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DELIVERY VECTORS FOR MINERALS AT EARLY LIFE STAGES OF MARINE FISH LARVAE (*Sparus aurata*)

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DELIVERY VECTORS FOR MINERALS AT EARLY LIFE STAGES OF MARINE FISH LARVAE (*Sparus aurata*)

Trabajo realizado en los laboratorios del Instituto Canario de Ciencias Marinas (ICCM) e Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA) de la Universidad de Las Palmas de Gran Canaria, España, bajo la dirección de la Dra. Mª Soledad Izquierdo López (Universidad de Las Palmas de Gran Canaria, España) y de la Dra. Carmen María Hernández Cruz (Universidad de Las Palmas de Gran Canaria, España).

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ABBREVIATIONS

ANOVA: Analysis of variance.

ARA: Arachidonic acid (20: 4n-6).

BHT: Butylated hydroxytoluene.

BMP2: Bone morphogenic protein 2.

bp: pair base.

ºC: Degree Celsius.

Ct: Cycle threshold.

cDNA: Complementary desoxiribonucleic acid.

cRNA: Complementary ribonucleic acid.

Cu: Cuivre.

Cu-Zn SOD: Cuivre-Zinc superoxide dismutase.

DEPC: Diethyl pyrocarbonate.

DHA: Docosahexaenoic acid (22:6n-3).

dNTPS: Deoxynucleotide triphosphates.

dah: Days after hatching.

dT: End-labeling of Oligonucleotides-Oligo.

DTT: Dithiothreitol.

dTTP: Deoxythymidine triphosphate.

dUTP: Deoxyuridine triphosphate.

DW: Dry weight.

EDTA: Ethylenediaminetetraacetic acid.

EPA: Eicosapentaenoic acid (20:5n-3).

FAME: Fatty acid methyl esters.

FAO: Food and Agriculture Organization.

Fe: Iron.

FEAP: Federación Europea de Productores de Acuicultura.

FIED: Flame ionization detector.

g: Gram.

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

GIA: Grupo de Investigación en Acuicultura.

GC: Gas chromatography.

GLC: Gas liquid chromatography.

GPx: Glutathione peroxidase.

GSH-Px: Glutathione.

h: Hour.

HUFA: Highly unsaturated fatty acid.

H&E: Haematoxilin & eosin.

ICCM: Instituto Canario de Ciencias Marinas.

IPTG: Isopropil-β-D-1-tiogalactopyranoside.

IUSA: Instituto Universitario de Sanidad Animal y Seguridad Alimentaria.

Kb: Kilo base.

KCl: Potassium Chloride.

kDa: Kilodaltons.

KOH: Potassium hydroxide.

Kg: Kilogram.

L: Liter.

Laczα: Lactose z gene.

LB: Lysogeny broth.

mg: Milligram.

min: minute.

ml: Milliliter.

mM: Milimol.

M-MLVRT: Moloney murine leukemia virus.

mm: millimeter.

Mn: Manganese.

Mn-SOD: Manganese superoxide dismutase.

mRNA: Messenger ribonucleic acid.

MT: Millions tones.

MW: Molecular weight.

ng: Nanogram.

NH2: Asparagine.

NRC: National Research Council.

OC: Osteocalcin.

pmol: picomole.

ppm: Parts per million.

PCR: Polymerase chain reaction.

pH: Potential hydrogen.

RNA: Ribonucleic acid.

rpm: Revolutions per minute.

RT-PCR: Quantitative time real time PCR.

rTH: Triphosphate.

SD: Standard deviation.

Se: Selenium.

sec: second.

SOC: Super optimal broth with catabolite repression.

SOD: Superoxide dismutase.

TAE: Tris-acetate-EDTA.

TmºC: Melting temperature.

t-RNA: transfer Ribonucleic acid.

µg: Microgram.

µl: Microliter.

µM: Micromol.

ULPGC: Universidad de Las Palmas de Gran Canaria.

UNG: Uracyl-N-glycosylase.

X-gal: Bromo-chloro-indolyl-galactopyranoside.

Zn-AA: Zinc-Amino acid.

Zn: Zinc.

LIST OF FISH SPECIES CITED

Atlantic salmon - *Salmo salar.*

Atlantic Cod - *Gadus morhua.*

Brook trout - *Salvelinus fontinali.*

Channel catfish - *Ictalurus punctatus.*

Cobia - *Rachycentron canadum.*

Common Carp - *Cyprinus carpio.*

Dover sole - *Solea solea.*

Eel - *Anguilla japonica*.

European sea bass - *Dicentrarchus labrax*.

Gibel carp - *Carassius auratus gibelio*.

Gilthead sea bream - *Sparus aurata*.

Grass carp - *Ctenopharyngodon idellus*.

Grouper - *Epinephelus malabaricus / Epinephelus coioides.*

Japanese flounder - *Paralichthys olivaceus*.

Rainbow trout - *Oncorhynchus mykiss / Salmo gairdneri.*

Red drum - *Sciaenops ocellatus.*

Red sea bream - *Pagrus major / Chrysophrus major*.

Tilapia - *Oreochromis niloticus / Oreochromis aureus.*

Yellowtail kingfish - *Seriola lalandi.*

Yellow catfish - *Pelteobagrus fulvidraco*.

RESUMEN

La cría larvario sigue siendo uno de los cuellos de botella en la producción acuícola de los peces marinos, eso es debido principalmente, al escaso desarrollo larvario, las altas mortalidades larvarias y la debilidad de las larvas a la hora de eclosionar. A pesar de la importancia de los minerales como ingredientes esenciales en la dieta de los peces, se sabe poco sobre sus requerimientos en la dieta de las larvas de peces marinos. La función fisiológica de los elementos traza: hierro, manganeso, selenio y zinc, es bien reconocida, son componentes de los fluidos corporales, cofactores en las reacciones enzimáticas, y constituyen las unidades estructurales de macromoléculas no enzimáticas, etc.

El objetivo general de este estudio fue determinar el efecto de la inclusión de cuatro minerales (Fe, Mn, Se y Zn), estos oligoelementos fueron suministrados a las larvas de cuatro formas diferentes: orgánicos, inorgánicos, nanometales o encapsulados por su mejor disponibilidad para las larvas, reduciendo también su lixiviación y su pérdida en el agua.

Los resultados demostraron que se requiere añadir Zn, Mn, Se y Fe a las microdietas para promover el crecimiento de las larvas, la mineralización ósea y la prevención de las anomalías esqueléticas. También, la no suplementación de estos minerales conduce a la baja regulación de la osteocalcina y de los genes Cu-ZnSOD y MnSOD, que denotan la deficiencia en Zn o Mn. La inclusión de Zn, Mn, Se y Fe bajo la forma orgánica (aminoácido quelato) promovió un máximo crecimiento, aumentó las reservas lipídicas, condujo a una temprana mineralización y previnieron las deformidades de los arcos branquiales. En general, la adición de los minerales en forma orgánica, inorgánica o nanometales redujeron considerablemente la supervivencia de las larvas, mientras que los minerales encapsulados sólo causaron bajas mortalidades. Estos resultados sugieren un efecto tóxico de alguno de estos minerales. Por otra parte, la mortalidad de las larvas se correlacionó con el contenido de la dieta en Mn o Zn. La toxicidad alimentaria del Mn es poco común en los peces y, en el presente estudio, una alta expresión del gene MnSOD denotó una mejor asimilación de Mn en los peces alimentados con la dieta encapsulada, la cual no mostró ninguna diferencia significativa en la supervivencia larvaria, en comparación con los larvas alimentadas con la microdieta no suplementada, lo que sugiere que la toxicidad no se relaciona con el Mn.

ABSTRACT

The hatchery phase remains as one of the production bottlenecks in marine fish aquaculture, mainly due to the poor development, high mortality rates and weakness of fish larvae at hatching. Despite the importance of trace minerals as essential ingredients in fish diets, little is known on mineral nutrition in marine fish larvae. Among trace elements, the physiological role of iron, manganese, selenium and zinc is well recognized, as components of body fluids, cofactors in enzymatic reactions, and structural units of non-enzymatic macromolecules, etc.

The overall objective of this study was to determine the effect of the inclusion of four minerals (Fe, Mn, Se and Zn) considering four types of delivery forms to supplement these trace elements: organic, inorganic, nanometals or encapsulated in order to increase their availability for the larvae and prevent leaching and water loss in the culture medium.

The results demonstrated that it is required to supplement early weaning diets with Zn, Mn, Se and Fe lead to promote larval growth, bone mineralization and prevention of skeleton anomalies. Non-supplementation of these minerals also leads to down-regulation of osteocalcin, Cu-ZnSOD and MnSOD genes, denoting the deficiency in Zn or Mn. The inclusion of Zn, Mn, Se and Fe in an organic form (amino acid-chelate) promoted maximum growth, increased larval lipid reserves, enhanced early mineralization and prevented branchial arches deformities. In general, addition of minerals in the form of organic, inorganic or nanometals forms markedly reduced fish survival, whereas encapsulated minerals only caused low mortalities. These results suggest the toxic effect of one or more of these minerals. Moreover, larval mortality was correlated with the dietary Mn or Zn contents. Dietary Mn toxicity is rare in fish and, in the present study, a higher MnSOD gene expression denoting a better assimilation of Mn was found in fish fed the encapsulated diet, which did not showed a significantly low survival, in comparison to the fish fed non-supplemented diet, suggesting that the toxicity was not related to the Mn.

1. INTRODUCTION

1.1 Present situation of Aquaculture production

Throughout the history of humanity, different ways of farming, grazing and ranching have been adopted to increase food production in order to cover the food requirements necessary for an exponentially growing world population. In this regard, the ocean has always been qualified as exploitable resource. Fishing has always been an old important activity that provides high quality nutrients source of proteins, essential fatty acids, vitamins and minerals, etc. Nevertheless, to satisfy the high demand of marine products, aquaculture is a feasible complement to wild captures. In fact, Aquaculture has accounted for 47.9% of total food fish supply in 2010, representing a continue increase from 42.7% in 2006 (FAO, 2011).

In this sense, Aquaculture is one of the most rapid developing animal production sectors in the world, reached 62.7 MT in 2011, up by 6.2% from 59 MT in 2010 (FAO, 2013).

However since 1988, in certain regions, growth rates of aquaculture productions wasn't so high and had not been enough to cover the decrease in capture fisheries. For instance, the leading countries in aquaculture development: Japan, Spain and France have shown a falling in the production during the last decades (FAO, 2011). Aquaculture currently produces almost half of the total supply of edible fish in the world (FAO, 2010). The FAO estimations expect for 2030, about 65% of the aquatic food coming from aquaculture. Moreover, the majority production of some aquatic species, such as mussels, clams, oysters, sea bream, sea bass, trout, tilapias and carps, comes almost entirely from aquaculture.

The future success of 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. Hence, to meet the increasing demands of seed production, it is necessary to improve the nutritional quality of its larvae, which still constitutes a major constraint for the development of marine fish species culture (Watanabe *et al*., 1983; Yúfera and Pascual, 1984; Izquierdo and Fernández-Palacios, 1997; Sargent *et al*., 1997; Izquierdo *et al*., 2000).

Over the last two decades, there are two important species, which have characterized the development of marine aquaculture in the Mediterranean Sea, and still require a larger expansion, which are the Gilthead sea bream (*Sparus aurata)* and the European sea bass

(*Dicentratchus labrax)* (Basurco and Abellán, 1999; FAO, 1999, Manual on Hatchery Production of Sea bass and Gilthead Sea bream).

In fact, while the production of these species is well controlled, the knowledge of their nutritional requirements in comparison with other species such as salmonids and carps is still incomplete (NRC, 2005). Particularly, larval rearing success is mostly affected by first feeding regimes and the nutritional quality of starter diets (Izquierdo *et al*., 2000).

1.2 Rearing techniques and production considerations

There are different techniques used over the history. In order to promote a successful and competitive aquaculture industry, we need to ensure the production of high quality and healthy fry. Survival rate production of fish larvae is variable and growth potential is in most cases not fully utilized (Shields, 2001; Conceição *et al*., 2003).

The hatchery phase remains as one of the production bottlenecks in marine fish aquaculture, mainly due to the poor development, high mortality rates (over 75%) and weakness of fish larvae at hatching. Marine fish species egg hatching is produced after some days, depending on water temperature, leading to a secondary embrionary stage (Sargent *et al.*, 2002). The problem consists in the production of small sized fish, and difficulties in acceptance of inert microdiets, that makes progress slow and difficult. One of the causes of high mortalities and quality problems commonly observed in larviculture is due to the lack of knowledge in nutritional requirements of fish larvae (Takeuchi, 2001; Bell *et al*., 2003; Cahu *et al*., 2003a; Kamler, 2008).

The Mediterranean production of marine fish culture has been increasing in several species, the gilthead sea bream is still the most cultivated species (Izquierdo, 2005). Demand for good quality fry has been increasing at a rate of 10% annually and the European annual fry production of sea bream and of sea bass respectively reached 497.7 and 457.7 millions in 2008 (FEAP, 2008). However, the success in mass production of juveniles is greatly affected by effective first feeding regimes and the high nutritional quality of starter diets (Kolkovski *et al*., 1993a; Sargent *et al*., 1997; Izquierdo *et al*., 2000; Hamre *et al*., 2013).

At present, the accepted practice worldwide for the intensive larval rearing of marine fish is the feeding with live prey during the first weeks, followed by a gradual weaning to a dry diet (Fernández-Diaz and Yúfera, 1997; Kolkovski *et al*., 1997; Kolkovski *et al.*, 2009). 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*., 1993b; Sargent *et al*., 1997).

The larval culture development of marine fish has been possible thanks to the use of live preys, mainly rotifers and *nauplii* and *metanauplii* of *Artemia*. 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 use of different strains of rotifers is due to the easiness of its mass production, its adequate dimensions to fit mouth size of early larvae, its planktonic habits, slow movements and the possibility to control its nutritional value for fish larvae (Barnabé, 1974). Moreover, the nutritional value of live preys varies notably according to its feed and environmental conditions such as light intensity and regimes, temperature, salinity, etc., risking the optimal nutrition of the larvae (Jones *et al*., 1993; Kolkovski *et al*., 1993b; Barnabé and Guissi, 1994; Rosenlund *et al*., 1997).

The importance of replacing live prey with inert food, either for intensive larval rearing or as a tool for nutritional studies using aquatic animals is well recognized (Yúfera *et al*., 1995).

1.3 Development of early weaning diets

During the last years, efforts have been made to improve the efficiency of microdiets in order to replace live feed: rotifers and *Artemia* known as complete/partial replacements for early larval stages of fish (Koven *et al*., 2001; Kolkovski *et al*., 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 proven to be successful en *Sparus aurata* (Saleh *et al*., 2012). 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 sea bream (*Pagrus major*) and Japanese flounder (*Paralichthys olivaceus*) (Kanazawa *et al*., 1989), and gilthead sea bream 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; Saleh, 2013).

However, inert diets are still not adequate when used exclusively to rear marine fish larvae from first feeding (Holt, 1993; Kolkovski *et al*., 1993b; Walford and Lam, 1993; Zambonino-Infante and Cahu, 1994; Kolkovski, 2001). Different factors external and

internal influence the efficiency of utilization of inert diets en marine fish larvae. The efficiency of utilization of feed particles by marine fish larvae is affected by many external and internal factors (Kolkovski, 2001, 2004 *et al*; Koven *et al*., 2001). To improve the quality of fish larvae, there are some optimal requirements:

- Reduced visual stimulus that inert diets induce in the larvae since they principally ingest living prey during the first days of development (Planas and Cunha, 1999).
- Characteristics of inert particles: structure, digestibility, nutritional value and stability (Planas and Cunha, 1999).

In addition, as the study of Kolkovski *et al*. (1995) proved that the use of *Artemia nauplii* combined with a microdiet enhances the peristaltic movement of the larval digestive tract providing microdiet ingestion and production of digestive enzymes.

Recent observation of Kolkovski *et al.* (2009) confirmed that the formulation of early weaning diets meets optimal nutritional requirements that satisfy larval physiological needs. However, the knowledge on the specific quantitative requirements of marine fish larvae is limited to few types of nutrients for some species (Hamre *et al*., 2013). For instance, several studies aimed to determine the requirements of essential fatty acids (Salhi *et al*., 1994; Izquierdo, 1996; Izquierdo *et al*., 2001; Roo *et al*., 2009) protein and lipid requirements (Salhi *et al*., 1995; Yúfera *et al*., 2005), taurine (Chen *et al*., 2005), phospholipids (Cahu *et al*., 2003b; Sandel *et al*., 2010; Saleh *et al*., 2012, 2013a, 2013b), vitamin A (Villeneuve *et al*., 2005; Mazurais *et al*., 2009), vitamin D (Darias *et al*., 2010) vitamin C (Merchie *et al*., 1997) and vitamin E (Atalah *et al*., 2008; Betancor *et al*., 2011). Studies on mineral quantitative and qualitative requirements for marine fish larvae are much more recent and scarce (Hamre *et al*., 2013).

1.4 Importance of trace minerals

Despite the importance of trace mineral as essential ingredients in fish diets, little is known on mineral nutrition in marine fish larvae. Aspects such as the larval capacity to absorb minerals from the water, the availability of different mineral forms, the high leaching rates from early weaning diets, among others, are not studied in depth in marine fish larvae and can markedly affect the determination of their mineral requirements. Common live preys used in commercial hatcheries such as rotifers are much lower in their mineral content than copepods (Hamre *et al*., 2008a; Busch *et al*., 2010) and their content in some of them such as Se are even lower than the requirements estimated for juveniles or adults (NRC, 2011).

INTRODUCTION

In juveniles, several studies have been aimed to determine nutritional requirements of trace elements in some species (Gatlin and Wilson, 1983; Watanabe *et al*., 1997), whereas the research carried out on other ones such as gilthead sea bream is still fragmentary (Serra *et al*., 1996; Carpenè *et al*., 1998). Minerals absorption from water put difficult the determination of requirements in fish, whereas the diet is a main source of essential elements (Lall, 2002). The ordinary metabolic activities in organism, such as in cells and tissues, need maintaining of characteristic concentrations and functional forms of the minerals. One of the main is the homeostatic mechanisms operating in the animal, catering for the fluctuations in dietary intake (Watanabe *et al*., 1997; Lim and Klesius, 2000). Minerals play an important role in the organism, it ensure the skeletal formation, the maintenance of colloidal systems, regulation of acid-base equilibrium and it is considered as an important biological compounds for hormones and enzymes. As a consequence, deficiency in minerals causes a biochemical, structural and functional pathologies. And this includes duration and degree of mineral deprivation (Watanabe *et al*., 1997; Lim and Klesius, 2000).

The most documents for importance of minerals are well developed for humans and some animals whereas many function and bioavailability of trace minerals in fish are still unclear. Among these, the physiological role of cobalt, copper, iron, manganese, selenium, zinc, chromium and iodine is well recognized, as components of body fluids, cofactors in enzymatic reactions, and structural units of non-enzymatic macromolecules, etc. However, the investigation to determine requirements of minerals in fish is complicated between dietary intake and waterborne mineral (Watanabe *et al*., 1997; Manera *et al*., 2000; Isani *et al*., 2004; NRC, 2011) (Table I).

Table I Quantitative requirements of trace minerals for fish (adapted from Watanabe *et al*., 1997; NRC, 2011)

Mineral	**Requirement** [a]
Iron	30-170
Copper	3-5
Manganese	2-20
Zinc	15-20
Cobalt	0.05-1.0
Selenium	0.15-0.7
Iodine	1-4

[a] Expressed as mg mineral kg^{-1} dry diet.

In order to ensure an optimal growth, any species requires very small quantities of trace metals (usually less than 100 mg kg^{-1} dry diet), and the excess of trace elements may

develop toxicity in organism. That's why we need to control storage and excretion of this parameter to balance levels of minerals in the body (Watanabe *et al*., 1997).

1.4.1 Selenium (Se)

Selenium (Se) is an essential micronutrient (Johansson *et al*., 2005), that can promote bone formation and mineralization (NRC, 2005), although also any element has the levels between requirement and toxicity (Chassaigne *et al*., 2002; Polatajko *et al*., 2006). Se is important in the fish diet for normal growth and physiological function. It is known as an integral part of the enzyme glutathione peroxidase (Rotruck *et al*., 1973), which optimize defense against oxidative damage of cytoplasmic structures by catalyzing the reduction of hydrogen peroxide to oxygen and water. Se in conjunction with vitamin E is essential to prevent nutritional muscular dystrophy (Betancor *et al*., 2013). This micronutrient protect from other heavy metals for example cadmium and mercury (Watanabe *et al*., 1997).

In general, Se deficiency can lead to oxidative stress in organs (Gatlin *et al.,* 1986; Bell *et al*., 1986, 1987) with reduction in weight gain, feed efficiency and glutathione peroxidase activity in rainbow trout (Hilton *et al*., 1980) and Atlantic salmon (Bell *et al*., 1987). In channel catfish, Se deficiency in diets showed reduced growth, poor feed efficiency and low GSH-Px activity (Wang and Lovell, 1997). The administration of Se-deficient diet containing 0.1 mg Se kg^{-1} and 500 IU vitamin E $kg^{-}1$ resulted mortality in salmon fry (Poston *et al*., 1976). The deficiency signs when Atlantic salmon fed a Se-deficient diet for 26 weeks included lethargy, loss of appetite, reduced muscle tone and mortality (Watanabe *et al*., 1997).

Different supplementation forms of Se may differ in their efficiency to fulfill Se requirements. For instance, selenomethionine has been claimed to be better absorbed than Se present in fishmeal (Bell and Cowey, 1989). Moreover, dietary selenomethionine can be stored in muscle, whereas other molecular forms such as sodium selenite cannot. In general, Se in selenite forms seem to be more available than selenate forms, these ones more available than selenomethionine which is more available than selenides and this one more than elemental Se (Betancor, 2012).

The dietary Se requirement, determined with sodium selenite, for best weight gain was 0.25 mg kg^{-1}diet for juvenile channel catfish (Gatlin and Wilson, 1984a), 0.35 mg kg^{-1}diet for rainbow trout (Hilton *et al*., 1980), 0.15 mg kg^{-1} for Atlantic salmon (Poston and Combs, 1979), 0.7 mg kg^{-1} for juvenile grouper (Lin and Shiau, 2005; NRC, 2011),

0.788 mg kg^{-1} for juvenile cobia (Liu *et al*., 2010) and 5.56 mg kg^{-1} for yellowtail kingfish (Le and Fotedar, 2013) (Table II).

Table II Optimum dietary Se levels for juveniles of different fish species

Species	Requirement	References
Ictalarus punctatus	0.25 mg kg^{-1}	Gatlin and Wilson, 1984a
Salmo salar	0.15 mg kg^{-1}	Poston and Combs, 1979
Salmo gairdneri	0.35 mg kg^{-1}	Hilton *et al*., 1980 NRC, 2011
Epinephelus malabaricus	0.7 mg kg^{-1}	Lin and Shiau, 2005 NRC, 2011
Rachycentron canadum	0.788 mg kg^{-1}	Liu *et al*., 2010
Seriola lalandi	5.56 mgkg^{-1}	Le and Fotedar, 2013

Toxic effects came from excessive quantities of Se that caused a decreased growth, feed efficiency and increased mortality. Levels above 13-15 mg Se kg^{-1}dry feed can be toxic (Hilton *et al*., 1980; Gatlin and Wilson, 1984a). Prolonged administration of 3 mg Se kg^{-1} diet also was detrimental (Hilton *et al*., 1980). Rainbow trout developed renal calcinosis when received over 10 mg Se kg^{-1} (Hodson and Hilton, 1983). However, deleterious effects on vertebrate skeletal tissue metabolism were caused by excessive intakes of this mineral (NRC, 2005). Cod larvae fed rotifers with Se-supplementation causes a trend of increased deformities in individual vertebra (Penglase *et al*., 2010) which could be caused by an alteration in skeletal mineralization in its ionic form, or via selenoenzymes as an antioxidant (Lall and Lewis-McCrea, 2007) or by regulating thyroid hormone ratios (Power *et al*., 2001).

Information about Se effects and requirements in fish larvae are much scarcer. Recent studies (Hamre *et al*., 2008b) have shown that Se content in rotifers is considerably lower (0.08-0.09 mg kg^{-1} DW) than both juvenile fish requirements (0.3-0.5 mg kg^{-1} DW) (NRC, 1993) and copepod levels (3-5 mg kg^{-1} DW) and, therefore, may be insufficient to meet Se requirements for larvae. Increase in Se contents in rotifers increased survival and GPx activity in Atlantic cod (*Gadus morhua*) (Hamre *et al*., 2008b; Penglase *et al*., 2010). Moreover, increase in Se levels from 1.3 to 6.27 μg mg^{-1} selenomethionine increased growth and reduced the incidence of muscle dystrophy in larval sea bass (Betancor *et al*., 2013). In diets for larval gilthead sea bream, Se increase from 1.7 to 11.65 mg kg^{-1} selenomethionine improved larval survival, stress resistance and bone mineralization (Saleh *et al.*, 2014).

1.4.2 Zinc (Zn)

Other important element in fish nutrition, is the Zinc (Zn) important for several processes (Lall, 1989) such as metabolism of numerous enzymes (Scarpa and Gatlin, 1992); which is an integral part of about 20 metalloenzymes, such as alkaline phosphatase, alcohol dehydrogenase and carbonic anhydrase (Watanabe *et al*., 1997); normal growth and skeletal development in humans and other animals including fish (Calhoun *et al*., 1974, 1975; Yamaguchi *et al*., 1987; Watanabe *et al*., 1997; Yamaguchi, 1998; Ovesen *et al.*, 2001; Yamaguchi and Fukagawa, 2005), when research on Zn-gene interactions has assigned a basic role for this element in controlling growth (Chesters, 1991). Supplementation of Zn in the diet of growing rat increased their bone strength (Ovesen *et al*., 2001).

Dietary Zn deficiency causes retardation of ectopic bone formation (Calhoun *et al*., 1975). In pregnant mammals, Zn deficiency causes congenital bone deformities in their offspring (Calhoun *et al*., 1974). In fish juveniles, Zn deficiency leads to growth retardation (Watanabe *et al*., 1997) and reduces protein and carbohydrate digestibility, probably due to a reduced carboxypeptidase activity (Ogino and Yang, 1978). Additional signs of Zn deficiency include eye lens cataract and erosion of fins and skin (Ogino and Yang, 1979; Hughes, 1985). Short-body dwarfism has been also pointed out as a main sign of Zn deficiency (Satoh *et al*., 1983a). Low growth in channel catfish fed diets low in Zn, reduces appetite, and levels of Zn and calcium in bone, as well as Zn in serum (Gatlin and Wilson, 1983). Dietary supplementation is considered a more significant source of minerals because relatively waterborne zinc and other trace elements are not sufficient to meet requirements of juveniles fish (NRC, 1993).

There are different forms of zinc that could affect its absorption and utilization as a supplement. It was reported that amino-acid-chelated-Zn (Zn-AA) is increasingly thought to be more bioavailable than inorganic forms of zinc, such as zinc carbonate, zinc sulfate, and zinc oxide (Ashemead, 1992), as observed in trout (Zn-AA) (Apines *et al*., 2001) and in catfish (Zn-proteinates) (Paripatananont and Lovell, 1997).

The zinc requirement has been estimated as 20 mg kg^{-1} feed for channel catfish (*Ictalurus punctatus*) (Gatlin and Wilson, 1983), 37-57 mg kg^{-1} feed for Atlantic salmon (*Salmo salar*) (Maage and Julshamn, 1993; Maage *et al*., 1993) and 40 mg kg^{-1} for carp *(Cyprinus carpio)* (Satoh *et al*., 1987b). The Zn requirement of rainbow trout *(Oncorhynchus mykiss*) is normally met by dietary levels of 15-30 mg kg^{-1}diet (Ogino

and Yang, 1978). Gatlin *et al*. (1991) reported that dietary Zn requirement of the red drum (*Sciaenops ocellatus*) was between 20 and 25 mg kg^{-1} diet (Table III).

Table III Optimum dietary Zn levels for juveniles of different fish species

Species	Requirement	References
Ictalurus punctatus	20 mg kg^{-1}	Gatlin and Wilson, 1983
Salmo salar	37-57 mg kg^{-1}	Maage *et al*.,1993 Maage and Julshamm, 1993
Cyprinus carpio	40 mg kg^{-1}	Satoh et *al*., 1987b
Oncorhynchus mykiss	15-30 mg kg^{-1}	Ogino and Yang, 1978
Sciaenops ocellatus	20-25 mg kg^{-1}	Gatlin *et al*., 1991

Inclusion up to several hundred mg/kg of Zn in rainbow trout diets did not exert any adverse effects (Wekell *et al*., 1983). In addition, any difference in growth or feed utilization, or any adverse effects on health did not affect young rainbow trout fed 15-600 mg kg^{-1}Zn supplementation diet (Watanabe *et al*., 1997). However, Manera *et al*. (2000) demonstrated an active interaction between the excess of minerals, such as Zn, and macrophage aggregates proliferation in Gilthead sea bream.

However, very few studies deal with the effects of dietary Zn in fish larvae. Since minerals are important for skeletal development of fish, mineral supplementation of hatchery live preys to reach the levels found in natural zooplankton may be a benefit for normal growth and skeletal development of cultured fish. Direct inclusion of Zn in the culture medium has failed to enrich rotifers in Zn (Matsumoto *et al*., 2009).

Recent studies mentioned that the of enriching *Artemia nauplii* in Zinc (Zn) and Manganese (Mn), has shown a significant improvement in growth performance of fish and has reduced occurrence of skeletal anomalies (Matsumoto *et al*., 2009; Nguyen *et al*., 2008).

1.4.3 Manganese (Mn)

Dietary manganese (Mn) is important, even in small quantities, for normal growth, reproduction and prevention of skeletal abnormalities in terrestrial animals and fish (Liu *et al*., 2013). Mn is essential for fish and is widely distributed in all body tissues. However, the most concentration of Mn is in the mitochondria than cytoplasm or other cell organelles, which is necessary for many functions such as the functioning of brain and for proper lipid and carbohydrate metabolism (Watanabe *et al*., 1997). Other Mn functions are as a cofactor for a large number of enzymes and forms metal-enzyme complexes or as an integral part of certain metalloenzymes in carbohydrate, lipid and

protein metabolism (Watanabe *et al*., 1997; Lall, 2002). Mn activates specific enzymes, the glycosyltransferase, kinases, transferases, hydrolases and decarboxylases (Watanabe *et al*., 1997) and leucine aminopeptidase in sole (Clark *et al*., 1987).

The deficiency in Mn has shown an increase in mortality, a reduction of growth and reproduction, a depression feed intake, an increase in skeletal abnormalities (dwarfism) and lens cataracts; and a decrease of Mn-superoxide dismutase (Mn-SOD) activities (NRC, 1993; Lall 2002). Poor growth is obtained in juveniles of rainbow trout and carp when the diets are low in Mn (Ogino and Yang, 1980), grass carp fingerling (Wang and Zhao, 1994), juvenile gible carp (Pan *et al*., 2008) and juvenile of cobia (Liu *et al*., 2013). In addition, insufficient dietary supply of Mn leads to a reduction of Mn content in bone (Satoh *et al*., 1983b) and the copper-Zn superoxide dismutase and Mn superoxide dismutase activities in cardiac muscle and liver are reduced in rainbow trout (Knox *et al*., 1981) and in tilapia (Lin *et al*., 2008). Short-body and cataracts of eye have been observed in rainbow trout and carp (Satoh *et al*., 1987c, 1991). Thus; Mn-deficient bones are considerably shortened and thickened (McDowell, 1992).

The use a chemical form of Mn to improve the biological availability has been given little attention during the last few decades. Mn sources signicantly influenced growth, whole-body and bone Mn contents, as well as Mn retention, but did not affect Mn absorption. Higher whole-body Mn contents was observed in rainbow trout fed the diet supplemented with acido amino-chelated Mn than others forms (Satoh *et al*., 2001). Thus, acido amino-chelated Mn has been demonstrated more bioavailable (Apines *et al*., 2004) than glass-embedded Mn and Mn sulfate in rainbow trout. Moreover, bioavailability of glass-embedded Mn and Mn sulfate are similar (Satoh *et al*., 2001). The result was comparable with the findings of Henry *et al*. (1989 and 1992) with broiler chicks and ruminants where Mn-methionine proved more bioavailable than Mn sulfate and Mn monoxide.

The requirement of Mn has been quantified in juveniles and fingerlings of some fish species, such as common carp (*Cyprinus carpio)* and rainbow trout *(Salmo gairdneri)* (Ogino and Yang, 1980), channel catfish (*Ictalurus punctatus*) (Gatlin and Wilson, 1984b), Atlantic salmon (*Salmo salar*) (Maage *et al*., 2000), gible carp (*Carassius auratus gibelio*) (Pan *et al*., 2008), tilapia species (*Oreochromis mykiss, Oreochromis aureus*) (Lin *et al*., 2008), grass carp (*Ctenopharyngodon idellus*) (Wang and Zhao, 1994) and grouper (*Epinephelus coicoides*) (Ye *et al*., 2009) at levels of 12–13, 12–13, 2.4, 7.5, 13.77, 7, 15 and 19 mg kg^{-1}diet, respectively (Table IV).

Table IV Optimum dietary levels of Mn for juveniles of different fish species

Species	Requirement	References
Cyprinus carpio *Salmo gairdneri*	13-12 mg kg^{-1}	Ogino and Yang, 1980
Ictalurus punctatus	2.4 mg kg^{-1}	Gatlin and Wilson, 1984b
Salmo salar	7.5 mg kg^{-1}	Maage *et al*., 2000
Carassius auratus gibelio	13.77mg kg^{-1}	Pan *et al*., 2008
Oreochromis mykiss *Oreochromis aureus*	7 mg kg^{-1}	Lin *et al*., 2008
Ctenopharyngodon idellus	15 mg kg^{-1}	Wang and Zhao, 1994
Epinephelus coicoides	19 mg kg^{-1}	Ye *et al*., 2009

Little information has been given about excess of Mn. The high dietary Mn may affect bone mineralization (Maage *et al*., 2000). But, Tan *et al*. (2012) demonstrated that yellow catfish grew normally when dietary Mn supplement was higher than requirement. Similar result reported that higher dietary Mn levels as 1000 mg kg^{-1} than requirement, did not affect the growth performance of grouper, indicating that the Mn has low toxicity in fish (Ye *et al*., 2009).

Despite only very few studies have been conducted in larvae, as it is the case for Zn, content of Mn in cultured live preys seems to be lower than in wild zooplankton and, accordingly, their enrichment with Mn and Zn promotes growth and reduces bone malformations (Matsumoto *et al*., 2009; Nguyen *et al*., 2008).

1.4.4 Iron (Fe)

Iron or Fe is important for several metabolic functions such as oxygen transport or neurological development (Fairbanks and Beutler, 1995). Although, fish can absorb soluble iron cross the gill membrane and intestinal mucosa (Roedar and Roedar, 1968). This element activates a large number of enzymes such as microsomal cytochromes, catalase, transferin, ferritin and flavin. It may participate in oxidation/reduction reactions and electron transport associated with cellular respiration (Lorentzen and Maage, 1999). Plasma is the principal carrier of Fe in blood, although Fe is also present in the erythrocytes hemoglobin (Watanabe *et al*., 1997). Routinely iron is added to fish feed in order to ensure good health and maximum growth (NRC, 1993).

Deficiency of Fe is common in diet human over the world (Qiao *et al*., 2013). In fish, iron deficiency induces anemia in brook trout (Kawatsu, 1972), yellowtail (Ikeda *et al*., 1973), red sea bream (Sakamoto and Yone, 1978a) and carp (Sakamoto and Yone, 1978b). However, the lower survival rate was observed in channel catfish due to iron

deficiency (Lim and Klesius 1997; Lim *et al*., 2000). Additionally, poor feed utilization and lowering of plasma Fe level and transferring saturation is found in channel catfish (Gatlin and Wilson, 1986a). A yellowish-white liver condition is found in carp fed Fe deficient diets (Sakamoto and Yone, 1978b). Finally, the hatching rate of rainbow trout eggs is also reduced, when the Fe content is low (Hirao *et al*., 1955).

However, only very few studies have been reported the chemical forms of Fe supplement in fish. Recent study reported that the bioavaibility of dietary from iron methionine was similar to that iron sulphate for weight gain and feed efficiency in juvenile cobia (Qiao *et al*., 2013). Similar results have been reported by Lim *et al*. (1996) en channel catfish and Mai and Tan (2000) en abalone.

En few species of fish have been determined the Fe requirement. Thus, vary dietary requirement has been estimated to be 150 mg kg^{-1}(ferrous chloride) for red sea bream (*Pagrus major*) (Sakamoto and Yone, 1978a), 170 mg kg^{-1} (Ferrous sulphate) for eel (*Anguilla japonica*) (Nose and Arai, 1979), 30 mg kg^{-1}(ferrous sulfate) for channel catfish (*Ictalurus punctatus*) (Gatlin and Wilson, 1986a), 60–100 mg kg^{-1} (ferrous sulphate) for Atlantic salmon (*Salmo salar*) (Andersen *et al*., 1996) and 85 mg kg^{-1} (ferrous sulphate) for tilapia (*Oreochromis niloticus* and *O. aureus*) (Shiau and Su, 2003) (Table V).

Table V Optimum dietary levels of Fe for juveniles of different fish species

Species	Requirement	References
Pagrus major	150 mg kg^{-1}	Sakamoto and Yone, 1978a
Anguilla japonica	170 mg kg^{-1}	Nose and Arai, 1979
Ictalurus punctatus	30 mg kg^{-1}	Gatlin and Wilson, 1986a
Salmo salar	60–100 mg kg^{-1}	Andersen *et al*., 1996
Oreochromis niloticus *Oreochromis aureus*	85 mg kg^{-1}	Shiau and Su, 2003

High level of Fe causes toxicity and reduces growth (Desjardins *et al*., 1987; Rasmussen, 1994). Also, it can increase virulence of fish pathogens (Rorvik *et al*., 1991; Fouz *et al*., 1994).

Iron has similar levels in rotifers and *Artemia* as in wild copepods (Hamre *et al*., 2013), and, therefore, no Fe enrichment seems to be required. At present, no information is available regarding the optimum levels of Fe in first feeding or early weaning diets for marine fish larvae.

1.5 Importance of different molecular forms of trace nutrients

There are several factors that affect the efficiency of minerals such as:

- The content and the chemical form of the nutrient.
- The particle size and digestibility of the diet.
- Nutrient interactions that may be synergistic or antagonistic.
- Physiological and pathological conditions of the fish (Watanabe *et al*., 1997).

However, fish meal and plant protein sources contain endogenous inhibitors such as tricalcium phosphate and phytate, respectively that inhibit minerals availability (Watanabe *et al*., 1980; Gatlin and Wilson, 1984c; Satoh *et al.*, 1987a, b, c, d, 1991; Paripatananont and Lovell, 1995). Among these factors, one of the factors that affect absorption and utilization of trace elements is their chemical form (Apines *et al*., 2004). Finally, to all these factors, the environment influence mineral bioavailability (Watanabe *et al*., 1997).

1.5.1 Inorganic forms

Supplementation of trace elements to animal diets, traditionally, has been achieved through the use of inorganic salts, such as sulfate and carbonate. However, continuous research have been made to improve its utilization both humans and animals because their relatively forms have a lower bioavailability (Apines *et al*., 2004).

1.5.2 Organic forms

The chelation of trace elements by organic compounds, as opposed to inorganic minerals salts, is referred to as ‘organic’ here onwards. The use of chelated minerals as more natural form of trace elements supplementation because the natural feed ingredients largely contains minerals bound to proteins and amino acids (Tucher and Tylor-Pickard, 2005). However, chelation influences the bioavailability of trace elements. A different natural chelating agent in foods, some that decrease bioavailability such as inositol hexaphosphate, or phytate, which forms insoluble complexes with many cations, including Zn and Fe (Lo *et al*., 1981; Spinelli *et al*., 1983) and other increases absorption of trace elements, such as amino acids and peptides (Paripatananont and Lovell, 1997). This latter type of chelation protects the trace element from forming insoluble compounds or complexes in the digestive tract or facilitates transfer of the element across membranes (Ashmead and Zunino, 1992). Thus, the metal chelate is absorbed from the gut intact, delivered to various parts of the body, and degraded at the site where the element is needed (Ashmead, 1992).

1.5.3 Encapsulated forms

Encapsulation is a process to separate a nutrient from its environment by which one material is coated by another material, consequently, to control its release and/or facilitate mixing and formulation (Risch, 1995). It used also to improve stability and cover up flavors, odors and taste in the pharmaceutical industry, but to modify physical characteristics of nutrients (minerals and vitamins) in the food industry (Risch, 1995). Encapsulation of nutrients has been realized by food ingredients such as starches and oligosaccharides. The increasingly use of encapsulated nutrients in the food industry determine the need to assess the nutritional effects and/or the bioavailability of encapsulated nutrient supplements (Bebe *et al*., 1999).

1.5.4 Nano forms

Actually, nanotechnology holds promise for nutrition because nanometer dimension exhibit novel properties different from those of both isolated atom and bulk material (Albrecht *et al*., 2006; Wang *et al*., 2007) such as great specific surface area, high surface activity, a lot of surface active centers and high catalytic efficiency (Gao and Hiroshi, 2005). Nanoparticles have been already used in pharmaceutical applications to increase bioavailability of drugs and target therapeutic agents of particular organs (Florance *et al*., 1995; Davda and Labhasetwar, 2002). It has been reported that nanoparticles showed new characteristics of transport and uptake and exhibited higher absorption efficiencies (Davda and Labhasetwar, 2002; Chithrani and Chan, 2007; Zha *et al*., 2008; Liao *et al*., 2010).

1.6 Objective

In view of the importance of micronutrients for fish metabolism and the lack of information on their importance for marine fish larvae, the overall objective of this study will be to determine the effect of the inclusion of four minerals essential for metabolism during larval development: Fe, Mn, Se or Zn, considering four types of delivery forms to supplement these trace elements: organic, inorganic, nanometals or encapsulated in order to increase their availability for the larvae and prevent leaching and water loss in the culture medium.

2. MATERIALS AND METHODS

2.1 Larvae

2.1.1 Gilthead sea bream larvae

Larvae were obtained from natural spawns from the gilthead sea bream broodstock of the Instituto Canario de Ciencias Marinas (Grupo de Investigación en Acuicultura (GIA), Las Palmas de Gran Canaria, Spain). Larvae (Initial total length 5.10±0.43mm, mean ±SD; dry body weight 0.12±0.03mg) previously fed rotifers (*Brachinous plicatilis*) enriched with DHA Protein Selco® (INVE, Dendermond, Belgium) until 20dah, were randomly distributed in 15 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 used had a volume of 200 L and a cylinder shape with a conical bottom and were made of light grey color painted fiberglass (Figure 1). All tanks were supplied with marine water (about 37ppm salinity) filtered by 50 μm mesh at an increasing rate of 0.4 – 1 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, oxygen and water quality were daily tested by using an Oxy Guard-handy beta instrument (Zeigler Bros, Gardners, USA). 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). Tanks were daily cleaned by hand between 17:00 and 19:00 with a hose by a siphon system (Figure 2).

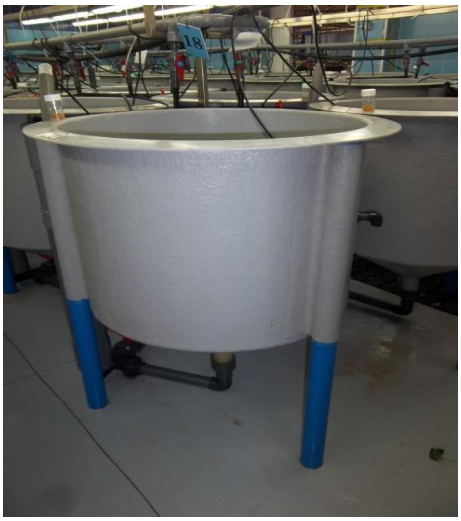


Figure 1: View of an experimental tank.

Figure 2: Experimental halls.

2.3 Diets and feeding

2.3.1 Microdiets

2.3.1.1 Microdiets formulation

The experimental diets, based on squid meal and krill oil having gelatin as a binder, were formulated to contain similar amounts of Fe, Mn, Zn and Se, provided as organic inorganic, nanoparticulated metals, or encapsulated minerals (Table VI). The desired lipid content was completed with a non essential fatty acid source, oleic acid (Merck, Darmstadt, Germany). Soybean lecithin (Acrofarma, Barcelona, Spain) containing around 50% of polar lipids was used as a source of phospholipids. The attractants mixture according to Kanazawa *et al.* (1989), the hydro and lipo-soluble vitamins mixture and minerals mixture according to Teshima *et al.* (1982). The protein source used was squid powder (Riber and Son, Bergen, Norway). To guarantee a best control

of the microdiet fatty acid profile, squid powder was defatted three consecutive times with a chloroform: meal ratio of 3:1 (Bentacor *et al*., 2012).

Minerals concentration was measured in diets. Samples were acidified in a microwave digestor (MarsXpress, CEM, Kamp-Lintfort, Germany)with 5 mL of 69% pure nitric acid, then poured after digestion 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 gas.

Diet C: Sin supplementation Fe, Mn, Se y Zn.

Diet O: Supplementation Fe, Mn, Se y Zn en organic form.

Diet I: Supplementation Fe, Mn, Se y Zn en inorganic form.

Diet N: Supplementation Fe, Mn, Se y Zn en nanometales form.

Diet E: Supplementation Fe, Mn, Se y Zn en encapsulated form.

Table VI Analyzed gross composition and contents of the different target minerals in the experimental diets

	Diet C	**Diet O**	**Diet I**	**Diet N**	**Diet E**
Lipid (%DW)	16.16±1	17.14±1.13	15.98±1.66	16.57±0.61	17.74±0.51
Moisture (%)	8.71±0.09	8.58±0.04	8.66±0.30	8.21±0.16	9.32±0.26
Ash (%)	6.04±0.01	6.2±0.03	5.76±0.64	5.31±0.57	5.82±0.02
Protein (%)	68.52±3.29	69.10±3.58	71.78±2.90	71.12±3.36	69.61±4.14
Minerals (mg/kg)					
Iron	340	440	310	330	370
Manganese	3.3	15	9.4	9.8	6.6
Zinc	86	110	110	96	88
Selenium	1.9	4.9	3.6	5.4	4.3

Data are means ± SD

2.3.1.2 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). The squid powder and water-soluble components of attractants and hydro-soluble vitamins were mixed well in a mortar, and then the lipids and fat-soluble 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 38 ºC for 24h (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 VI).

2.3.2 Larval feeding

Diets were manually supplied every 45 min from 9:00 to 19:00. Larvae were fed with rotifers once a day during the first two days of experimental feeding at a density of 1 individual 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 was determined calculating the percentage of gut occupation of the microdiet by image analysis in pictures of 30 larvae /tank (Figure 3). For such study, pictures of the abdominal cavity of 30 larvae per tank were taken (Figure 4) (Leica Wild M3Z, Optotek, California, USA).

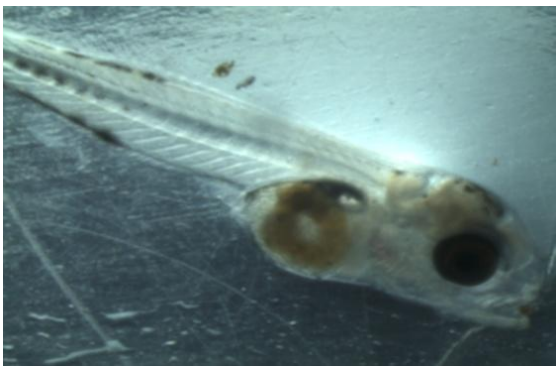


Figure 3: Observation under the binocular microscopic; Magnification 2X.

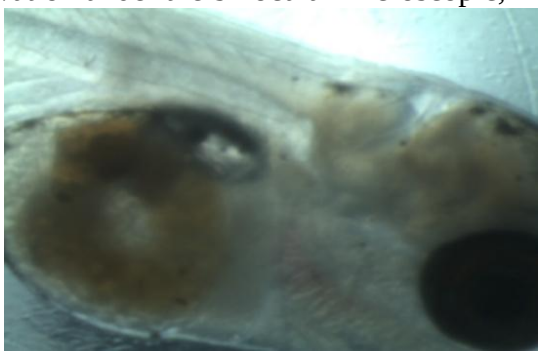


Figure 4: Picture of the abdominal cavity; Magnification 5X.

2.4 Sampling

2.4.1 Biological parameters

At the beginning (13 May 2013) and at the end (5 June 2013) of each experiment with the period of 24 days, samples of alive larvae unfed during the night were taken to determine total length (30 larvae of each tank) and whole body weight (10 larvae of each tank). At the end of the experiment, 20 alive fed larvae were sampled for the activity test.

2.4.2 Proximate analysis

Finally, to analyze biochemical composition, all the remaining larvae in each tank, after a starving period of 1 day, were collected, washed with distilled water and kept at -80ºC in air free labeled plastic sampling bags until analysis. Prior to the beginning of the dietary experiments, sea bream larvae samples were taken to analyze initial biochemical composition.

2.4.3 Molecular biology

At the final sampling points, around 100 mg of unfed sea bream larvae were collected, washed in Diethyl pyrocarbonate (DEPC) water and conserved in 500 µl of RNA Later (Sigma-Aldrich, Madrid, Spain) overnight at 4ºC, then RNA Later was removed and samples kept at -80ºC until RNA extraction.

2.4.4 Activity test and survival

At the end of the experiment 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 24h. Final survival was calculated by individually counting all the alive larvae $tank^{-1}$ at the end of the experimental trials.

2.4.5 Growth

Growth was determined by measuring total length and dry body weight. Total length of 30 anesthetized (with formol 1%) 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. Finally, the biomass was determined by this equation:

Biomass (mg) = [(2100x Number of survival larvae of each tank)/100] x Dry weight.

2.5 Biochemical analysis

2.5.1 Proximate analysis

2.5.1.1 Moisture

Moisture content was determined by oven drying until constant weight at 110ºC, with a first 24h drying period, followed by 1h 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 desiccators. Moisture was expressed as a percentage of the weight according to Official Methods of Analysis (A.O.A.C., 1995), using the following equation:

$$Moisture\ (\%) = \frac{100 - (B - A) - (C - A)}{B - A}$$

Where:

A = Weight of empty flask

B = Weight of wet sample + flask

C = Weight of dry sample + flask

2.5.1.2 Ash

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

2.5.1.3 Proteins

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.

2.5.1.4.1 Total 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.

2.5.1.4.2 Fatty acid methyl esters preparation and quantification

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 16h at 50ºC. Afterwards, fatty acid methyl esters were extracted with hexane: diethyl ether (1:1, v/v) and purified by adsorption chromatography on NH2 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: 0.32mm; Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180ºC for the first 10 min, increasing to 215ºC at a rate of 2.5ºC 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 Whole mount staining for skeleton studies

Staining procedures with alizarin red (Table VII) were conducted to evaluate the skeletal anomalies and vertebral mineralization following previous methods (Izquierdo *et al*., 2013) modified from previous studies (Vandewalle *et al*., 1999).

Table VII Staining protocol

STEP	DURATION	Significance of the step	Solutions
Alcian Blue (8GX)	2 hours	Proteoglycan (cartilage) Staining	For 100 ml: 80 ml alchol 95% 20 ml glacial acetic acid 10 mg Alcian blue
Ethanol 95%	1 hour	Hydratation	For 100 ml: 95 ml absolute ethanol 5 ml distilled water
Ethanol 95%	1 hour		
Ethanol 95%	1 hour		
Ethanol 75%	1 hour		For 100 ml: 75 ml absolute ethanol 15 ml distilled water
Ethanol 40%	1 hour		For 100 ml: 40 ml absolute ethanol 60 ml distilled water
Ethanol 15%	1 hour		For 100 ml: 15 ml absolute ethanol 75 ml distilled water
Distilled water	1 hour or all night		
Trypsine solution	1 hour	Tissue digestion	90 mg pancreas porcine trypsine; 70 ml distilled water 30 ml saturated solution of $Na_2B_4O_7$
Alizarine red	1 hour and a half	Calcium (bone) staining	1g L^{-1} alizarine red In a 0.5% KOH solution
Glycerin KOH 1:3	12 to 24 hours	Clearing	For 100 ml: 25 ml Glycerin 75 % KOH (0.5%)
Glycerin KOH 1:1	12 to 24 hours		For 100 ml: 50 ml Glycerin 50 mL KOH (0.5%)
Glycerin KOH 3:1	12 to 24 hours		For 100 ml: 75 ml Glycerin 25 ml KOH (0.5%)
Pure glycerine and some grains of thymol		Storage	

2.7 Molecular biology studies

All processes for molecular biology studies, except RNA extraction and GPx gene expression, were performed during a stay at the University of Insubria, Department of Biotechnology and Lifer Sciences (Varese, Italy). Studied genes were Cu-Zn superoxide dismutase (Cu-Zn SOD), Mn superoxide dismutase (Mn-SOD), Osteocalcin (OC), Bone morphogenetic protein 2 (BMP2) and glutathione peroxidase (GPx), gene sequences were reconfirmed prior to its use for quantitative real time PCR (RT-PCR) (Table VIII). Primers of these genes were ordered to Eurofins (Ebersberg, Germany).

Table VIII Genes studied in the present research

Gene	Accession number
Cu-Zn Superoxide dismutase	JQ308832.1
Mn Superoxide dismutase	JQ308833.1
Osteocalcin	AF048703.1
Bone morphogenetic protein 2	JF261172.1
Glutathione peroxidase	DQ524992

2.7.1 Total RNA extraction

Total RNA extraction 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 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, 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 3ml of RNase-free water.

The quality and quantity of RNA were analyzed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.7.2 Synthesis of cDNA

Complementary DNA (cDNA) is typically generated from messenger RNA (mRNA) (except GPx) by action of a retroviral reverse transcriptase which reverse transcribes a single strand molecule of RNA into single stand cDNA. 3 μg of total RNA was reverse transcribed into cDNA in a volume of 12 μl, including 1 μl of oligo dT16 primer (50 pmol) and 1 μl of 10 mM deoxynucleotide triphosphates (dNTPS). This mix was heated

at 65ºC for 5 min, chilled on ice and then 4 µl of 5x reverse transcription buffer, 2 µl 0.1 M dithiothreitol (DTT), 1 µl RNAse out and 1 µl of Moloney murine leukemia virus (M-MLVRT) added. After incubation at 37ºC for 50 min, reaction was stopped by heating at 75ºC for 15 min (Figure 5).

Figure 5: The thermomixer for incubation reaction.

In the case of gene GPx, synthesis of complementary desoxiribonucleic 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 PBR322 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 β-actin as the house-keeping gene in a final volume of 20 µl per reaction well, and 100 ng of total RNA reverse transcribed to complementary cDNA. The PCR conditions were the following: 95ºC for 3 min 30 sec followed by 40 cycles of 95ºC for 15 sec, 61ºC for 30 sec, and 72ºC for 30 sec; 95ºC for 1 min, and a final denaturing step from 61ºC to 95ºC for 10sec. Data obtained were normalized and the Livak method ($2^{-\Delta\Delta Ct}$) 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) (Table IX). Thus, the following steps do not apply for the GPx gene.

2.7.3 Cloning and sequencing

To amplify selected primers sequences, touch up PCR was performed (except GPx). The selection of this specific kind of PCR was due to the wide variety of melting temperatures of the primers (Table IX). In this sense, with a touch up PCR, the initial temperature of 55ºC increases to 0.5ºC in each cycle, arriving to a final temperature of 95ºC. A total of 33 cycles (Eight touchup) of the PCR amplification were performed for all primer sets, using an automated Thermal Cycler (Mycycler, Bio-Rad, Italy) (Figure 6).

Table IX Oligonucleotide primer sequences and melting temperatures used for PCR to produce standard curves

Oligo name	Sequence (5'→3')	TmºC
Cu-Zn SOD _T3	caattaaccctcactaaagggCGTTCATTTTGAGCAG GAGA	71.4
Cu-Zn SO antisense	CCTCCTTTTCCCAGGTCATC	59.4
Mn-SOD_T3	caattaaccctcactaaagggTGGTCAAATACGCAGG TGTG	72.4
Mn-SOD antisense	CTCGCCTCCTCCGTTTGG	60.5
OC_T7	gtaatacgactcactatagggGGCAGCCATCTGTACT T	73.4
OC antisense	GGTCCGTAGTAGGCCGTGTA	61.4
BMP2_T7	gtaatacgactcactatagggCTCAGGTGTTGCTGGA AGGT	73.4
BMP2 antisense	TTGCCTTTCAGACTGGCCAG	59.4
GPx _T7	TCCATTCCCCAGCGATGATGCC	-
GPx antisense	TCGCCATCAGGACCAACAAGGA	-

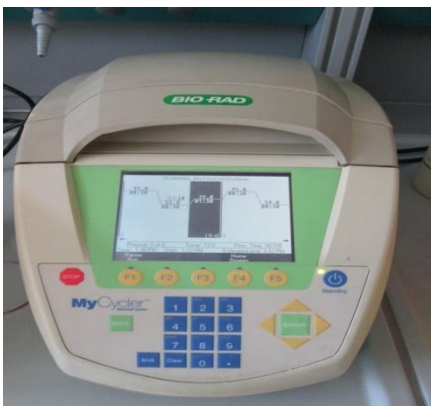


Figure 6: The automated Thermal Cycler

An aliquot of 3 µl of the resulting cDNA was amplified using 25 µl GoTaq Green Master Mix 2×(Promega, Milan, Italy) in 50 µl of final volume containing 1µl de primer T7 or T3 (50µM) and 1µl de oligo antisense (50 µM).

MATERIALS AND METHODS

A total of 33 PCR amplification cycles (Eight touchup) were performed for all primer sets, using an automated Thermal Cycler (MyCycler, BioRad, Milan, Italy). Each sample was then electrophoresis on a 1.5% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer (BioRad, Milan, Italy) and bands were detected by ethidium bromide (Figure 7). Samples ran with a 100 bp+1.5 Kb DNA ladder to control molecular weight of DNA and with a negative control consisting of reaction mixture without cDNA, confirming in that way the absence of genomic contamination.

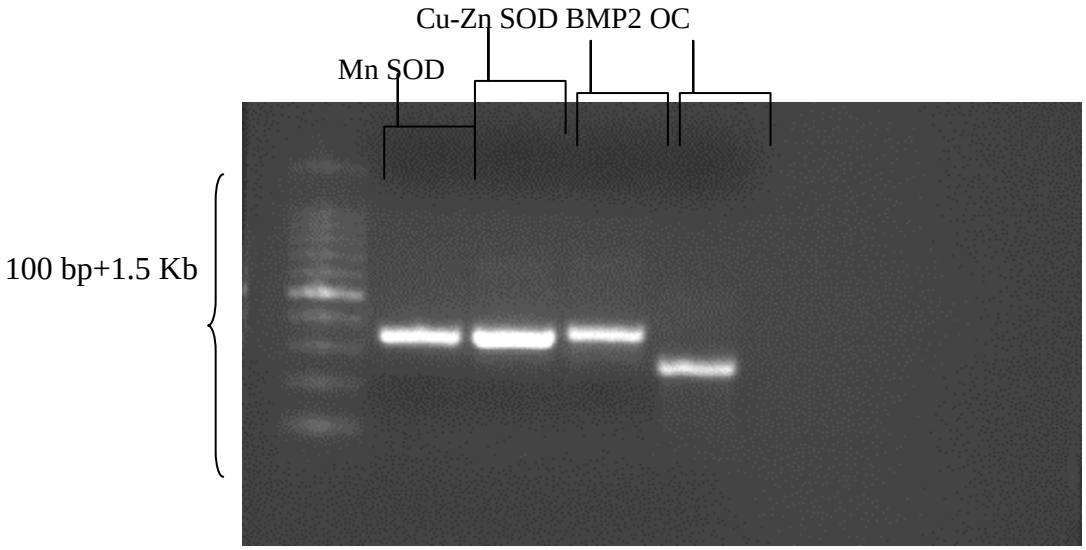


Figure 7: Agarose gel showing PCR products of each designed primer**.**

Each PCR product was purified by cutting a DNA fragment of the predicted molecular weight from the gel and processing it with NucleoSpin Gel and PCR Clean-up (MACHEREY-NAGEL, Milan, Italy) according to the manufacturer's instructions. After gel slice was dissolved it was placed on one SV Minicolumn and incubated one minute at room temperature. Columns were then centrifuged at 11000 x *g* for 1min, liquid discarded and washed with 700µl of Membrane Wash Solution diluted with 95% ethanol. After centrifugation the mini column was placed on a new microcentrifuge tube and eluted with 30 µl of Nuclease-Free water. The purified PCR product concentration was then measured using a SmartSpec Plus spectrophotometer (Bio-Rad, Milan, Italy). The amount of PCR product to include in the cloning reaction was calculated using the following formula:

$$ng\ Insert = \frac{ng\ Vector \times Kb\ Insert}{Kb\ Vector} \times Insert{:}\ vector\ molar\ ratio$$

Where:

ng vector= 50 ng

Kb vector= 3.0 Kb

MATERIALS AND METHODS

Insert: Vector molar ratio= 5:1

Kb size of insert = In this case the genes had a length of 331bp, so 0.33Kb.

The cloning vector used in this study was pGEM®-T Easy cloning vector system (Promega, Milan, Italy; Figure 8). The ligation of the PCR product was performed by 1 µl of T4 DNA ligase activity associated with 1 µl of the commercial cloning vector preparation and 5 µl of 2×Rapid ligation Buffer. The cloning reaction was performed overnight at 4ºC and next day 100 µl of *Escherichia coli* bacteria (Promega, Milan, Italy) was added to this mixture and incubated in ice for 20 min. A heat shock was then performed in a water bath (42ºC) without shaking for 45 sec and tubes immediately returned to ice for 2 min. 900 µl of SOC medium (Sigma) was then added and the mixture was incubated at 37ºC for 2h on a shaker mixer. The mixture containing the transformed cell was then spread on a 2 selective LB Agar plates per gene and incubated overnight at 37ºC. The selective LB Agar plates contained ampicilin (100 µg ml^{-1}), X-gal and IPTG. As a result, only the cells containing the plasmid which contained ampicilin resistant gene would grow. Moreover, the selection of cells containing a ligated PCR fragment was based on the colorimetric metabolic reaction of X-gal by Laczα gene, as the ligation of the PCR product would result in disruption of the Laczα reading frame leading to the production of white colonies. Three white colonies per gene were solved in 20 µl of RNase-Free Water, being used 5 µl of this solution to make a new PCR (Figure 9).

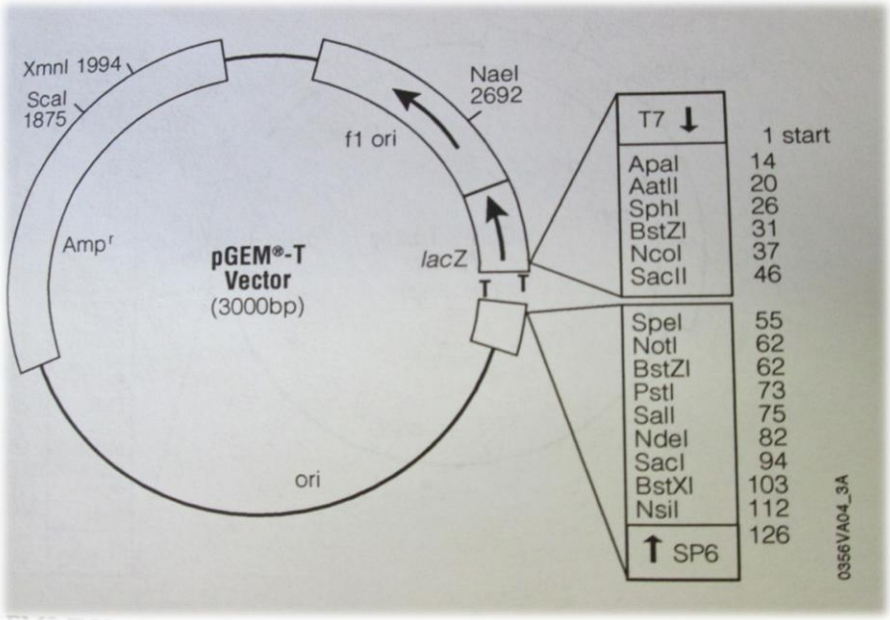


Figure 8: pGem – T Vector Map and sequence reference points.

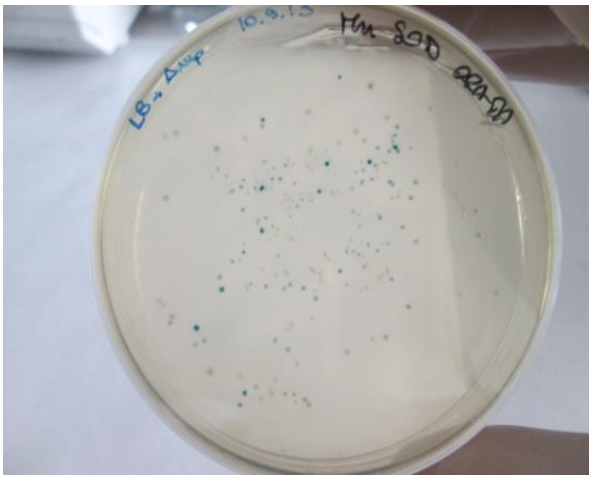


Figure 9: The selective LB Agar plates (White and Blue colonies).

According to the quality of the bands, one colony will be chosen and grown overnight at 37ºC in 10 ml of LB Agar containing 10 µl of ampicilin. The plasmid vector was purified using the NucleoSpin Plasmid Kit (MACHEREY-NAGEL) as described in the manual. The column was eluted with 50 µl of RNase-Free Water, concentration calculated with a SmartSpec Plus spectrophotometer (Bio-Rad, Milan, Italy) and conserved at -20ºC. Extracts were dried up and subsequently sent to BMR Genetics (Padova, Italy) for its gene sequencing in both directions (T7 and SP6) (Figure 10).

Cu-Zn-SOD

caattaaccctcactaaagggCGTTCATTTTGAGCAGGAGAGTGAGTCAGCACCTGTGACGCTCACAGGAGAAATCAAA
GGGCTTACTCCCGGTGAGCATGGCTTCCATGTCCATGTATTTGGAGACAATACAAATGGGTGCATCAGTGCAGGCCCT
CACTTCAATCCCCATGGTAAGAATCATGGCGGTCCTACTGATGCAGAGAGGCACGTTGGAGACCTGGGCAACGTGAC
TGCAGGAGCAGATAATGTTGCCAAGATAGACATCACGGACAAGATGCTCACTCTCAGTGGGCCCTTGTCCATCATTG
GCAGAACCATGGTGATCCACGAGAAGGTAGATGACCTGGGAAAAGGAGG

Mn-SOD

caattaaccctcactaaagggTGGTCAAATACGCAGGTGTGCAGCCAGCCTGAGCCAAACTTTGAACCAGGTAGC
TGCATCGAGGCAGAAGCACACGCTCCCTGACCTGACCTACGACTATGGTGCCCTGGAGCCCCACATCAACGCAGAGA
TCATGCAGCTGCACCACAGCAAGCACCATGCCACATATGTCAACAACCTCAACGTTACAGAGGAGAAATATCAGGAG
GCACTAGCAAAGGGAGATGTGACGACACAGGTTGCCCTCCAGCCTGCTCTGAAGTTTAATGGAGGAGGCCACATTAA
TCACACCATCTTCTGGACAAACCTGTCGCCAAACGGAGGAGGCGAG

OC

ATGAAGACTCTGGCCTTCCTGGTTCTCTGCTCCCTGGCAGCCATCTGTCTGACTTCAGATATGAAGACTTTGGCCATCC
TGGTTCTCTGCTCCTTGGCAGTCATCTGTCTGACTTCAGATGCCTCCACTGGCTCCCAGCCTGCCAGTGACAACCCTGC
TGATGAGGGTATGTTCGTGGAGGCCTCCGCTGGCTCCCAGCCTGCCAGTGACAACCCTGCTCAGGAGGGTTTGTTTGT
GGAGAGGGACCAGGCCTCCGCAGTGGTGAGACAGAAGAGAGCGGCTGGACAGCTGTCCCTCACTAGGGAGCAGGCC
TCCACCGTGGTGAGACAGAAGAGAGCTGCTGGAGACTTATCCCTGACACAGCTGGAGAGCCTGAGAGAAGTGTGTG
AGCTCAACCTGGCTTGCGAGCACATGATGGACCAGCTGGAGAGAGGTGTGTGAGGCCAACCTGGCTTGT
GAGGACACGATGGACACTGAGGGAATCATCGCTGCCTACACGGCCTACTACGGACCGATCCCCTACTA

BMP2

gtaatacgactcactatagggCTCAGGTGTTGCTGGAAGGTGCCACGGGACTTATCCCTGAGGTCGGCCGGAGGAAGTAC
AGCGAATCTGGGAAGCAAACCCCACAGCAGTCGGAGAGCTTCCTCAACGAGTTTGAGCTTCGGCTTCTCAATATGTTT
GGACTGAGGCGCAGGCCGACCCCGAGCAAGCAAGCCGTGGTGCCGCAGTACATGGTGGACCTTTACCGCATGCACTC
AGCGAACGGAGACCACAGCACTAAACGACCCAAGAGCATGGGGAAGCACGCAGAGAGAGCCGCCAGCAAGGCCAA
CACGATTAGAAGCTTTCACCATGAAGAGTCTATGGAGGCCCTGGCCAGTCTGAAAGGCAA

Figure 10: Obtained nucleotide sequences from sea bream genes and predicted amino acid sequences. Arrows indicate the position and sequences of primers used to amplify the cDNAs.

2.7.4 Quantitative real-time RT-PCR

2.7.4.1 Generation of in vitro-transcribed cRNAs for standard curves

The number of each target gene transcript copies could be absolutely quantified by comparing them with a standard graph constructed using the known copy number of mRNAs of each gene. For this, a forward and a reverse primer were designed based on the mRNA sequences of *Sparus aurata*. These primer pairs were used to create templates for the *in vitro* transcription of cRNAs for each gene. The forward primers were engineered to contain a T7 or a T3 phage polymerase promoter gene sequence to their 5' end and used together with the reverse specific primer in a conventional RT-PCR of total sea bream larvae RNA. RT-PCR products were the checked on a 1.5% agarose gel stained with ethidium bromide, cloned using pGEM®-T Easy cloning vector system (Promega, Milan, Italy) and subsequently sequenced in the SP6 direction.

In vitro transcription was performed using T7 or T3 RNA polymerase and other reagents supplied in the Promega RiboProbe in Vitro Transcription System kit according to the manufacturer's protocol.

The molecular weight (MW) of the in vitro-transcribed RNAs was calculated according to the following formula:

$$MW = [(n^o \text{ of A bases}) \times 329.2) + (n^o \text{ of U bases}) \times 306.2) + (n^o \text{ of C bases}) \times 305.2) + (n^o \text{ of G bases}) \times 345.2)] + 159.$$

Spectrophotometer at 260 nm gave a concentration of each cRNA. Therefore, the concentration of the final working solutions were calculated and expressed as n° of molecules μl^{-1}.

2.7.4.2 Generation of standard curves

The cRNAs produced by in vitro transcription were used as quantitative standards in the analysis of experimental samples. Defined amounts of cRNAs at 10-fold dilutions were subjected in once to real-time PCR using one-step TaqMan Core Reagents (Applied Biosystems, Italy), including 1X Taqman buffer, 3 mM MnOAc, 0.3 mM deoxynucleotide triphosphates (dNTP) except deoxythymidine triphosphate (dTTP), 0.6 mM deoxyuridine triphosphate (dUTP), 0.2 μM GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) forward primer, 0.1 μM GAPDH reverse primer, 0.1 μM GAPDH Probe, 0.1 units rTH DNA polymerase, and 0.01 units AmpErase UNG enzyme in a 30 μl reaction. AmpErase® uracil-N-glycosylase (UNG) is a 26-kDa recombinant enzyme encoded by the *Escherichia coli* uracil-N-glycosylase gene. UNG acts on single-and double-stranded dU containing DNA. It acts by hydrolyzing uracil-glycosidic bonds at

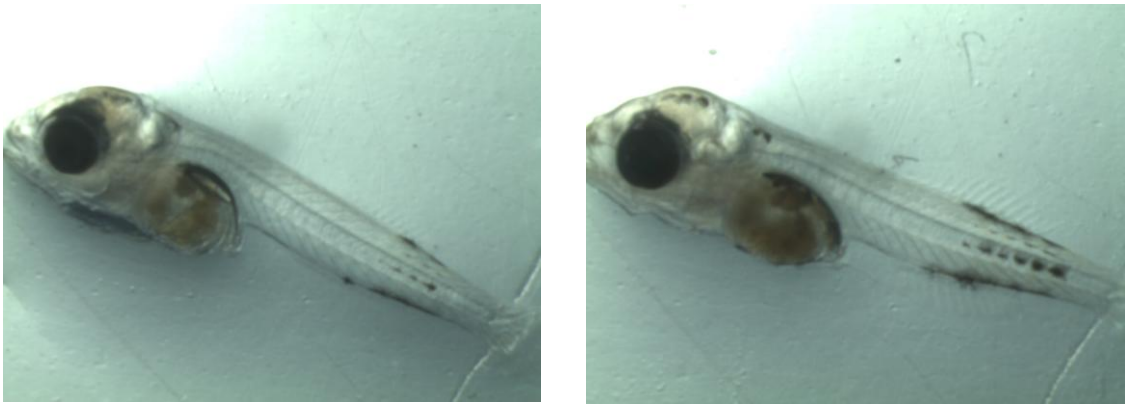
dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA. For TaqMan® assays, AmpErase® UNG treatment can prevent the reamplification of carry over PCR products from previous PCR reactions. When dUTP replaces dTTP in PCR amplification, AmpErase® UNG treatment can remove up to 200,000 copies of amplicon per 50µl reaction. RT- PCR conditions were: 2 min at 50°C, 30 min at 60°C, and 5 min at 95°C, followed by 40 cycles consisting of 20 s at 94°C, 1 min at 62°C. The Ct (cycle threshold) values obtained by amplification were used to create standard curves for target genes.

2.8 Statistical analysis

All data were tested for normality and homogeneity of variances with Levene´s test, and treated using one-way ANOVA. Means 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 (skeletal anomalies, mineralization of the column, total survival and activity test survival) were arc cosine transformed before performing any statistical test.

3. RESULTS

All experimental microdiets were well accepted by larvae as denoted by the image analysis of the guts (Figure 11).



(A) **(B)**

(C) **(D)**

(E)

Figure 11: Observation of gut larvae for different dietary treatments under the binocular microscopic: Diet C (A); Diet O (B); Diet I (C); Diet N (D); Diet E (E); Magnification 2X.

RESULTS

At the end of the trial, survival of 44 dah larvae was significantly (P<0.05) higher in fish fed diet C without supplementation of Se, Zn, Mn or Fe, than fish fed organic (Diet O), inorganic (Diet I) or nano-minerals (Diet N) (Table X; Figure 12). Besides, survival of fish fed encapsulated minerals (Diet E) was not significantly different to that of fish fed diet C (Figure 12). Therefore, the lowest survival was detected in larvae fed the other three different forms of minerals in diets O, I, and N, with values of 7.22%, 7.46% and 8.84 %, respectively (P < 0.05) (Table X; Figure 12). Besides, the survival rate was also negatively correlated to the dietary Mn (P=0.047; P<0.05; Figure 13) and less significantly, to the Zn contents (P=0.052; Figure 14). As a consequence of the high mortality, the growth of fish fed these three diets (O, I and N) in terms of total length did not significantly differed among them and was higher (P < 0.05) than in fish fed diets C and E (Table X; Figure 15). Regarding body weight, the highest growth was obtained in larvae fed diet O and the lowest in those fed diet N (Table X; Figure 16). Finally, the biomass in larvae fed diets C and E did not significantly differed among them and was higher (P<0.05) than fish fed diets O, I and N (Table X; Figure 17).

Table X Gilthead sea bream larvae performance after 24 days of feeding microdiets with different mineral sources (mean ± SD)

	44 dah				
	Diet C	**Diet O**	**Diet I**	**Diet N**	**Diet E**
Survival (%)	22.19±9.13^{b}	7.22±3.63^{a}	7.46±2.39^{a}	8.84±4.26^{a}	15.65±5.49^{ab}
Total Length (mm)	8.19±0.25^{a}	9.21±0.29^{b}	8.88±0.25^{b}	9.00±0.32^{b}	8.08±0.54^{a}
Dry Weight (mg)	0.80 ±0.09^{a}	0.99 ± 0.02^{b}	0.86± 0.12^{ab}	0.78 ±0.07^{a}	0.80 ±0.04^{a}
Survival after activity test (%)	59.69±10.24^{ab}	62.50±17.68^{ab}	34.94±10.13^{a}	65.79±3.72^{b}	62.54±15.16^{ab}
Biomass (mg)	8020,15± 4.22E+03^{b}	3152.24± 1.57E+03^{a}	2770.78± 7.95E+02^{a}	3028.69± 1.47E+03^{a}	5483.05± 1.70E+03^{ab}

Values (mean ± standard deviation) with the same letters are not significantly different (P<0.05).

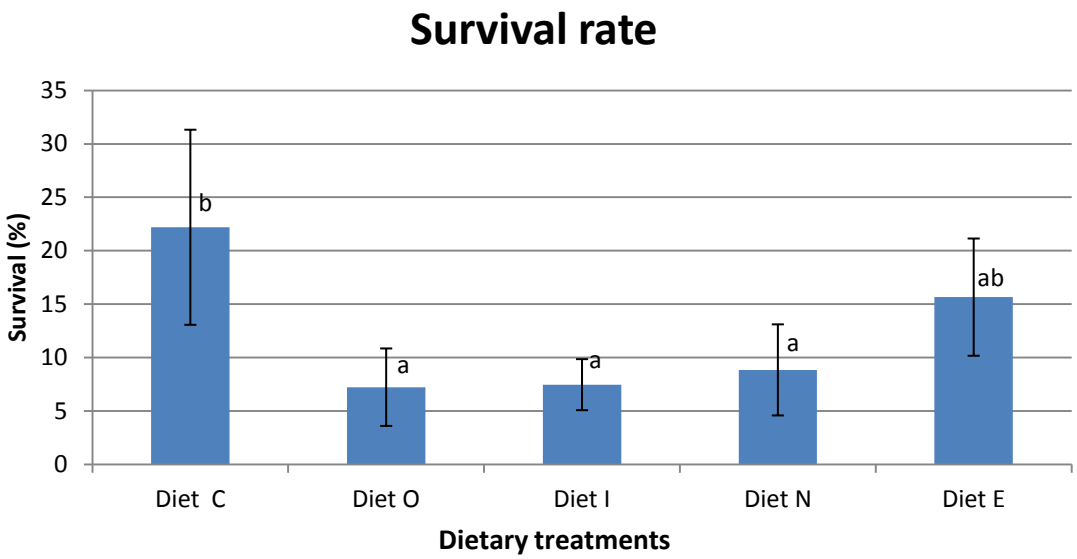


Figure 12: Survival rate of gilthead sea bream larvae (44 dah) after 24 days of feeding the experimental diets containing different mineral sources. Values (mean ± standard deviation) with the same letters are not significantly different (P<0.05).

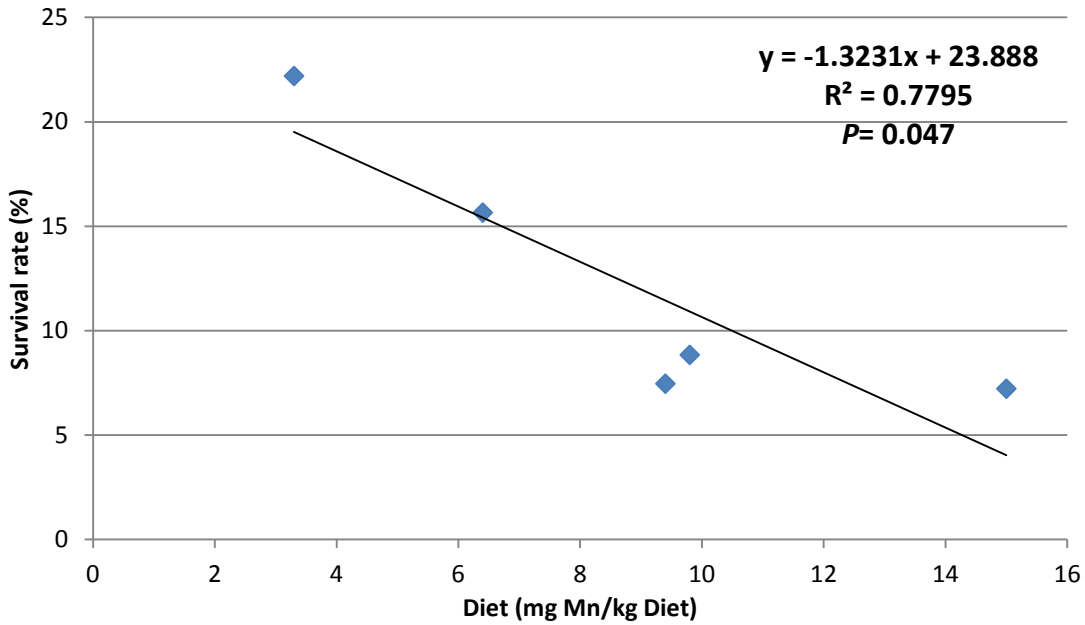


Figure 13: Correlation between dietary Mn contents and survival rates (%) of larvae (44 dah) after 24 days of feeding the experimental diets containing different mineral sources (P<0.05).

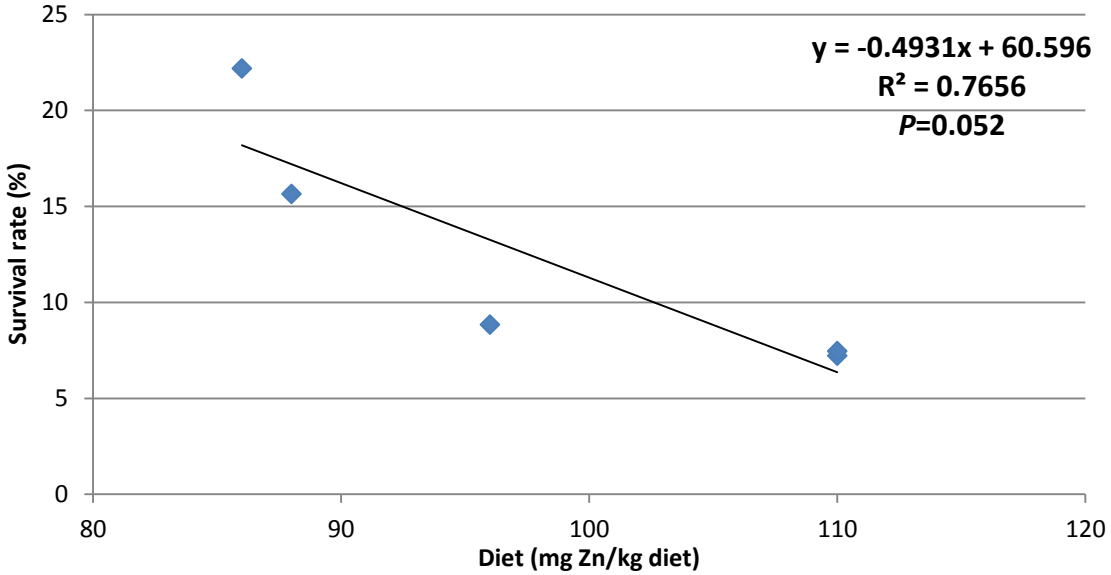


Figure 14: Correlation between dietary Zn contents and survival rates (%) of larvae (44 dah) after 24 days of feeding the experimental diets containing different mineral sources (P<0.1).

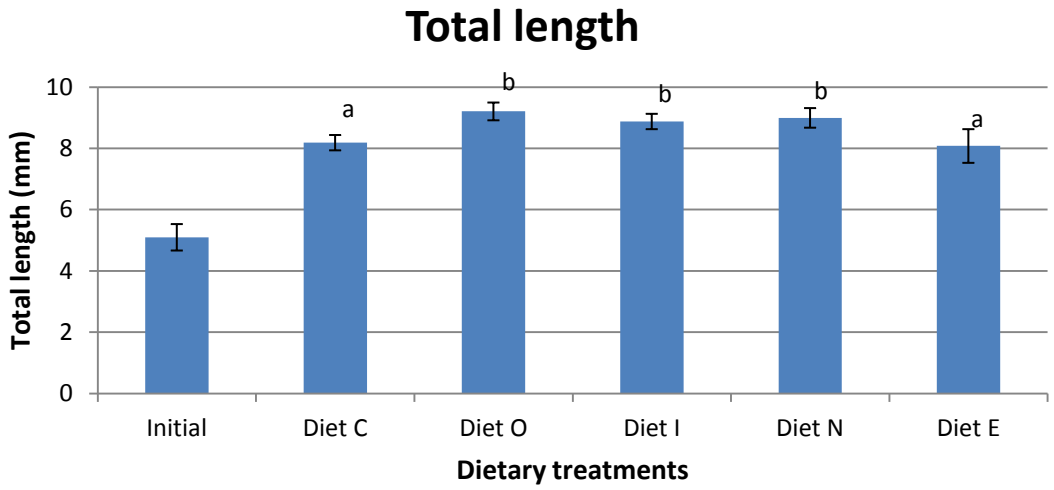


Figure 15: Total length of gilthead sea bream larvae (44 dah) after 24 days of feeding the experimental diets containing different mineral sources. Values (mean ± standard deviation) with the same letters are not significantly different (P<0.05).

RESULTS

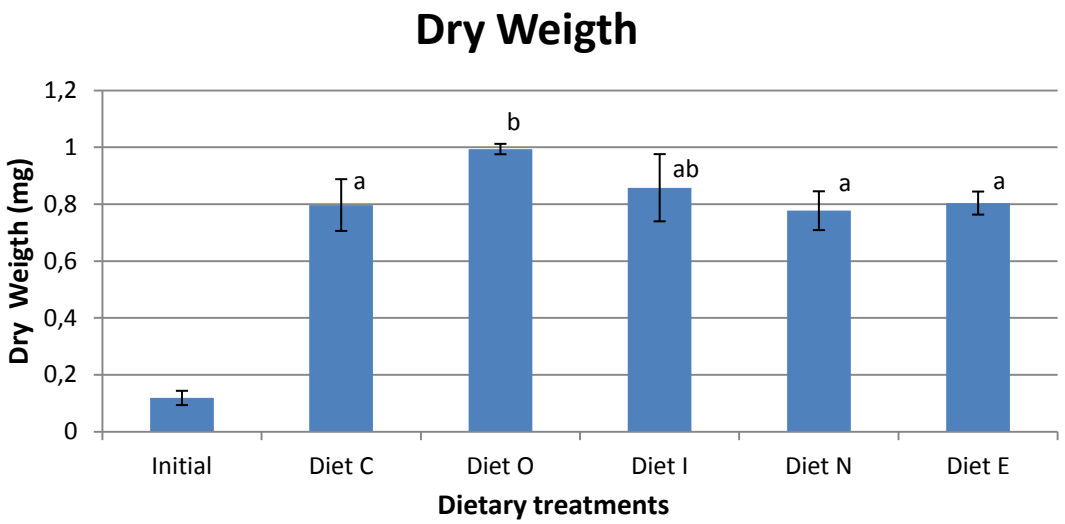


Figure 16: Dry weight of gilthead sea bream larvae (44 dah) after 24 days of feeding the experimental diets containing different mineral sources. Values (mean ± standard deviation) with the same letters are not significantly different (P<0.05).

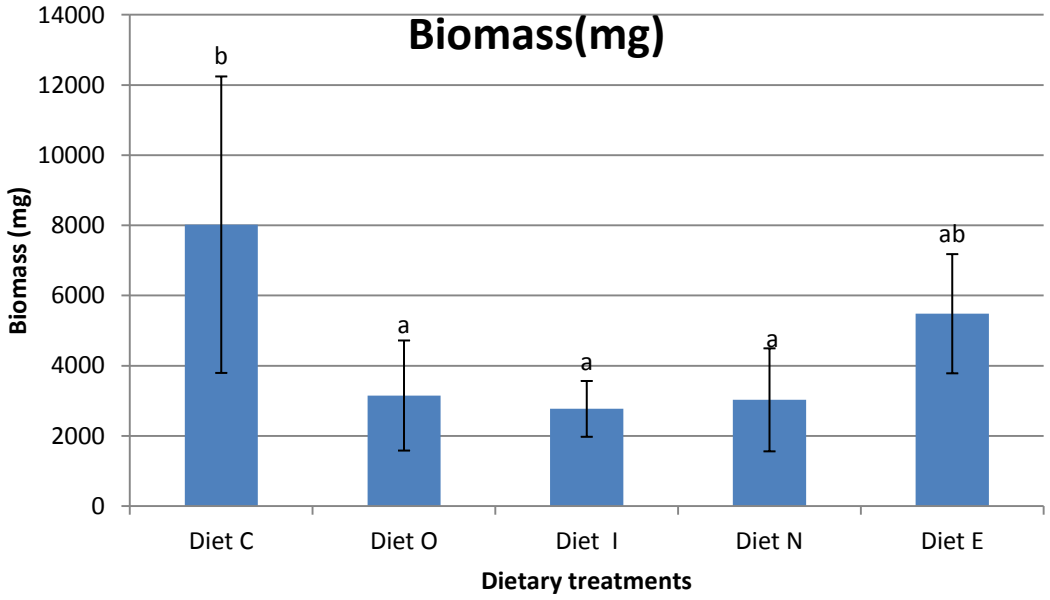


Figure 17: Biomass (mg) of gilthead sea bream larvae (44dah) after 24 days of feeding the experimental diets containing different mineral sources. Values (mean ± standard deviation) with the same letters are not significantly different (P<0.05).

Larvae submitted to air exposure showed a significantly (P < 0.05) lower survival when they were fed with inorganic minerals (34.94%) (Table X; Figure 18) than when fed diet N. In turn, survival of N larvae was not different than of larvae fed diets E, O and C, being 65.79 %; 62.54%; 62.50% and 59.69%, respectively.

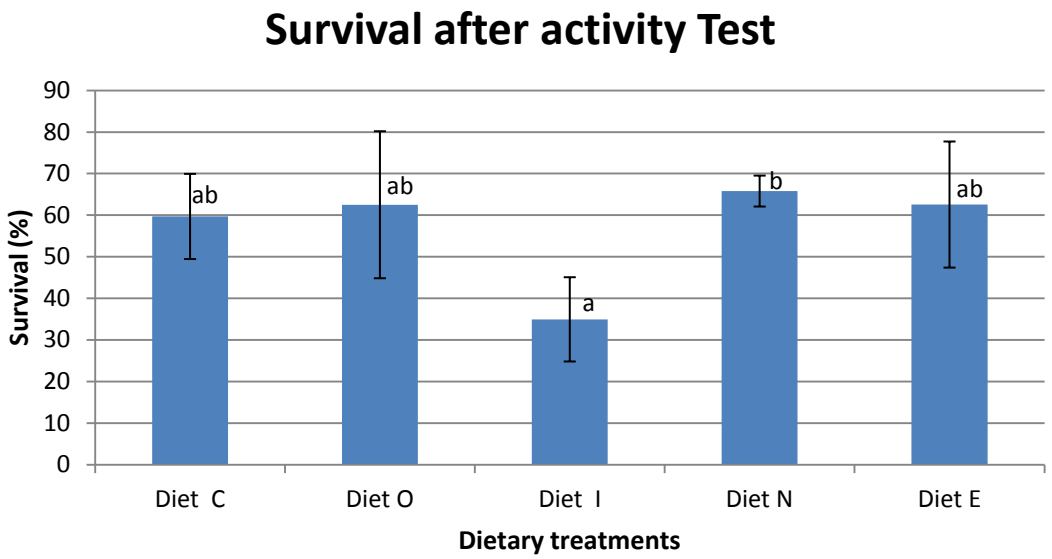


Figure 18: Survival rate (24 hours after activity test) of gilthead sea bream larvae (44dah) after 24 days of feeding the experimental diets containing different mineral sources. Values (mean ± standard deviation) with the same letters are not significantly different ($P<0.05$).

The effects of the different treatments on skeletal mineralization were analyzed considering the average number of mineralized vertebrae in each size class (Figure 19), and the average percentage of individuals with a complete mineralization of the vertebral column (Figure 20). Considering the number of mineralized vertebrae from all the larvae studied (6-14 mm) the highest values were found in fish fed the organic minerals (Diet O), followed by those fed nanometals and encapsulated minerals (Diets N and E) (Figure 19). Further analysis of each given size class showed that in the smaller size classes (6-8 and 8-10 mm) larvae fed diet O had the highest number of mineralized vertebrae, whereas in the larger size classes (10-12 and 12-14 mm) larvae fed diets E and N showed the highest mineralization (Figure 19). In most size classes the lowest number of mineralized vertebrae was found in fish fed either inorganic minerals (Diet I) or non-supplemented diet (Diet C). In agreement, the ossification degree was significantly highest in fish fed diet O, followed by diet N (Figure 20), whereas the lowest percentage of larvae with a complete vertebral mineralization was found in fish fed diets C and I.

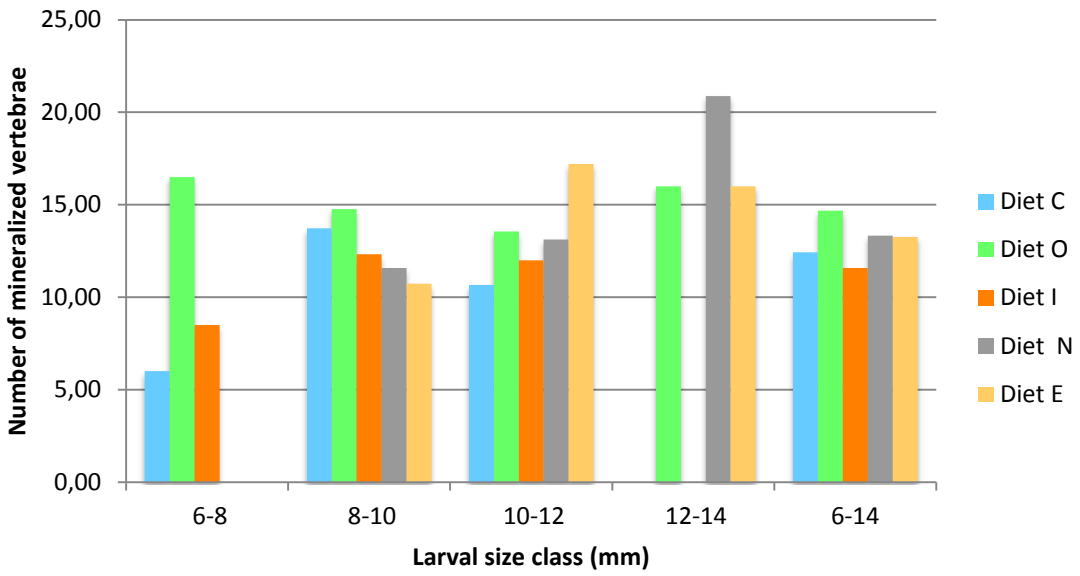


Figure 19: Average number of mineralized vertebrae for each size class in 44 dah larvae fed different mineral sources.

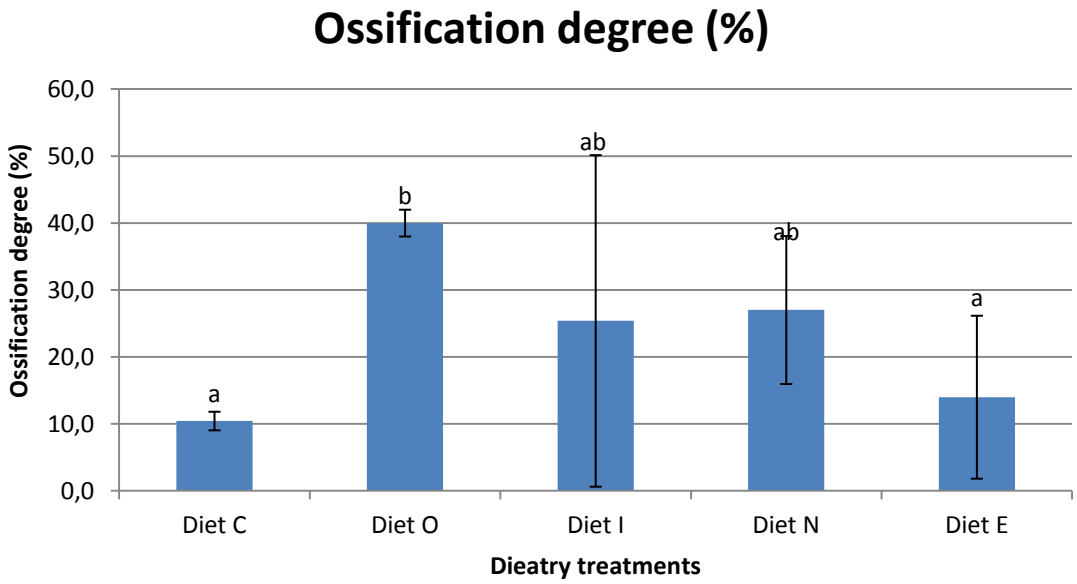


Figure 20: Bone mineralization frequency expressed as the average percentage of individuals with a completely mineralized vertebral column (%) in 44 dah gilthead sea bream larvae fed different mineral sources. Values (mean ± standard deviation) with the same letters are not significantly different (P<0.05).

Generally very high percentages of skeletal anomalies were detected along the study (from 50 to 80%). However, considering the incidence of total anomalies no significant differences (P<0.05) were found among larvae fed the different mineral sources (Figure

21). More specifically, deformities in the pre-haemal region were significantly higher in fish fed inorganic minerals in comparison to larvae fed diets C and E (Figure 21).

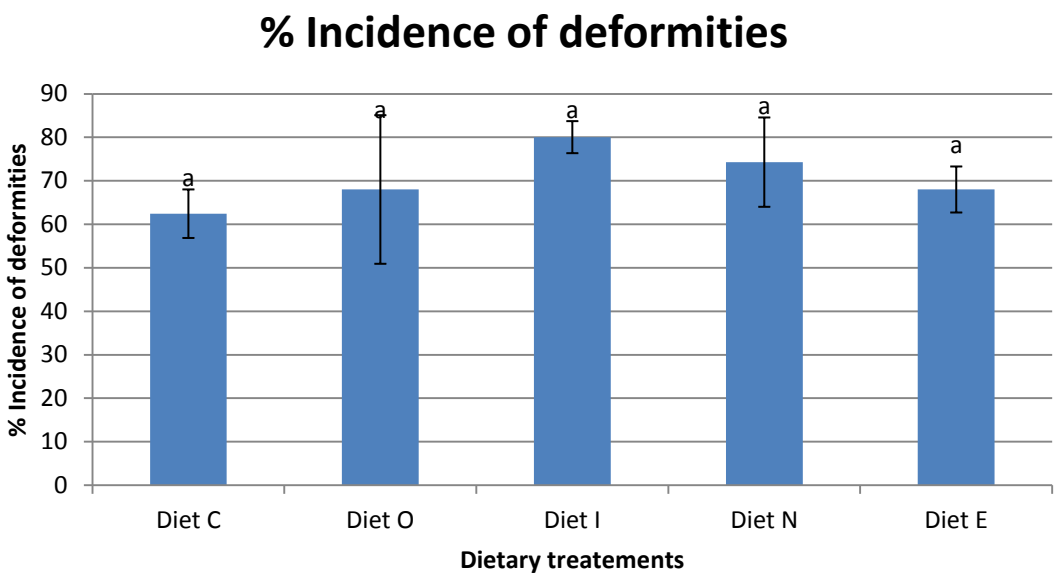


Figure 21: Gilthead sea bream larvae incidence of deformities frequency (%) at 44 dah from the different dietary treatments. Values (mean ± standard deviation) with the same letters are not significantly different (P<0.05).

Deformities of branchial arches in larvae fed diets E and C (42.32%; 38.29% respectively) were on average, the most represented en all experiment groups (Figure 23), followed by deformities pre-haemal (32.38%) (Figure 22 and 23) en group fed diet I (Elongation en vertebral (Figure 24 B), neurospine of pre-haemal vertebrae (Figure 24 B)). Skull deformities, particularly neurospine of cephalic vertebrae (Figure 24 A), were identified in all experimental groups especially in larvae fed diets O and I with values 21.63% and 19.78%, respectively. Other bone anomalies, such as haemal deformities presented especially by (reduction in length vertebrae (C4), neurospine (C5), haemal spine (C6)) (Figure 24C) were present in all experimental groups fed of larvae sea bream. In summary, branchial arches deformities were significantly higher in fish fed diets C and E, whereas anomalies related to vertebral elongation were significantly highest in fish fed diet I (Figure 23).

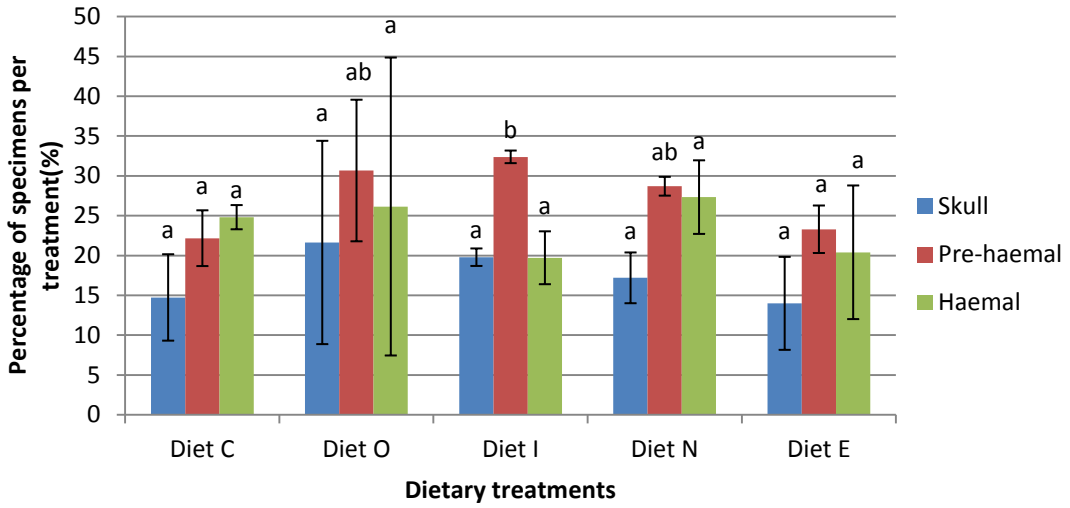


Figure 22: Skeletal anomalies in the different larval regions (not including branchiostegals) in 44 dah larvae fed different mineral sources. Values (mean ± standard deviation) with the same letters for the same body region are not significantly different (P<0.05).

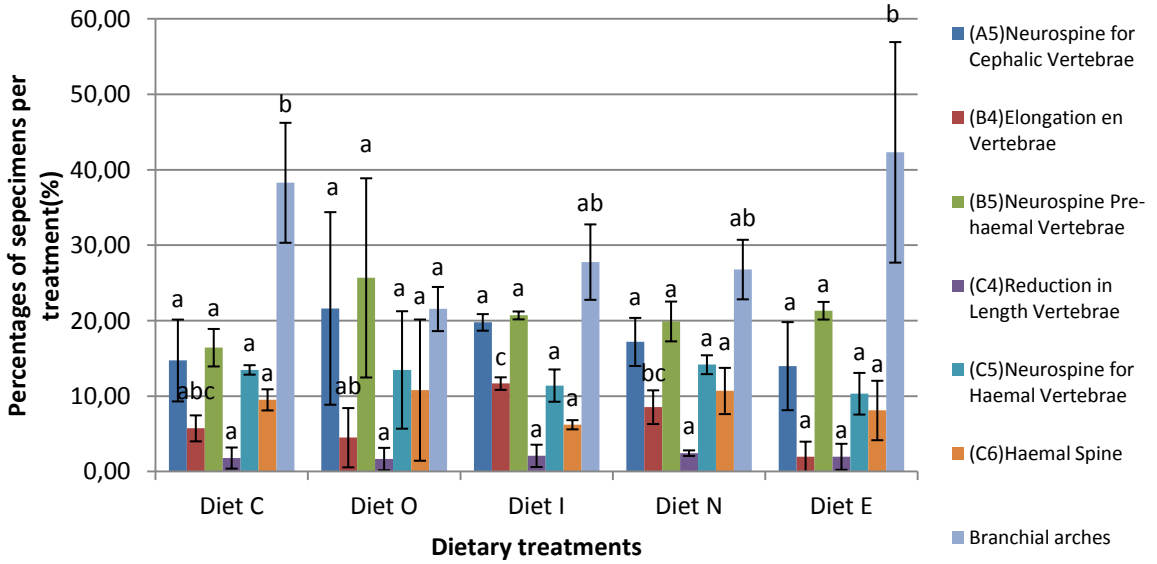
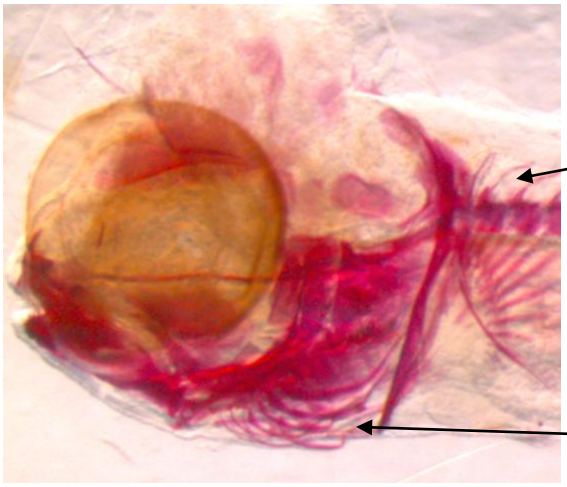
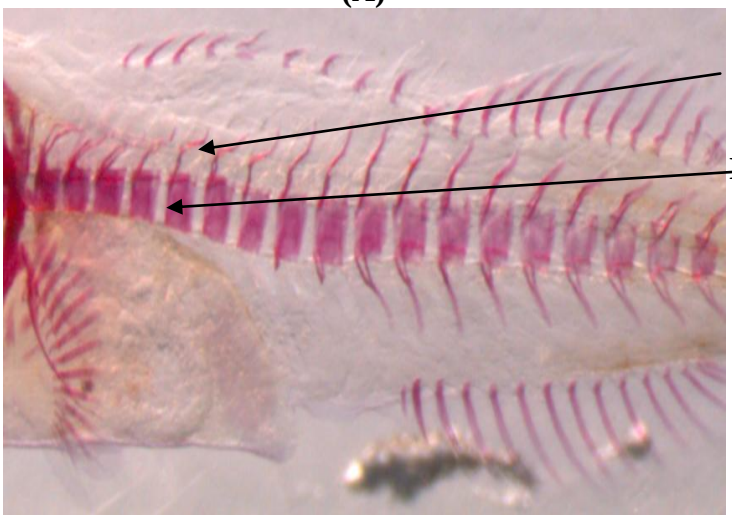


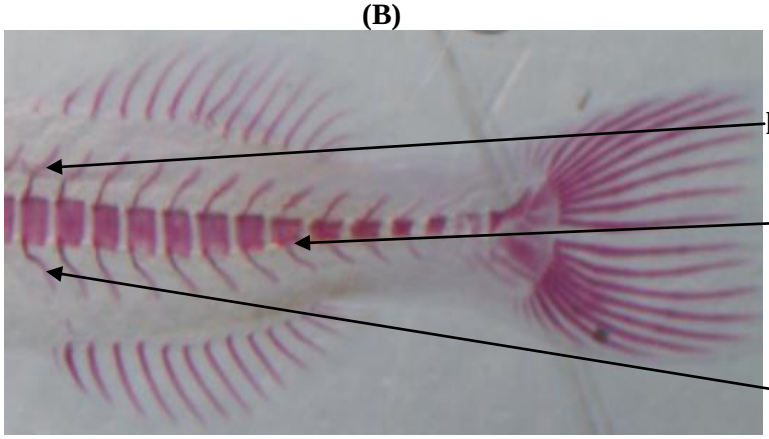
Figure 23: Incidence of specific anomalies in 44 dah larvae fed different mineral sources. Values (mean ± standard deviation) with the same letters for the same body region are not significantly different (P<0.05).



(A)



(B)



(C)

Figure 24: Characteristic skeletal anomalies found in sea bream larvae: Branchiostegal ray and neurospine anomalies for cephalic vertebrae (A); Neurospine anomalies and elongation in pre-haemal vertebrae (B); Neurospine and haemal spine anomalies and

reduction in haemal vertebrae length (C). Treatment: DH. Alizarin red staining; Magnification 5X.

Total lipid content of sea bream larvae showed significantly higher values in those fed diets O, C and I (19.60%, 16.93% and 15.69% respectively), than in fish fed nanometals and encapsulated minerals with 13.47% and 13.31%, respectively (Table XII; Figure 25). A significant correlation was found between larval total lipid contents and dry weight (P=0.070; P<0.1; Figure 26).

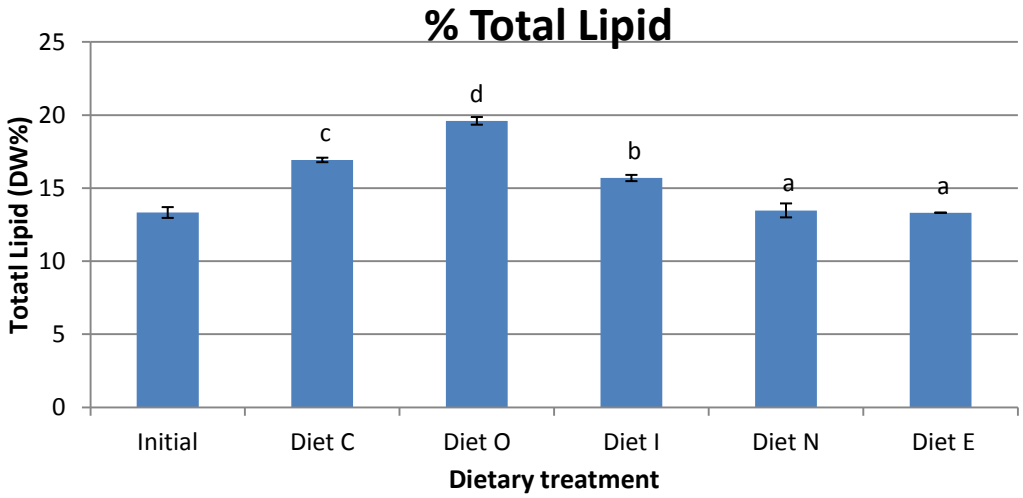


Figure 25: Total lipid (%DW) of gilthead sea bream larvae (44 dah) following 24 days of feeding the experimental diets containing different types of minerals. Values (mean ± standard deviation) with the same letters are not significantly different (P<0.05).

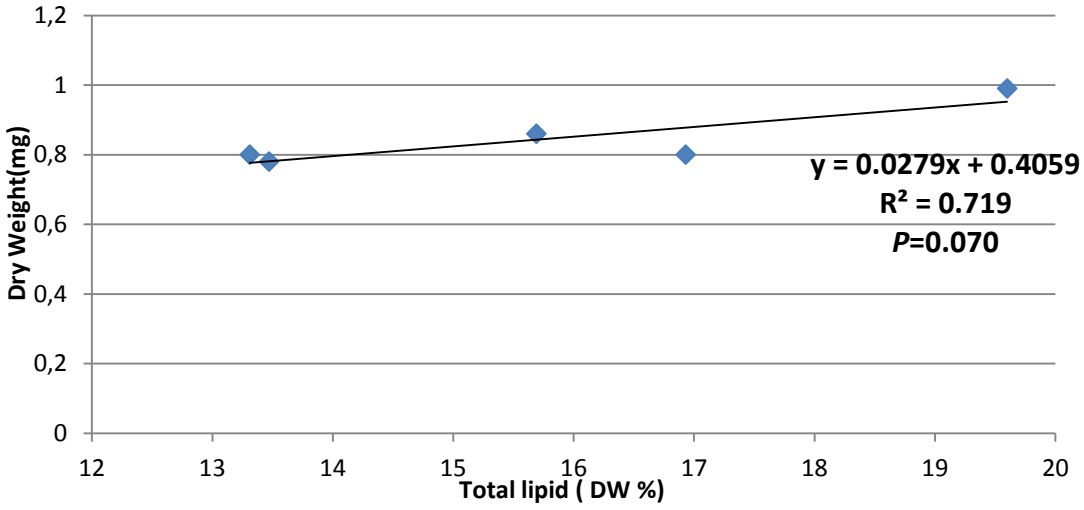


Figure 26: Correlation between larval dry body weight and total lipid content of 44 dah larvae fed different mineral sources (P<0.1).

RESULTS

All diets were isoproteic, isoenergetic, isolipidic. Diets fatty acids compositions were similar in saturated and monounsaturated content, as well as in polyunsaturated fatty acids contents (Table XI). Total lipid content of sea bream larvae did not vary among dietary treatments; moreover, fatty acid composition of the larvae generally reflected the fatty acid composition of the diet (Table XII).

RESULTS

Table XI Fatty acid composition (% total identified fatty acids) of the experimental diets

	Diet C	Diet O	Diet I	Diet N	Diet E
14:0	9.38	9.94	9.50	9.58	9.28
14:1n-5	0.18	0.22	0.19	0.19	0.18
14:1n-7	0.16	0.16	0.15	0.15	0.16
15:0	0.37	0.40	0.39	0.39	0.36
15:1n-5	0.07	0.07	0.07	0.07	0.07
16:0iso	0.06	0.06	0.06	0.05	0.06
16:0	22.62	24.32	23.50	23.68	22.24
16:1 n-7	5.83	6.00	5.90	5.92	5.76
16:1n-5	0.23	0.24	0.24	0.24	0.23
16:2n-6	0.49	0.50	0.49	0.50	0.50
16:2n-4	1.24	1.13	1.16	1.22	1.17
17:0	0.17	0.16	0.17	0.16	0.17
16:3n-4	0.16	0.16	0.16	0.16	0.15
16:3n-3	0.28	0.30	0.29	0.29	0.28
16:3n-1	0.28	0.27	0.26	0.25	0.29
16:4n-3	0.64	0.56	0.60	0.58	0.67
16:4 n-1	0.03	0.03	0.02	0.03	0.03
18:0	2.71	3.05	3.16	3.10	2.73
18:1 n-9	10.18	10.76	10.19	10.28	9.96
18:1 n-7	5.85	5.99	5.86	5.91	5.74
18:1 n-5	0.28	0.28	0.27	0.28	0.27
18:2n-9	0.06	0.05	0.05	0.06	0.06
18:2 n-6	1.43	1.51	1.38	1.41	1.43
18:2n-4	0.06	0.06	0.06	0.06	0.06
18: 3n-6	0.21	0.21	0.21	0.21	0.21
18:3n-4	0.05	0.04	0.05	0.05	0.05
18:3 n-3	0.99	0.96	0.93	0.93	0.99
18:3n-1	0.01	0.01	0.01	0.01	0.01
18:4 n-3	2.80	2.43	2.52	2.49	2.89
18:4 n-1	0.04	0.04	0.04	0.04	0.05
20:0	0.11	0.12	0.12	0.12	0.12
20:1 n-9	0.12	0.13	0.13	0.13	0.14
20: 1n-7	2.68	2.91	3.02	3.04	2.65
20: 1n-5	0.32	0.33	0.32	0.33	0.32
20: 2n-9	0.01	0.01	0.01	0.01	0.01
20:2 n-6	0.12	0.13	0.14	0.13	0.12
20:3n-9+n-	0.03	0.03	0.03	0.03	0.03
20:3 n-6	0.04	0.04	0.04	0.04	0.04
20:4 n-6	0.66	0.67	0.71	0.70	0.68
20: 3n-3	0.24	0.25	0.28	0.27	0.25
20:4 n-3	0.30	0.27	0.28	0.27	0.30
20:5 n-3	14.19	12.40	13.06	12.95	14.50
22:1 n-11	0.87	0.88	0.85	0.85	0.84
22:1 n-9	0.16	0.17	0.16	0.16	0.15
22:4 n-6	0.10	0.10	0.10	0.10	0.10
22:5 n-6	0.14	0.14	0.16	0.15	0.14
22:5 n-3	0.41	0.37	0.39	0.38	0.41
22:6 n-3	12.65	11.14	12.33	12.02	13.20
Total Saturados	35.37	37.99	36.84	37.03	34.90
Total Monoenoicos	26.91	28.14	27.36	27.57	26.45
Total n-3	32.51	28.69	30.68	30.20	33.48
Total n-6	3.19	3.31	3.23	3.24	3.21
Total n-9	10.55	11.14	10.56	10.67	10.34
Total n-3HUFA	27.79	24.44	26.34	25.91	28.66
EPA/ARA	21.35	18.41	18.41	18.57	21.33
ARA/EPA	0.05	0.05	0.05	0.05	0.05
DHA/EPA	0.89	0.90	0.94	0.93	0.91
DHA/ARA	19.03	16.54	17.38	17.23	19.42
Oleico/DHA	0.80	0.97	0.83	0.85	0.75
Oleico/n-3HUFA	0.37	0.44	0.39	0.40	0.35
n-3/n-6	10.18	8.67	9.51	9.31	10.42
EPA/ARA	21.35	18.41	18.41	18.57	21.33
ARA/EPA	0.05	0.05	0.05	0.05	0.05

RESULTS

Table XII Total lipid level (%DW) and fatty acid composition (% total identified fatty acid) from sea bream larvae at 20 dah and after 24 days of feeding the experimental diets (44dah)

	Initial larvae	Diet C	Diet O	Diet I	Diet N	Diet E
Total Lipid (%DW)	13.33± 0.37	16.93± 0.15^{c}	19.60 ±0.26^{d}	15.69± 0.21^{b}	13.47 ± 0.48^{a}	13.31± 0.01^{a}
14:0	0.90	2.96	2.69	2.38	2.88	2.86
14:1n-5	0.23	0.07	0.10	0.08	0.08	0.07
14:1n-7	0.01	0.03	0.04	0.03	0.03	0.03
15:0	0.62	0.31	0.36	0.34	0.34	0.30
15:1n-5	0.04	0.03	0.03	0.03	0.03	0.03
16:0iso	0.21	0.05	0.06	0.05	0.05	0.05
16:0	18.27	24.20	24.66	24.92	24.67	23.71
16:1 n-7	3.36	2.96	2.99	2.70	2.98	2.86
16:1n-5	0.46	0.18	0.19	0.17	0.19	0.18
16:2n-6	0.04	0.13	0.11	0.09	0.12	0.12
16:2n-4	1.19	2.33	1.99	2.14	2.05	2.34
17:0	0.27	0.07	0.06	0.08	0.08	0.07
16:3n-4	0.38	0.19	0.25	0.22	0.22	0.19
16:3n-3	0.11	0.22	0.21	0.21	0.22	0.22
16:3n-1	1.81	0.72	0.80	0.97	0.80	0.69
16:4n-3	0.37	0.35	0.38	0.38	0.37	0.36
16:4 n-1	0.17	0.18	0.21	0.22	0.20	0.18
18:0	11.62	7.83	9.03	9.03	8.11	7.63
18:1 n-9	12.69	10.74	14.19	11.54	11.45	10.79
18:1 n-7	3.17	4.51	4.30	4.16	4.50	4.48
18:1 n-5	0.32	0.23	0.24	0.23	0.25	0.22
18:2n-9	0.79	0.03	0.19	0.12	0.11	0.07
18:2 n-6	8.11	1.07	2.42	1.18	1.15	1.10
18:2n-4	0.05	0.08	0.08	0.07	0.08	0.08
18: 3n-6	0.37	0.09	0.34	0.10	0.10	0.08
18:3n-4	0.12	0.08	0.08	0.09	0.08	0.08
18:3 n-3	0.92	0.40	0.49	0.32	0.38	0.40
18:3n-1	0.00	0.00	0.00	0.00	0.00	0.00
18:4 n-3	0.08	0.80	0.52	0.48	0.68	0.79
18:4 n-1	0.04	0.02	0.01	0.03	0.01	0.01
20:0	0.27	0.24	0.29	0.28	0.25	0.24
20:1 n-9	0.26	0.06	0.09	0.06	0.07	0.06
20: 1n-7	1.08	1.53	1.64	1.37	1.58	1.48
20: 1n-5	0.38	0.26	0.27	0.24	0.25	0.25
20: 2n-9	0.24	0.01	0.03	0.02	0.02	0.01
20:2 n-6	0.61	0.12	0.24	0.15	0.14	0.12
20:3n-9+n-	n.d	0.01	0.01	0.01	0.01	0.01
20:3 n-6	0.58	0.07	0.08	0.08	0.07	0.07
20:4 n-6	4.53	1.47	1.57	1.78	1.57	1.53
20: 3n-3	0.25	0.18	0.24	0.18	0.20	0.18
20:4 n-3	0.37	0.33	0.28	0.32	0.32	0.30
20:5 n-3	4.29	10.41	8.41	9.61	10.53	10.71
22:1 n-11	0.28	0.14	0.12	0.08	0.14	0.12
22:1 n-9	0.40	0.74	0.84	0.70	0.74	0.79
22:4 n-6	0.42	0.13	0.14	0.14	0.13	0.14
22:5 n-6	0.59	0.26	0.26	0.30	0.27	0.27
22:5 n-3	1.93	1.09	0.92	0.99	0.97	1.08
22:6 n-3	16.80	22.08	17.55	21.37	20.52	22.61
Total Saturados	31.95	35.61	37.11	37.02	36.33	34.82
Total Monoenoicos	22.66	21.49	25.03	21.38	22.28	21.37
Total n-3	25.13	35.86	28.99	33.85	34.20	36.64
Total n-6	15.26	3.35	5.15	3.80	3.56	3.44
Total n-9	14.38	11.59	15.35	12.44	12.39	11.73
Total n-3HUFA	23.65	34.09	27.40	32.47	32.54	34.88
EPA/ARA	0.95	7.09	5.37	5.40	6.71	6.99
ARA/EPA	1.05	0.14	0.19	0.19	0.15	0.14
DHA/EPA	3.92	2.12	2.09	2.22	1.95	2.11
DHA/ARA	3.71	15.05	11.21	12.02	13.07	14.75
Oleico/DHA	0.76	0.49	0.81	0.54	0.56	0.48
Oleico/n-3HUFA	0.54	0.31	0.52	0.36	0.35	0.31
n-3/n-6	1.65	10.70	5.63	8.90	9.61	10.64

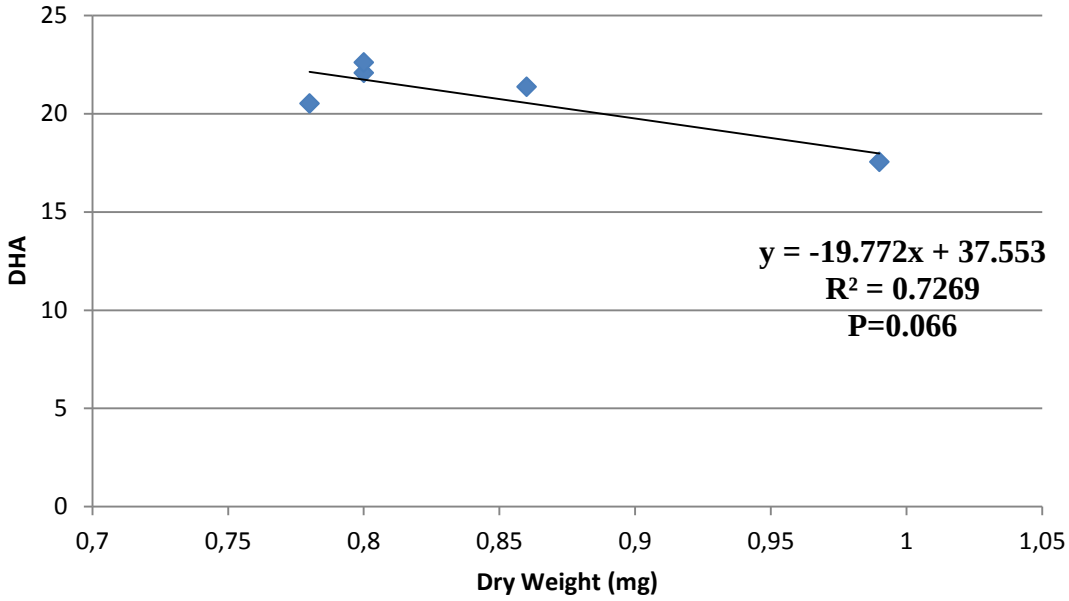


Figure 27: Correlation dry weight and DHA of larvae (44dah). Values (mean ±standard deviation) (P<0.1).

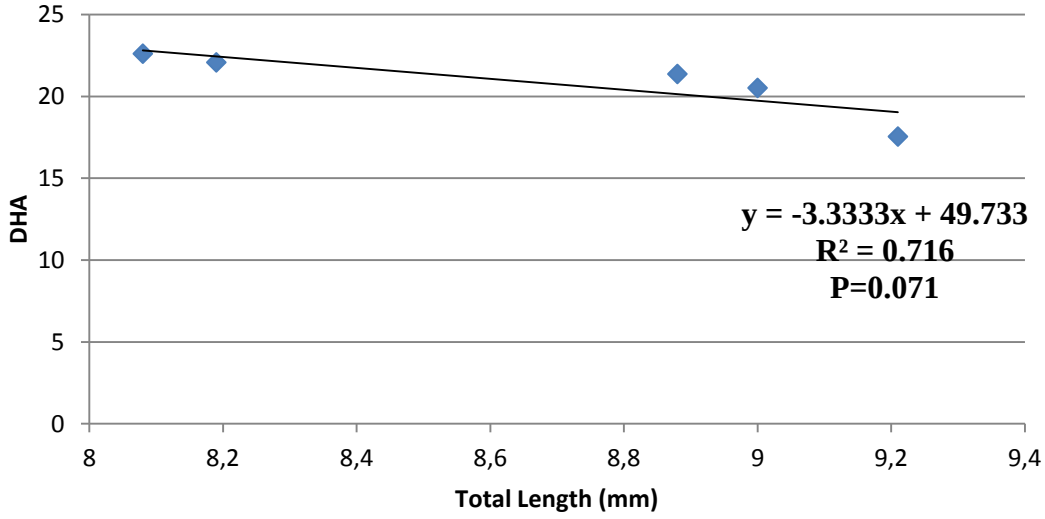


Figure 28: Correlation larval DHA content and the total length of larvae (44 dah). Values (mean ±standard deviation) (P<0.1)

The mRNA expression of Cu-ZnSOD was low in larvae fed diet C, increased by the supplement of minerals in diets, particularly in the inorganic form. Thus, the Cu-ZnSOD mRNA copy number was significantly higher (P <0.05) in larvae fed diet I than in larvae from diet C (Figure 29). No significant correlation was found between the dietary Zn levels and the Cu-ZnSOD gene expression, suggesting a different delivery or absorption rate of this mineral with the different dietary sources provided. Similarly, the

lowest MnSOD expression was detected in larvae fed diet C and the highest in larvae fed diet E, supplemented with encapsulated minerals (Figure 30). No significant correlation was found between the dietary Mn levels and the MnSOD gene expression, suggesting a different delivery or absorption rate of this mineral with the different dietary sources provided.

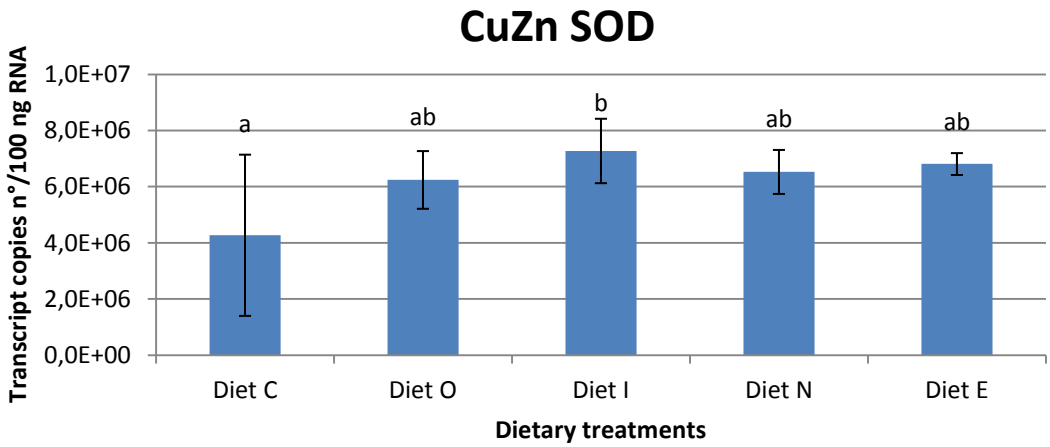


Figure 29: Cu-ZnSOD expression levels measured by real-time PCR in *Sparus aurata* larvae when were fed diets experiments, mRNA copy number was normalized as a ratio to 100 ng total RNA. Values (mean ± standard deviation) with the same letters are not significantly different (P<0.05).

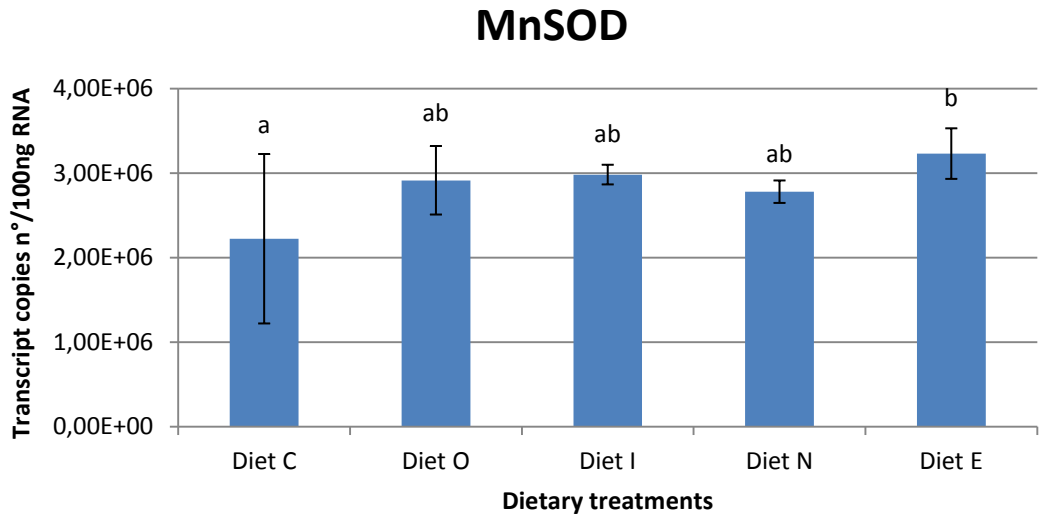


Figure 30: MnSOD expression levels measured by real-time PCR in *Sparus aurata* larvae when were fed diets experiments, mRNA copy number was normalized as a ratio to 100 ng total RNA. Values (mean ± standard deviation) with the same letters are not significantly different (P<0.05).

RESULTS

No significant differences were found in GPx gene expression (Figure 31). Thus, the increase in Se levels from 1.9 mg/kg in diet C without Se supplementation to 4-5 mg/kg in the other diets supplemented with different delivery vectors for Se did not significantly affected GPx expression.

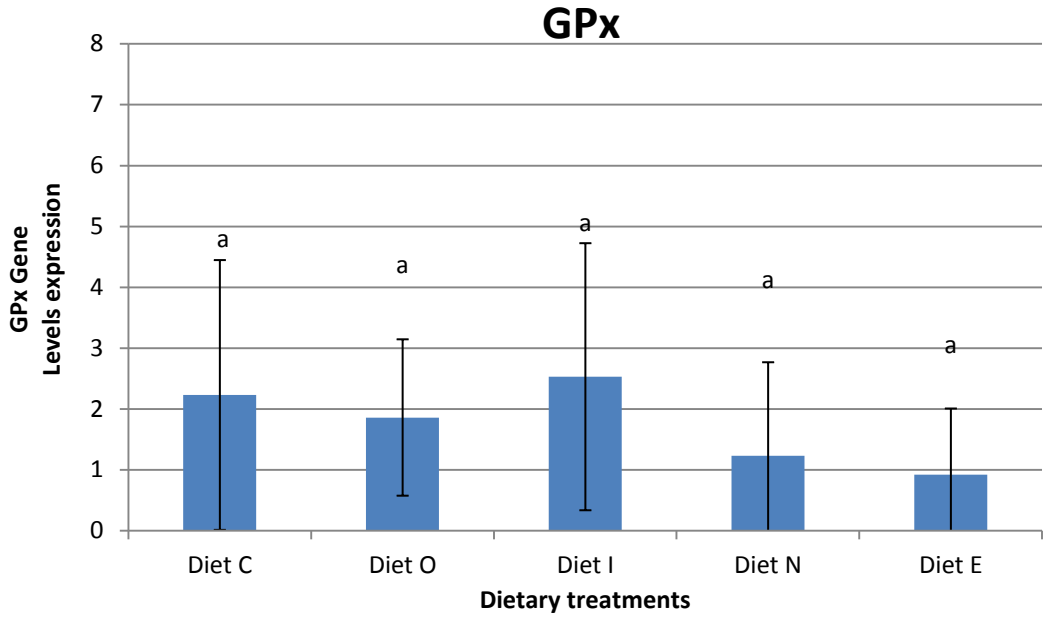


Figure 31: GPx gene expression level measured by real-time PCR in sea bream larvae. Values (mean ±standard deviation) with the same letters are not significantly different ($P<0.05$).

Bone morphogenetic protein 2 (BMP2) expression levels were similar for all the larvae and no statistical differences were observed (Figure 32). Regarding Osteocalcin gene, OC mRNA copy number was significantly ($P<0.05$) higher in larvae fed diets O and N than in larvae fed diets C and E. OC expression was elevated in larvae with higher ossification degree, the highest number of mRNA copies being found in larvae fed diet O (Figure 33). Therefore, a significant correlation was found between OC gene expression and larva ossification degree ($P=0.060$; $P<0.1$; Figure 34).

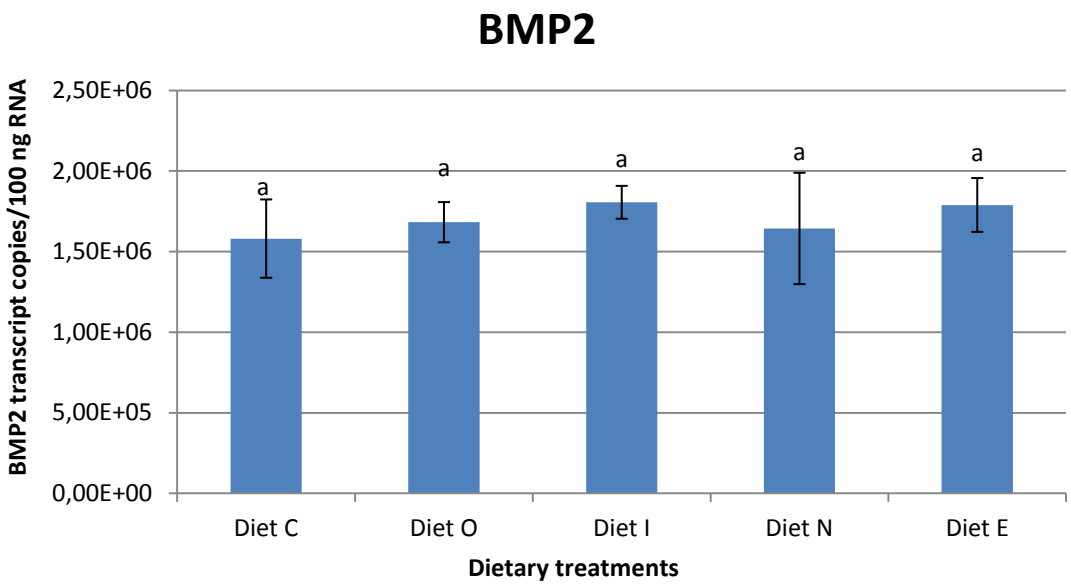


Figure 32: BMP2 expression levels measured by real-time PCR in *Sparus aurata* larvae when were fed diets experiments, mRNA copy number was normalized as a ratio to 100 ng total RNA. Values (mean ± standard deviation) with the same letters are not significantly different (P<0.05).

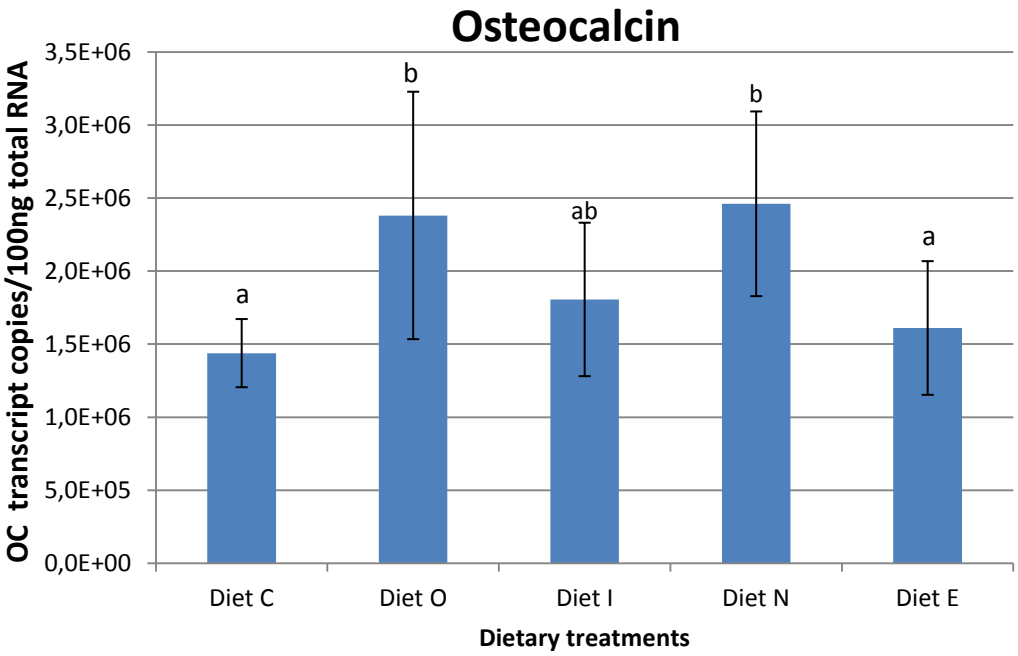


Figure 33: OC expression levels measured by real-time PCR in *Sparus aurata* larvae when were fed diets experiments, mRNA copy number was normalized as a ratio to 100 ng total RNA. Values (mean ± standard deviation) with the same letters are not significantly different (P<0.05).

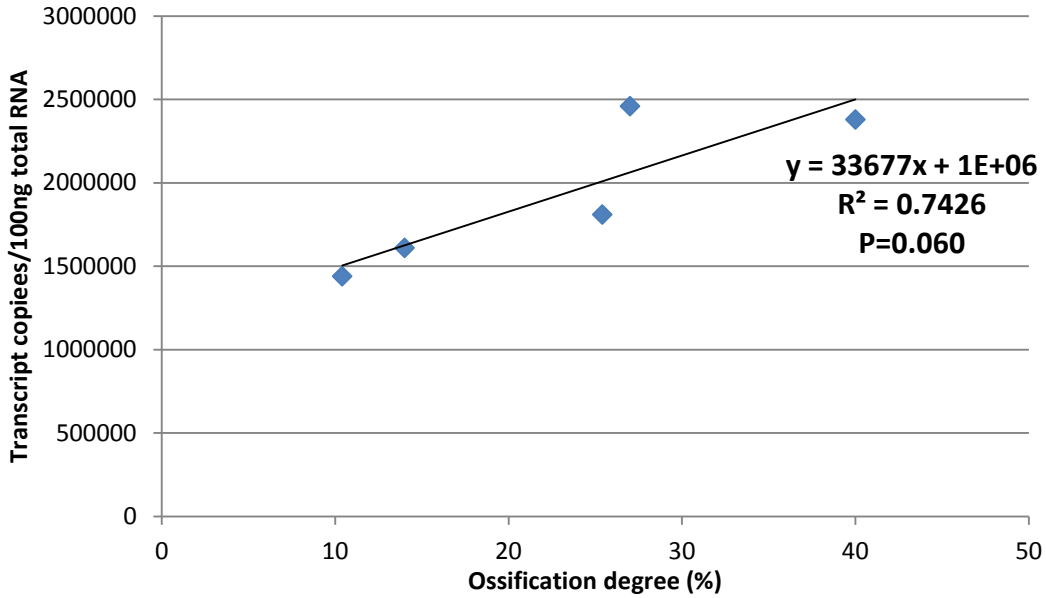


Figure 34: Correlation ossification degree (%) and Osteocalcin expression levels. Values (mean ± standard deviation) (P<0.1).

4. DISCUSSION

There is an almost complete lack of information about the mineral requirements of marine fish larvae (Hamre *et al*., 2013). In order to define the quantitative requirements for minerals in microdiets, it is necessary to determine the most appropriate dietary delivery form to ensure their adequate bioavailability.

Feeding 20-44 dah gilthead sea bream larvae with diets without supplementation of Zn, Mn, Se and Fe lead to poor growth, low bone mineralization and high incidence of anomalies in the branchial arches, together with a down-regulation of OC, Cu-ZnSOD and MnSOD genes, suggesting a deficiency in some of these minerals. The low Se levels did not seem to be the cause for this growth reduction. Despite the addition of Se may increase weight gain and SGR in other fish species (Liu *et al*., 2010; Le and Fotedar, 2013), the lack of significant differences in the expression of GPx gene in the present study suggests that there was not an important effect of supplementation of Se in fish growth regardless the delivery form employed, since the GPx transcript expression level is a sensitive biomarker for Se intake (Betancor *et al*., 2012; Pacitti *et al*., 2013; Saleh *et al*., 2014). Moreover, in previous studies with similar conditions to the present trial, elevation of Se levels from 2 to 6 mg/kg did not affected gilthead sea bream larvae growth (Saleh *et al*., 2014). Regarding the Fe levels, in gilthead sea bream juveniles (Dominguez *et al*., in press) supplementation of Fe with the same delivery vectors than in the present study, despite markedly altering haematocrit, did not affected fish growth. Similarly, growth reduction in fish fed non-supplemented diet could be related to the lack of minerals other than Fe. Indeed, the lack of either Zn or Mn has been found to reduce growth in fish (Liu *et al*., 2013) and alter bone formation (Satoh *et al*., 1992; Watanabe *et al*., 1997), in agreement with the lower OC expression obtained in larval sea bream fed non-supplemented diets in the present study. Moreover, the down-regulation of Cu-ZnSOD and MnSOD could be indicating a deficiency in these minerals. Therefore, the lack of both Zn and Mn could be the main reason for the poor growth and particularly low mineralization and increased deformities in the larvae fed the non-supplemented diet.

Inclusion of the target minerals in an encapsulated inorganic form did not improved growth, mineralization or anomalies deficiencies, suggesting the low availability of these minerals if they are encapsulated. Nevertheless, expression of MnSOD was

significantly higher in larvae fed the encapsulated minerals, suggesting and improve availability of Mn by this delivery vector. These results are in agreement with the lower MnSOD activities in cardiac muscle and liver found in rainbow trout fed low Mn levels (Knox *et al*., 1981).

The inclusion of Zn, Mn, Se and Fe in an organic form (amino acid-chelate) as a source of trace elements for *Sparus aurata* larvae promoted maximum growth, increased larval lipid reserves, enhanced early mineralization and prevented branchial arches deformities. On the contrary, feeding larval sea bream with inorganic forms of these minerals was less effective than organic minerals in improving larval weight or bone mineralization in comparison to the non-supplemented diet. Moreover, in these larvae the lipid content was even lower than in the non-supplemented fish, as well as resistance to stress, and fish showed even higher bone anomalies in the pre-hemal region. In other vertebrates, the use of chelate trace elements has resulted in improved growth (Hill *et al*., 1986; Wekedekind and Baker 1989; Wedekind *et al*., 1992; Paripatananont and Lovell, 1995; Puchala *et al*., 1999; Tan and Mai, 2001; Apines *et al*., 2003). Amino acid chelates of cooper, iron, manganese, and zinc have been shown to have higher bioavailability for chicks, rats, sheep, pigs and cattle (Ammerman *et al*., 1995). Micronutrients, particularly Zn and Mn are known to be essential for growth both animals and humans (Burch *et al*., 1975; Castillo-Duran and Uauy, 1988; Watanabe, 1988; Dorup *et al*., 1991; Ninh *et al*., 1995, 1996; Semrad, 1999). Thus, improved growth in larvae fed organic minerals could be associated to a better utilization of organic Zn or Mn, since both minerals have been found to positively affect these parameters in fish (Watanabe *et al*., 1997; Satoh *et al*., 2001; Lin *et al*., 2008; Liu *et al*., 2013). Moreover, Mn chelated with amino-acid complexes induces a higher growth rate for rainbow trout than the inorganic manganese sulfate (Satoh *et al*., 2001; Apines *et al*., 2003). Besides, found the bioavailability of organic chelated zinc for growth of channel catfish is 3 times higher than that of inorganic zinc sulfate (Paripatananont and Lovell, 1995) and shows a higher digestibility (Paripatananont and Lovell, 1997). Studies with mammals have shown that chelation of minerals to amino acids may increase their absorption rate in the intestine (Ashmead, 1992), although other factors may be also responsible for the higher bioavailability of organic trace elements compared to others forms (Wang and Lovell, 1997). The increased mineralization and reduced bone anomalies found in sea bream fen organic minerals could be also related to an increase efficiency of organic Mn and Zn, since both minerals are known to affect those

parameters (Lall, 2002). Moreover, Zn and Mn may promote normal skeletal development red sea bream larvae (Nguyen *et al*., 2008). Thus, bone Mn concentration increases with dietary Mn in channel catfish (Gatlin and Wilson, 1984b), in grass carp (Wang and Zhao, 1994), Atlantic salmon (Lorentzen *et al*., 1996; Maage *et al*., 2000) and gibel carp (Pan *et al*., 2008). In this sense, a stimulatory of Zn on bone formation and mineralization in vitro and in vivo has been reported (Yamaguchi *et al*., 1987). The possible mechanism by which Zn stimulates bone growth is that Zn directly activates aminoacyl-tRNA synthetase in osteoblastic cells and it stimulates cellular protein synthesis (Yamaguchi, 1998). Moreover, Zn inhibits osteoclastic bon re-sorption by inhibiting osteoclast-like cell formation from marrow cells (Yamaguchi, 1998). Zinc can influence bone mineralization either directly, as divalent cation acting on nucleation and mineral accumulation, or indirectly, as a co-factor of enzymes involved in the process like alkaline phosphatase (Gomez *et al*., 1999). A clear correlation between expression osteocalcin gene and the mineralization degree was observed in bone mineralization of larvae sea bream. In humans, the role of Zn in bone formation involves the activation of bone alkaline phosphatase, osteoblast tyrosine kinase and RNA synthetase (Yamaguchi and Hashizime, 1994). An in vitro study in rats demonstrated that Zn increased the protein components in the femoral-diaphyseal and metaphyseal tissues, and enhanced bone growth in collaboration with insulin-like growth factor I (Ma and Yamaguchi, 2001).

Regarding the reduction in the occurrence of branchial arches anomalies, a negative significant correlation was found with expression of GPx gene, which was the lowest in fish fed organic Se. In fish, bone can develop directly into bone (intramembranous bone) or from cartilaginous precursors (cartilage replacement bone) (Kohno and Taki, 1983) as branchial arches. Indeed, Se deficiency has been found to cause osteochondropathy, damage to this type of bones, in other vertebrates. Different studies suggest that skeletal anomalies develop at the time of chondrogenesis and osteogenesis at early larval stages and that may manifest late in development (Faustino, 2002; Grotmol *et al*., 2005; Imsland *et al*., 2006). In addition, Betancor *et al.* (2012) demonstrated that organic selenium had some protective effect on osteoblasts, promoting bone mineralization in larvae sea bass. Indeed, inclusion of selenium seemed to help to reduce the incidence of skull deformities (cartilage replacement bone as branchial arches) in sea bass larvae (Betancor *et al*., 2012). Besides, increased dietary organic Se levels led to promoted expression of bone formation and mineralization

genes (Saleh *et al*., 2014). Therefore, organic Se could be more effective in organic than inorganic forms to prevent these type of anomalies.

Feeding inorganic minerals significantly increased Cu-ZnSOD in comparison to fish fed non-supplemented diet. Cu-ZnSOD, an antioxidant enzyme, is a sensitive marker of Cu status (Gatlin and Wilson, 1986b; Harris, 1992; Hari *et al*., 1998). Nevertheless, in some studies Zn levels did not cause any significant alteration in the activities of antioxidant enzymes such as SOD (Pathak *et al*., 2002). Cu-ZnSOD is a product of the SOD1 gene and is largely cytosolic but based on studies in yeasts; it seems to protect both the cytosolic and mitochondrial components from oxidative damage (Gralla and Kosman, 1992; Sturtuz *et al*., 2001). In the present study, the expression of Cu-ZnSOD in fish fed inorganic minerals was not significantly different than in those fed organic Zn, despite in previous studies expression of was also higher when Zn was chelated (Apines *et al*., 2003).

Addition of Zn, Mn, Se and Fe in the form of nanometals did not enhanced growth, larval lipid contents and did not prevented branchial arches deformities, in comparison to the diet without supplementation of these minerals. However, it improved stress resistance, mineralization and OC expression. Previous studies have pointed out the interest of nano-selenium for its high bioavailability and low toxicity, due to its high effectiveness to prevent oxidative stress (Wang *et al*., 2013). Therefore, the significantly higher stress resistance found in sea bream fed nanometals in comparison to inorganic minerals could be related with a higher efficiency of nano-Se than inorganic Se in the stress prevention. Moreover, this hypothesis agrees well with the better mineralization found in these fish and the role of Se as discussed above.

In general, addition of minerals in the form of organic, inorganic or nanometals forms markedly reduced fish survival, whereas encapsulated minerals only caused slight mortalities. These results suggest the toxic effect of one or more of these minerals. These mortalities could not be related to the Se levels since the elevation of Se from 2 to 6 mg/kg did not affected larval sea bream survival in our previous studies with very similar conditions to the present trial (Saleh *et al*., 2014). Besides, the reduction in growth rate is the most sensitive indicator of chronic selenosis in vertebrates (Goehring *et al*., 1984; Hamilton *et al*., 1990) whereas in the present study, good growth was observed in fish with high mortalities associated to mineral supplementation. Therefore no evident signs of Se toxicity could be detected in the present study. Since the

mortality was correlated with the dietary Mn or Zn contents, it could be related with any of these minerals.

The toxicity of excessive Mn appears to cause antagonism in mineral metabolism particularly with Fe, P and Ca (McDowell, 2003). For instance, grouper fed 1000 mg kg^{-1} of Mn had significantly lower whole body Fe (Ye *et al*., 2009). Besides, a negative correlation between whole body P and dietary Mn levels has been also (Maage *et al*., 2000). Moreover, whole body contents in Ca, P and Fe are also obtained in fish fed increasing Mn dietary levels (Tan *et al*., 2012). However, no previous studies have been reported about dietary Mn toxicity on fish. In agreement, in the present study, the higher MnSOD gene expression that suggests a better assimilation of Mn was found in fish fed the encapsulated diet and this fish did not showed a significantly lower growth in comparison to the fish fed non-supplemented diet, what suggest that the toxicity was not related to the Mn. Regarding Zn, gilthead sea bream juveniles fed a diet supplement with 900mg Zn kg^{-1} feed had a significantly lower weight than fish fed with a control diet (60.9 mg Zn kg-^{1}); however, no evident sign of toxicity was present, indicating that possible molecular damages could be present at a sub lethal level (Serra *et al*., 1996; Carpenè *et al*., 1999).

Numerous theories have been proposed to explain the differences between the toxicity of natural diets and laboratory-prepared feeds. One explanation is that the metals in natural diets are 'biologically incorporated' into prey organisms in forms that are more easily absorbed by fish and therefore more toxic (Clearwater *et al*., 2002). For example, some of the metals in invertebrates will be bound to proteins or other organic molecules, whereas most laboratory prepared diets are supplemented with metals in an inorganic form. But, the bioavailability of dietborne metals in these forms is unknown. Similarly, there is no evidence that increased dietborne metal bioavailability results in significantly increased toxicity to fish (Clearwater *et al*., 2002).

5. CONCLUSIONS

Along the studies in this thesis the following general conclusions could be drawn:

1. It is necessary to supplement weaning diets for gilthead sea bream larvae with Se and Fe, and particularly with Zn and Mn, to avoid poor growth, low bone mineralization and high incidence of anomalies in the branchial arches.
2. Non-supplementation of Zn, Mn, Se and Fe down-regulates the expression of osteocalcin, Cu-ZnSOD and MnSOD genes, denoting a deficiency in some of these minerals.
3. Inclusion of the target minerals in an encapsulated inorganic form did not improved growth, mineralization or anomalies deficiencies, suggesting the low availability of these minerals if they are encapsulated.
4. The inclusion of Zn, Mn, Se and Fe in an organic form (amino acid-chelate) as a source of trace elements for *Sparus aurata* larvae promoted maximum growth, increased larval lipid reserves, enhanced early mineralization and prevented branchial arches deformities.
5. The increased mineralization and reduced bone anomalies found in sea bream fed organic minerals could be related to an increase efficiency of organic Mn and Zn. A clear correlation between expression osteocalcin gene and the mineralization degree was observed in bone mineralization of larvae sea bream.
6. Feeding larval sea bream with inorganic forms of these minerals was less effective than organic minerals in improving larval weight or bone mineralization in comparison to the non-supplemented diet.
7. Addition of Zn, Mn, Se and Fe in the form of nanometals did not enhanced growth or larval lipid contents and did not prevented branchial arches deformities, in comparison to the diet without supplementation of these minerals. However, it improved stress resistance, mineralization and OC expression.
8. In general, addition of minerals in the form of organic, inorganic or nanometals forms markedly reduced fish survival, denoting the toxic effect of one or more of these minerals. Encapsulated minerals only caused little mortality in agreement with a lower availability of the minerals.

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