dinoflagellate *C. cohnii* and eustigmatophyceae *N. gaditana* are effective sources of essential fatty acid in gilthead seabream larvae early weaning diets. However, *N. gaditana* alone is not sufficient to replace fish oil, but could be used to complement possible EPA deficiencies in gilthead sea bream larvae.

4) Inclusion of 1.5% MOS in early weaning diets on gilthead sea bream larvae from 15 dph improves feed acceptance, survival, growth and fatty acid utilization.

5) The content of trace minerals in early weaning diets for gilthead sea bream markedly affects larval performance. Whereas inorganic Cu and Zn, as well as organic Se must be supplemented in squid meal and krill oil based diets, Mn basal contents are sufficient and this mineral should not be supplemented to avoid a potential toxic effect (10 mg/kg).

9.1. OBJECTIVES

MEJORA DE LA CALIDAD NUTRICIONAL DE LAS DIETAS PARA LARVAS DE DORADA: ALIMENTOS PARA ROTIFEROS Y DIETAS PARA DESTETE TEMPRANO

Dado que una adecuada nutrición de las larvas sigue siendo uno de los principales cuellos de botella para la producción de suficientes cantidades de juveniles de dorada de alta calidad, el objetivo principal de esta tesis fue el mejorar la calidad nutricional de las dietas para larvas. Para lograr este objetivo, la tesis abordó dos fases bien diferenciadas y críticas de la producción de larvas de dorada: alimentación inicial y destete hacia dietas inertes. Dado que los rotíferos deben utilizarse durante la primera alimentación de larvas de dorada, el primer objetivo específico fue estudiar el efecto de sus diferentes posibles presentaciones en la producción de rotíferos y su calidad nutricional desde el punto de vista de su composición bioquímica. Para mejorar la calidad nutricional de las dietas de destete, se consideró la inclusión de microalgas ricas en ácidos grasos esenciales, como alternativas al aceite de pescado, junto con la suplementación de probióticos o minerales.

Por lo tanto, para lograr el objetivo principal se formularon los siguientes objetivos:

1- Evaluar el efecto de diferentes presentaciones comerciales a base de rotíferos en el rendimiento de crecimiento, la composición de aminoácidos y ácidos grasos de los rotíferos (Capítulo 3)

2- Determinar el valor nutricional de los aceites de origen unicelular (aceites de microalgas) ricos en DHA ARA o EPA como sustitutos del aceite de pescado en las dietas de destete temprano para larvas de dorada (capítulos 4 y 5).

3- Determinar el potencial efecto positivo de la incorporación de oligosacáridos de mananos en las dietas de destete temprano de larvas de dorada sobre el rendimiento de crecimiento, composición de ácidos grasos, resistencia al estrés y morfología intestinal.

4- Evaluar el efecto de la inclusión de diferentes minerales en las dietas de destete temprano para larvas de dorada sobre el rendimiento de crecimiento, la deposición de minerales y los marcadores moleculares del estado oxidativo y de desarrollo óseo.

9.2 Resúmenes de los experimentos

9.2.1 Capítulo 3:

Valor nutricional y rendimiento del rotifero *Brachionus plicatilis, Müller* 1786 cultivado con diferentes ingredientes y tecnologías de producción a escala comercial.

Resumen

El rotífero *Brachionus plicatilis* es el primer alimento vivo utilizado en larvicultura para diferentes especies de peces marinos. Los rotíferos son comúnmente alimentados con dietas comerciales y especies de microalgas recién cultivadas. Estas dietas difieren en su composición bioquímica y propiedades físicas así mismo, su tecnología de producción y los protocolos de alimentación varían en gran medida entre las distintas instalaciones productoras. El objetivo del presente estudio ha sido determinar el efecto de tres formas diferentes de *Nannochloropsis oculata* y dietas comerciales de uso común en el crecimiento y composición bioquímica de rotíferos producidos en condiciones comerciales. Este estudio se realizó en la Akvatek Marine Fish Culture Company (İzmir, Turquía), donde las microalgas se cultivaron en fotobioreactores tubulares y la biomasa de microalgas se almacenó atomizada o en forma de pasta fresca. Los rotíferos fueron alimentados con seis tipos diferentes de ingredientes: Algome® (*Schizochytrium* sp. seca), ProteinPlus® (PP), inactive beaker's yeast® (INBY), *N. oculata* atomizada (SDN) o *N. oculata* recién cultivada (FN). Los rotíferos se alimentaron con las dietas experimentales 4 veces al día durante 14 días de cultivo semi-contínuo. La densidad de siembra inicial de los rotíferos fue de 600 ind/ml^{-1}. Durante el experimento, se contabilizaron diariamente la biomasa de rotíferos total, la proporción de huevos y el número de huevos transportados por hembra. También se analizaron los perfiles de composición proximal, ácidos grasos y aminoácidos de las dietas de rotíferos y de la biomasa. El mayor crecimiento se obtuvo en rotíferos alimentados con la dieta SDN (p<0.05), mientras que los rotíferos alimentados con PP mostraron los contenidos más altos de ∑n-3, ARA, EPA y DHA (p<0.05). El perfil de aminoácidos de los rotíferos se mejoró mediante la utilización de las dietas INBY y SDN. En general, los resultados indicaron que N. *oculata* atomizada fue la mejor presentación para la producción de biomasa de rotíferos a largo plazo. Sin embargo, el perfil nutricional de los rotíferos mejora notablemente al alimentar con las dietas PP, INBY y SDN.

9.2.2 Capítulo 4:

Reemplazo del aceite de pescado por diferentes productos a base de microalgas en microdietas para destete temprano de dorada (*Sparus aurata*, L.).

Resumen

El objetivo de este estudio fue determinar si ingredientes a base de algas, ricos en DHA o ARA pueden reemplazar completamente el aceite de pescado en microdietas para larvas de peces marinos, concretamente en dorada. También, si una suplementación adicional con EPA puede mejorar aún más el rendimiento larvario. Para ello, se alimentaron larvas de dorada de 20 días de edad de una longitud total media de 5.97±0.4 mm y un peso seco corporal medio de 0.12 ± 0.001 mg con cinco microdietas ensayadas por triplicado: una dieta de control a base de aceite de sardina; una dieta incluyendo AquaGrow® DHA (dieta DHA) para sustituir completamente el aceite de sardina; una dieta incluyendo AquaGrow® ARA (dieta ARA); una dieta conteniendo ambos productos, AquaGrow® DHA y AquaGrow® ARA para sustituir completamente el aceite de pescado; y una dieta conteniendo ambos productos, AquaGrow® DHA y AquaGrow® ARA junto con una fuente de EPA. También se realizaron pruebas de actividad frente a cambios de temperatura, exposición al aire y salinidad para detectar la resistencia de las larvas al estrés. Al final del experimento, las supervivencias finales no difirieron entre los grupos. El DHA producido por microorganismos fue capaz de reemplazar completamente el aceite de pescado en las dietas de destete para doradas sin afectar la supervivencia, el crecimiento o la resistencia al estrés, mientras que la inclusión del ARA producido por microorganismos no mejoró el rendimiento de las larvas. Además, la adición de EPA a las dietas con reemplazo total de aceite de pescado por DHA y ARA producido por microorganismos, mejoró significativamente el crecimiento en términos de peso corporal y longitud total. Los resultados de este estudio revelaron el buen valor nutricional del DHA producido por microorganismos como reemplazo del aceite de pescado en las dietas de destete para doradas, sin una suplementación complementaria de ARA. Sin embargo, la suplementación dietética de EPA parece ser necesaria para promover aún más el rendimiento larvario.

9.2.3 Capítulo 5:

Nannochloropsis gaditana y *Crypthecodinium cohnii,* dos microalgas como fuente alternativa de ácidos grasos esenciales en el destete temprano de la dorada

Resumen

El dinoflagelado heterotrófico *Crypthecodinium cohnii* y la eustigmataceae fotoautotrófica *Nannochloropsis gaditana* fueron evaluados como sustituto parcial o total del aceite de pescado en microdietas de destete temprano para dorada. Larvas de 20 días de edad se alimentaron con cuatro microdietas: una dieta control basada en aceite de pescado, dos dietas que incluyen *N. gaditana* (11%) o *C. cohnii* (8%), como sustitución del aceite de pescado (dietas N y C, respectivamente), una última dieta que combina *N. gaditana* (5.5%) y *C. cohnii* (5.5%) junto con aceite de pescado (5.5%, dieta N+C). Al final de los 17 días de tratamiento, la supervivencia no difirió entre grupos, pero el crecimiento en longitud total y peso seco de las larvas alimentadas tanto con aceite de pescado como con los tratamientos incluyendo *C. cohnii* fue superior el de las alimentadas con *N. gaditana* ($p<0.05$). El análisis de ácidos grasos confirmó que *C. cohnii* y *N. gaditana* fueron utilizadas como fuente de ácidos grasos esenciales en las microdietas para larvas la dorada. Los resultados del presente estudio revelaron que el ácido eicosapentaenoico (EPA; 20:5*n*-3) suministrado por *N. gaditana* permite mantener la supervivencia de las larvas, pero que la disponibilidad de ácido docosahexaenoico (DHA; 22:6*n*-3) suministrado por *C. cohnii* es necesario en las dietas de destete de dorada para mantener un crecimiento óptimo de las larvas.

9.2.4 Capítulo 6:

Efecto de la inclusión de oligosacaridos de manano en microdietas de destete temprano en el crecimiento, supervivencia y morfología intestinal de larvas de dorada (*Sparus aurata).*

Resumen

El destete temprano de larvas de peces marinos con dietas inertes retrasa la maduración intestinal y reduce las tasas de crecimiento en comparación con la alimentación basada en presas vivas. En juveniles y adultos de varias especies de peces marinos, se ha demostrado que la inclusión de manano oligosacáridos (MOS) en las dietas mejora la integridad y funcionalidad intestinal, sin embargo, aún no se han abordado los efectos de la inclusión de MOS en las microdietas para larvas de dorada (*Sparus aurata*, L.). Por lo tanto, este estudio evalúa los efectos de la inclusión de MOS en las microdietas sobre la supervivencia, el crecimiento, la integridad intestinal, la aceptación del alimento y la calidad de la larva de la dorada. Para ese propósito, se alimentaron larvas de dorada de 16 días con cuatro niveles de MOS (Biomos, Alltech, Nicholasville, KY, USA) con microdietas de destete temprano conteniendo 0 g kg^{-1} MOS, 0.5 g kg^{-1} MOS, 1.5 g kg^{-1} MOS y 2 g kg^{-1} MOS respectivamente. La inclusión de MOS en las dietas no afectó a los porcentajes finales de aceptación en las larvas de dorada (p>0.05). Sin embargo, la suplementación con MOS se correlaciono con un aumentó en la supervivencia, dosis dependiente (P = 0.026). Tras 15 días de tratamiento, MOS aumentó el contenido larvario completo (p<0.01) del ácido araquidónico, eicosapentaenoico y docosahexaenoico. Las larvas de dorada alimentadas con 2 g kg^{-1} MOS presentaron una mayor proporción de células caliciformes en comparación con las larvas alimentadas con los otros tratamientos. En general, los resultados sugieren que la inclusión de MOS en las microdietas de destete temprano para dorada mejora la utilización de ácidos grasos esenciales y puede promover el crecimiento y la supervivencia final.

9.2.5 Capítulo 7:

Efecto de la inclusión de minerales en las microdietas de destete temprano en crecimiento, supervivencia y expresion génica de larvas de dorada (*Sparus aurata).*

Resumen

A pesar de la importancia de los microminerales para el desarrollo normal de las larvas de peces marinos, estudios previos han demostrado que el contenido de estos nutrientes en las presas vivas utilizadas en la larvicultura es mucho menor que en copépodos. Además, estudios anteriores de nuestro grupo han demostrado la importancia de una inclusión de Zn, Mn y Se en las microdietas de destete temprano para el crecimiento larvario, y sugirieron la toxicidad potencial de uno de estos elementos. El objetivo del presente estudio fue determinar el efecto de la inclusión dietética de manera aislada de Zn, Mn, Se y Cu, su combinación (Control +) o su ausencia (Control -). Estas 6 dietas se administraron por triplicado a larvas de dorada de 22 días durante tres semanas. Al final del ensayo, la supervivencia fue significativamente más baja (p <0.05) en los peces alimentados con todos estos minerales (dieta C +, 17.16 ± 7% media ± SD), seguida de la de Mn (dieta Mn, 21.91±7). La mayor supervivencia se obtuvo mediante la suplementación con Cu (dieta Cu, 35.27±15), seguida de la dieta control negativo (dieta C-, 34.58 ± 9). Cu y Se mejoraron significativamente el crecimiento de larvas, en términos de longitud total y peso corporal, en comparación con los peces alimentados con la dieta C-, mientras que los peces alimentados con Mn y C + mostraron el menor crecimiento. La suplementación con Zn o Cu aumentó significativamente CuZnsod, mientras que la expresión de *gpx* también se incrementó significativamente en los peces alimentados con las dietas Se y C +. Otras enzimas relacionadas con el estrés oxidativo no fueron afectadas. La composición de ácidos grasos tanto de las microdietas como de las larvas fue homogénea, a excepción de unos valores significativamente más altos de ARA/EPA y más bajos el DHA/EPA alimentando con la dieta Cu y justamente lo opuesto alimentando las larvas con la dieta C +. Los resultados señalaron la importancia de la suplementación con Cu y con Se y Zn en las dietas de destete temprano para doradas, así como el posible efecto tóxico de Mn. Se están realizando estudios adicionales para determinar los niveles óptimos de estos nutrientes en las dietas para larvas de peces marinos.

9.3 Conclusiones Generales

1) *Nannochloropsis oculata* producida en los criaderos en fotobioreactores y en presentación atomizada es el alimento de elección frente a *Nannochloropsis oculata* fresco, Protein Plus, levadura inactiva o Algamac para el cultivo de rotíferos, pero debe ir seguido de un enriquecimiento para optimizar su valor nutricional para las larvas de peces marinos.

2) Los aceites de origen unicelular con alto contenido de DHA obtenidos del dinoflagelado marino *Crypthecodinium cohnii* son un buen sustituto del aceite de pescado en dietas de destete para doradas, sin una suplentación de ARA, pero deben complementarse con EPA para promover aún más el rendimiento larvario.

3) Los aceites de origen unicelular a partir del dinoflagelado *C. cohnii* y la eustigmatophyceae *N. gaditana* son fuentes efectivas de ácidos grasos esenciales en las dietas de destete temprano de larvas de dorada. Sin embargo, *N. gaditana* de manera individual no es suficiente para reemplazar el aceite de pescado, pero podría usarse para complementar las posibles deficiencias de EPA en larvas de dorada.

4) La inclusión de MOS en un 1.5% en las dietas de destete temprano en larvas de dorada de 15 dph mejora la aceptación del alimento, la supervivencia, el crecimiento y la utilización de ácidos grasos.

5) El contenido en microminerales en las dietas de destete temprano para dorada afecta el rendimiento larvario. Mientras que el Cu y Zn inorgánico, así como el Se orgánico deben complementarse con dietas basadas en harina de calamar y aceite de krill, los contenidos basales de Mn son suficientes y este mineral no ser complementado para evitar un posible efecto tóxico (10 mg / kg).

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