MASTER OFICIAL EN CULTIVOS MARINOS

VI MASTER INTERNACIONAL EN ACUICULTURA

Las Palmas de Gran Canaria, España

2008-2010

Effects of dietary fatty acids on patterns of early mineralisation and bone formation in the axial skeleton of *Sparus aurata* (Linnaeus, 1758)

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TESIS PRESENTADA Y PUBLICAMENTE DEFENDIDA PARA LA OBTENCION DEL TITULO DE MASTER OFICIAL EN CULTIVOS MARINOS

Las Palmas de Gran Canaria
a 20 de septiembre de 2010

MASTER OFICIAL EN CULTIVOS MARINOS

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Organizado conjuntamente por la Universidad de Las Palmas de Gran Canaria (ULPGC), el Instituto Canario de Ciencias Marinas (Gobierno de Canarias) y el Centro Internacional de Altos Estudios Agronómicos Mediterráneos (CIHEAM), a través del Instituto Agronómico Mediterráneo de Zaragoza (IAMZ)

Effects of dietary fatty acids on patterns of early mineralisation and bone formation in the axial skeleton of *Sparus aurata* (Linnaeus, 1758)

MARIA SCOLAMACCHIA

Trabajo realizado en los laboratorios del Instituto Canario de Ciencias Marinas (ICCM) y 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 Lopez (Universidad de Las Palmas de Gran Canaria, España) y del Dr. P. Eckhard Witten (University of Ghent, Belgíca).

Presentado como requisito parcial para la obtención del Título oficial de Máster Universitario en Cultivos Marinos otorgado por la Universidad de Las Palmas de Gran Canaria y del Diploma de Master of Science en Acuicultura otorgado por el Centro Internacional de Altos Estudios Agronómicos Mediterráneos (CIHEAM).

[…] A los estudiantes y estudiantas queremos pedirles que estudien y luchen. Que sin dejar de luchar terminen sus estudios, que se vayan de la universidad. que no se queden en ella. Que la universidad, con todo y ser universal, es limitada, que allá afuera hay también otro universo y son necesarios y necesarias ahí para que luchen ahí. Que allá afuera estamos nosotros y muchos otros como nosotros. Que con nosotros tienen un lugar y no un número. Que no hagan de la juventud que tienen el pretexto para intentar hegemonizar y homogeneizar al otro alumno, al otro profesor, al otro trabajador, al otro diferente.

A los profesores y profesoras, a los investigadores e investigadoras:

Les queremos pedir que enseñen a aprender. Que vean y enseñen a ver todo, incluyéndonos a nosotros, con espíritu crítico y científico. Que enseñen y se enseñen a ver al otro, porqué verlo es respetarlo, y respetar al otro es respetarse a uno mismo. Que no permitan que su trabajo de docencia e investigación sea tasado según la lógica mercantil, donde importa el volumen de cuartillas y no los conocimientos que se producen, donde sólo vale la firma al pie del desplegado en apoyo al señor rector, donde el criterio para que un proyecto tenga presupuesto es el número de horas invertido en audiencias y cortejos a funcionarios grises y analfabetas. Que no hagan del saber un poder que pretenda hegemonizar y homogeneizar al otro profesor, al otro investigador, al otro alumno, al otro trabajador [...].

Desde el discurso del Sub Comandante Marcos a la UNAM

21 MARZO 2001 –Mexico D.F. - Mexico

A Pierluigi, Pino y Maria Teresa
Mis primeros maestros

AGRADECIMIENTOS

Tengo que agradecer mucha gente que ha permitido de una manera u otra al desarrollo de este trabajo y mi formación.

Gracias primero a la doctora Marisol Izquierdo, que me ha permitido desarrollar este trabajo, para sus miles de ideas y para empujarme siempre a buscar soluciones independientes. Al Prof. Eckhard Witten para el material bibliográfico suministrado y todas las sugerencias de gran apoyo en tema de huesos. Gracias a Juan Socorro para la introducción a la "mística" tinción de huesos y a Javi Roo para el material biológico, los consejos y el asesoramiento a lo largo de todo mi cultivo larvario (que bueno es el ruido de la bomba que arranca eh!?). Gracias a Mª José Caballero, para los consejos en los temas de histología, en principio totalmente obsuros para mi. Gracias a Domi (¡y a su mamá!) para los cinnamon buns fuente inagotable de azúcar en las noches de tinción. Gracias a los maestros que se alternaron a lo largo de nuestras clases teóricas.

Muchísimas "thankyous" a todos los becarios (¡precarios!) que trabajan en el ICCM y en el IUSA, para la ayuda práctica y moral que me dieron en estos meses: Mónica, Silvia, Alex, Tibi, Vane, Lahousseine, Moha, Davinia, Fefi, Juanito, Bea, Besay, Fran, Valentina, Lore, Lita…ustedes me hacen creer que la ciencia tiene un futuro y cuanto es fundamental la unión del grupo.

Mil gracias a los equipazos técnicos de la nave del ICCM y del IUSA que nunca me hicieron faltar sonrisas, ayuda incondicionada tabaco y café! Ada, Dailos, Rubén, Lorena, Yaiza, Desi y Desi, Johnny, Eyad, Emma, Ruth y Guaci, Carmen, Yure y Silvia.
Gracias a los "Carontes" del ICCM: Martín, Cristina, Gera, Jose para acogerme también en los horarios mas improbables. Gracias a Teri, Eulogia y María para la compañía en las tardes al microscopio y para las miradas cariñosas mientras ensuciaba el suelo.

Mil gracias a mis compañeros de piso "ampliado": Marko, Pico, Bruno, Rayco, Diogo y Catarina para las cenas a la luz de la farola de Calle Ercilla.
Gracias a todos mis compañeros de master: Esther, Nati, Dailos, Antonio, Islam, Filiz, Alex, Mohamed, para permitirme de conocer miles lugares del mundo sin moverme de Canarias (!!), y en particular a Fati mi primer contacto en Canarias (sin ti no hay ni sol ni madrugada!!!...y sobre todo sin ti no hubiera sido capaz ni de tomar una guagua!!!) . Gracias a mi compi de piso el "DUDE" para soportarme en cualquier condición y ser el mejor ejemplo de amistad y optimismo.

Gracias al Presidente de mi región Nichi Vendola para seguir financiando la movilidad de los jóvenes estudiantes por lo medio del proyecto Bollenti Spiriti que también financió parte de mi estancia aquí en el extranjero.

Gracias a mi familia, en cualquier lugar del mundo se encuentre ahora.

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ABBREVIATIONS

2-ODDs: di-oxygenase enzyme family.

A: *Artemia* sp.

AA: ascorbic acid (vitamin C).

AAs: aminoacids.

A_0: Instar I (nauplius) stage of *Artemia* sp.

A_1: Instar II (metanauplius) stage of *Artemia* sp.

Adm.: administration (regarding live food administration to the larvae).

a.h.: anterior height. Refers to the height of vertebral bodies.

ANOVA: Analysis of variance.

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

B.C.: Before Christ.

BHT: butylated hydroxytoluene, an industrial antioxidant.

BMP-4: bone morphogenic protein 4.

Cell.: cells of phytoplankton.

dah: days after hatching.

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

DH: DHA high treatment.

DHE: DHA high treatment supplemented with vitamin E up to 15000 mg kg^{-1} of the original product.

DL: DHA low treatment.

D:L: dark:light, indicate the hours of light and dark during larval rearing.

DM: DHA medium treatment.

DME: DHA medium treatment supplemented with vitamin E up to 15000 mg kg^{-1} of the original product.

DW: dry weight.

EFA: essential fatty acids.

e.g.: *exempli gratia* (for example).

EPA: eicosapentaenoic acid.

FAAs: free aminoacids.

FAMEs: fatty acids methyl esters.

FAO: Food and Agriculture Organization.

GC: gas chromatography.

Gla: γ-carboxiglutamate.

Hox: homebox genes family.

HPLC: high pressure liquid chromatography.

HUFAs: highly unsatured fatty acids.

ICCM: Instituto Canario de Ciencias Marinas.

Ins (1, 4, 5) P3: D-myo-inositol 1,4,5 triphosphate.

LSK: lordosis-scoliosis-khyphosis

MDA: Malonaldhehyde.

n-3 HUFA: highly unsatured fatty acids of n-3 series (20 or more carbon atoms).

P: phosphorous.

Pax: paired box genes.

PCR: principal caudal fin rays.

PE: phosphatidylethanolamine.

PGE_2: prostaglandine E_2.

p.h.: posterior height. It refers to the height of vertebral bodies.

PI: phosphatidylinositol.

PL: phospholipids.

PPAR-γ: proliferator peroxisome activated receptors.

PUFAs: polyunsatured fatty acids.

RARs: retinoic acid receptors.

ROS: reactive species of oxygen.

Rot.: rotifers.

RXRs: retinoid X receptors.

SCR: secundary caudal fin rays.

s.d.: standard deviation.

Shh: sonic hedgehog gene family

SL: standard length (from the tip of the snout up to the peduncle or, in the case of larvae, up to the point of flexion of the notochord).

Sox: Sry HMG box genes (Sry – High Mobility Group – box).

Svct1: vitamin C transporter (soluble carrier family 23, member 1).

TAG: triacylglicerols.

TBARS: thiobarbituric acid reactive substances.

TFA: total fatty acids.

TL: total length (from the tip of the snout up to the end of the caudal fin).

TRPV6: transient receptor potential cation channel, subfamily V, member 6 (transcellular mediator of calcium uptake from the intestinal lumen).

V.B.: vertebral body.

v.c.: vertebral column.

VDR: vitamin D receptor.

Vit : vitamin.

VM : vitamin mix.

Vs: *versus* (in contrast of).

v/v: volume on volume (mix considering the volumes instead of the weights of the components).

WMS: Whole mount staining. It refers to the staining of entire larvae without processing for microtome cutting.

w/w: weight on weight.

All physical measures were expressed according to the IS (Système International d' Unités).

LIST OF FISH SPECIES CITED

Atlantic salmon – *Salmo salar*

Ayu - *Plecoglossus altivelis*

Barramundi – *Lates calcifer*

Channel catfish – *Ictalurus punctatus*

Cod – *Gadus morhua*

Common carp – *Cyprinus carpio*

Common dentex - *Dentex dentex*

Common pandora – *Pagellus erythrinus*

European sea bass – *Dicentrarchus labrax*

Gilthead sea bream - *Sparus aurata*

Haddock - *Melanogrammus aeglefinus*

Halibut - *Hippoglossus hippoglossus*

Japanese flounder – *Paralichthys olivaceus*

Milkfish – *Chanos chanos*

Rainbow trout – *Oncorhyncus mykiss*

Red porgy – *Pagrus pagrus*

Sharpsnout seabream - *Diplodus puntazzo*

Striped trumpeter – *Latris lineata*

White sea bream – *Diplodus sargus*

Yellowtail flounder – *Limanda ferruginaea*

Zebrafish – *Danio rerio*

RESUMEN

Las deformidades esqueléticas en los peces de cultivo representan unos de los principales problemas que aun ralentizan el desarrollo del sistema acuícola. En particular la nutrición ha sido señalada entre los principales factores que más pueden afectar durante las primeras fases de vida larvaria al desarrollo y la calidad del producto final.

En este trabajo, se testaron diferentes protocolos para la visualización del hueso y el desarrollo osteológico en larvas de dorada. Después, se estudió el efecto de enriquecedores de rotíferos con diferentes niveles de DHA y vit E sobre el desempeño biologico, su composición bioquímica, la presencia de deformidades óseas y la mineralización de la columna.

La doble tinción de huesos y cartílagos se mostró potencialmente dañina en la visualización de los cuerpos vertebrales, mientras que parece no afectar los elementos del cráneo. El patrón de osificación de larvas de doradas criadas en sistema de mesocosmos, fue semejante al descrito anteriormente. En todos los ejemplares examinados, la mayoría de la columna está constituida por vértebras hemales y pre-hemales, pero a medida que la larva se desarrolla, las vértebras craneales y caudales representan un porcentaje creciente de la columna.

La alimentación con diferentes tratamientos pareció no afectar la supervivencia total y después del test de actividad, ni significativamente el crecimiento total en términos de longitud, aunque, deficiencias en DHA implicaron menor peso seco. La composición de los ácidos grasos reflejó el contenido de los rotíferos hasta 15 dah, mientras que su efecto disminuió después del destete.

Altos niveles de DHA sin apropiados niveles de vit E implicaron larvas con un mayor porcentaje de deformidades (10%) en particular de la boca, mientras los

alimentados con niveles comerciales de DHA, con o sin suplemento de vit E, presentaron principalmente anomalías de las espinas neurales o hemales. Bajos niveles de DHA incrementaron la incidencia de cifosis y también un número inferior de vértebras mineralizadas por clase de talla. Los ejemplares alimentados con altos niveles de DHA suplementados con vit E presentaban un desarrollo mas rápido de los elementos esqueléticos.

ABSTRACT

Fish deformities are one of the principal bottlenecks for the expansion of the aquaculture sector. Nutrition has been indicated as one of the principal factors that may affect larval quality up to the final product quality.

In this experimental work staining protocols for bone and cartilage and osteological development of sea bream larvae was carried out. In a second phase, the effect of different levels of DHA and vit E in rotifers were evaluated on the biological performances, the occurrence of skeletal deformities and on the mineralization process on the same species.

Double staining of bone and cartilage resulted potentially harmful for the visualization of the vertebral bodies of sea bream larvae even though it seems to affect less cranial structures. Ossification pattern of larvae bred in a mesocosm system resulted comparable to that reported in literature for this and other Sparids' species. In all specimens, since low SL, haemal and pre-haemal vertebrae constituted the majority of the vertebral column, even though, as the specimen grow, cranial and caudal vertebrae account for an increased percentage of the column.

Feeding sea bream larvae with rotifers with different content of DHA and vit E did not affect overall or after stress survival or growth even though low levels of DHA presented larvae with lower dry weight. Rotifers' fatty acids composition was reflected in larvae up to 15 dah while no significant correlation was found thereafter. High percentages of DHA in rotifers induced a higher percentage of skeletal deformities (10%) in particular regarding the jaw region, while DM and DME groups showed higher percentages of haemal and neural spines defects. Low levels of DHA presented higher presence of khyphosis. This last group

showed also delay in axial skeleton mineralization in opposition to larvae fed high levels of DHA supplemented with vit E.

1. INTRODUCTION

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1.1 AQUACULTURE PRODUCTION

Aquaculture has been the world's fastest growing food production system on the past decades, with a global production that increased from 0.6 millions mT in 1950 by weight, to 65.2 millions mT in 2007 (APROMAR, 2009). In the Mediterranean basin extensive marine fish farms date back to the 6th century B.C. Aquaculture is increasingly replacing fish from fisheries due to declining wild fish stocks and the continuously increasing demand (Figure 1).

This rapid increase in production could be accomplished by the improved control of fish reproduction, the development of optimised diets and technological innovations that allowed the fast development of land- and sea-based aquaculture facilities (UNEP/MAP/MED POL, 2004).

Gilthead sea bream Europe

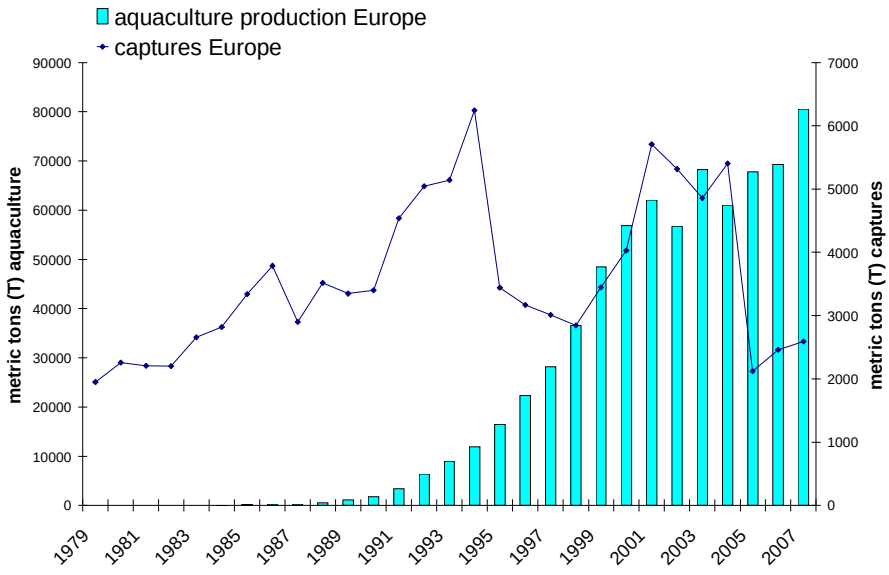


Figure 1 Aquaculture production *versus* captures of gilthead sea bream in Europe from 1979 to 2007 (data elaborated from FAO FISHSTAT Plus).

Notwithstanding the fact that the principal marine finfish species in South Europe aquaculture (bream [*Sparus aurata*] and bass [*Dicentrarchus labrax*]) are being produced in high quantities, various bottlenecks still limit their production, principally linked to the obtention of high quality eggs and juveniles. Besides genetic improvement programs for sea bass and sea bream are scarce at a mass production level and according to Mylonas and Papasolomontos (2008) the reproduction of these species relies significantly on the use of wild broodstock. Marine fish larvae are generally poorly developed at hatching, and undergo important functional and morphological changes during the larval period, such as in visual, nervous, digestive and skeletal systems (Faustino and Power, 1998; Roo *et al.*, 1999; Izquierdo *et al.*, 2000; Benítez-Santana *et al.*, 2007). Therefore, larvae are very sensitive and suffer high mortality rates (>75%) during their early life history. The stress associated to culture conditions is probably related to the lack of knowledge about the specific nutritional requirements and optimal environmental conditions. As a result, fry quality can be poor and larvae may suffer several disorders such as skeletal anomalies or immune suppression (Planas and Cuhna, 1999). Indeed, despite the important advances in the development of inert diets for larvae (Roo, 2009), the production of marine larvae strongly depends on the reproduction of a simplified trophic chain, based on the use of microalgae, rotifers and Artemia. Therefore, parallel to larval production it is required to maintain costly facilities to produce live prey for larval feeding (Roo, 2009). Thus, the improvement of rearing larval efficiency remains to be one of the principal objectives of the aquaculture industry in order to continue to be competitive. Prevention of skeletal malformations in larvae and juvenile fish is an important part of the effort to

improve culture conditions. New research initiatives to the study of this topic have been implemented over the past years. Examples are EU fund projects and research networks like FINEFISH and LARVANET. Fish farmers are developing increased awareness of the consequences of impaired animal welfare not only for ethical issues (animal welfare), but also for utilitarian purposes, since malformed fish are generally not sold to consumers. Downgrading of malformed fish represents a major source of financial losses both, for hatcheries and growers. Stressed, malformed fish consume more food, leading to poor food conversion efficiency and therefore to low production efficiency (Hansen *et al.*, 2009).

1.1.1 GILTHEAD SEA BREAM (*Sparus aurata*, L. 1758) – BIOLOGICAL AND ECOLOGICAL FEATURES

The gilthead sea bream *Sparus aurata* is a perciform fish, belonging to the family *Sparidae* and to the genus *Sparus* (Table I).

Table I Systematic position of *S. aurata* (Fisher *et al.*, 1987)

Kingdom	*Animalia*
Phylum	*Chordata*
Class	*Actinopterigi*
Order	*Perciformes*
Family	*Sparidae*
Genus	*Sparus*
Species	*S. aurata*

Wild populations of gilthead sea bream are common in the Mediterranean (where it is the only species of its genus) and in Atlantic coasts from Great Britain to Senegal, while it is rare in Black sea (Figure 2). Being eurythermal and euryaline, this species can be found both in marine and in brackish waters (estuaries and coastal lagoons), in particular during the early life stages. Born at open sea in wintertime, fingerlings migrate to the coast in early spring in search for food and milder temperatures (trophic migration). Being very sensitive to low temperatures (minimum 4°C), in late autumn they return to open sea where the adults bred (Moretti *et al.*, 1999).

Sea bream is a benthopelagic species that can be found on rocky grounds, sandy or seaweed-covered bottoms, isolated or in small groups. Juveniles can be found at depths of up to 30 m and adults up to 150 m deep. The structure of wild populations remains unclear but there is some evidence for

fragmentation into sub populations, suggesting a genetic isolation of populations in the Western Mediterranean from the populations in the Atlantic Ocean and in the Adriatic Sea (De Innocentis *et al.*, 2004).

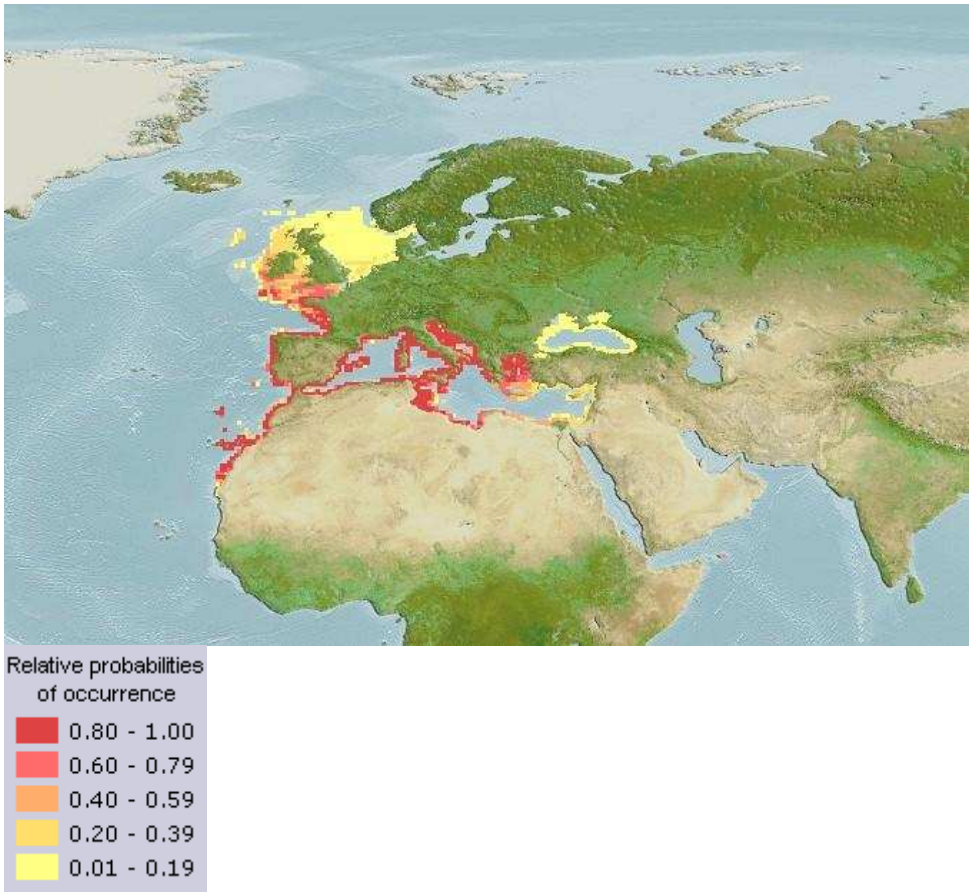


Figure 2 Distribution of gilthead sea bream natural populations (from Froese and Pauly, 2010).

Sea bream is a hermaphroditic protandric species with asynchronous ovarian development. They spawn at 24 hours intervals for up to three months under captivity conditions (Barbaro *et al.*, 1997). The species reproduce in wintertime, generally from October to December. Maturing males under farming conditions can be found after the first year of life. Sexual inversion usually occurs between the second and the third year (about 30 cm total length) depending on the social structure of the population, but it can also occur later (Bruslé-Sicard and Fourcauld, 1997; Kissel *et al.*, 2001). Eggs are

pelagic and translucent with a diameter of about 1 mm, and normally bearing a single oil droplet (Moretti *et al.*, 1999; Fischer *et al.*, 1987).

Adults (Figure 3) have an oval body shape, very high and laterally compressed. The colour is silver grey with a big dark spot at the beginning of the lateral line that cover also the upper part of the opercular bone. A gold and black band is found between the eyes, the dorsal fin is blue-grey with a median black line. The caudal one is grey-greenish white with black tips.

The head profile is convex with small eyes. The cheeks are covered with scales and the pre-opercular bone is scale less. The mouth has the mandible shorter than the upper jaw. Both jaws show canine (4-6) and molariform teeth, in 2-4 series in the upper jaw and 3-4 series, of which 1-2 are notably bigger, in the lower jaw. The dorsal fin has 11 hard and 13 soft rays, while the anal fin has 3 hard and 11-12 soft rays. The pectoral fins are long and pointed, the pelvic are shorter. The axial skeleton of gilthead sea bream generally consists of 23 vertebrae plus the urostyle, 23 neural arches and spines of which 1 is modified (the 23rd) 13 haemal arches and spines of which the 13th is the parahypural (Faustino and Power, 1998). At the dorsal side, three pairs of epineural bones are also present, while in the ventral side from the third vertebra downwards, five pairs of pleural ribs can be recognized. The caudal fin endoskeleton and its osteological development have been described both by Faustino and Power (1998) and by Koumoundourous and collaborators (1997a). When it is completely formed, it normally contains 5 hypurals, and some accessory cartilage elements, 3 epurals and 2 uroneurals. The principal caudal rays (PCR) are 18 (8 supported by hypurals 1 to 2 more 9 supported by hypurals 3 to 5), while the

secondary fin rays (SCR) are 20 (10 in the upper part and 10 in the lower part).

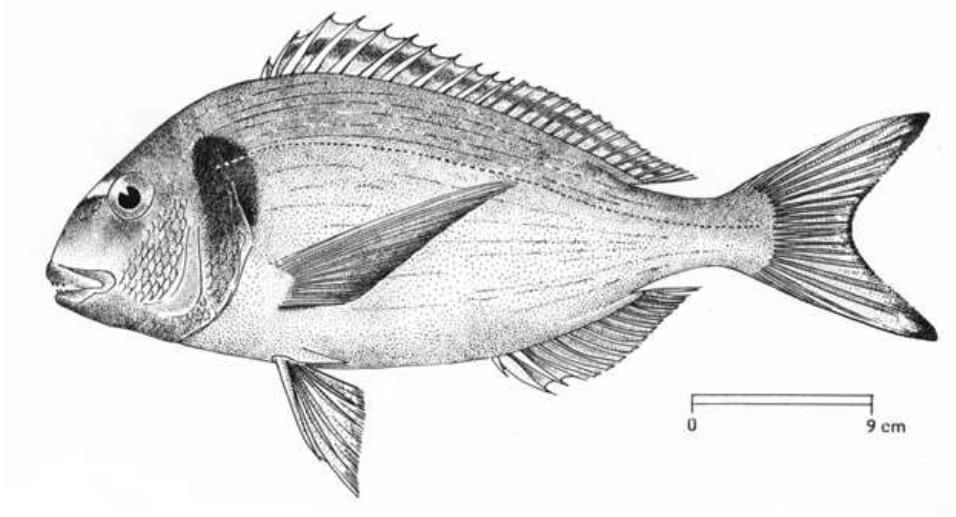


Figure 3 Adult specimen of gilthead sea bream (*Sparus aurata*) [from Moretti *et al.*, 1999].

1.1.2 PRODUCTION DATA

Since 1979, production of gilthead sea bream covers almost the entire aquaculture production of Sparids in the European Community (Figure 4), Spain being the second largest producer (18,000 metric tons in 2007, 16% of Europe production after Greece (51%) and followed by Italy with 6% (Figure 5). Lower quantities of this species are produced by France, Croatia and Portugal, while new productors are represented by Albania and Bosnia (FEAP, 2008; APROMAR, 2009).

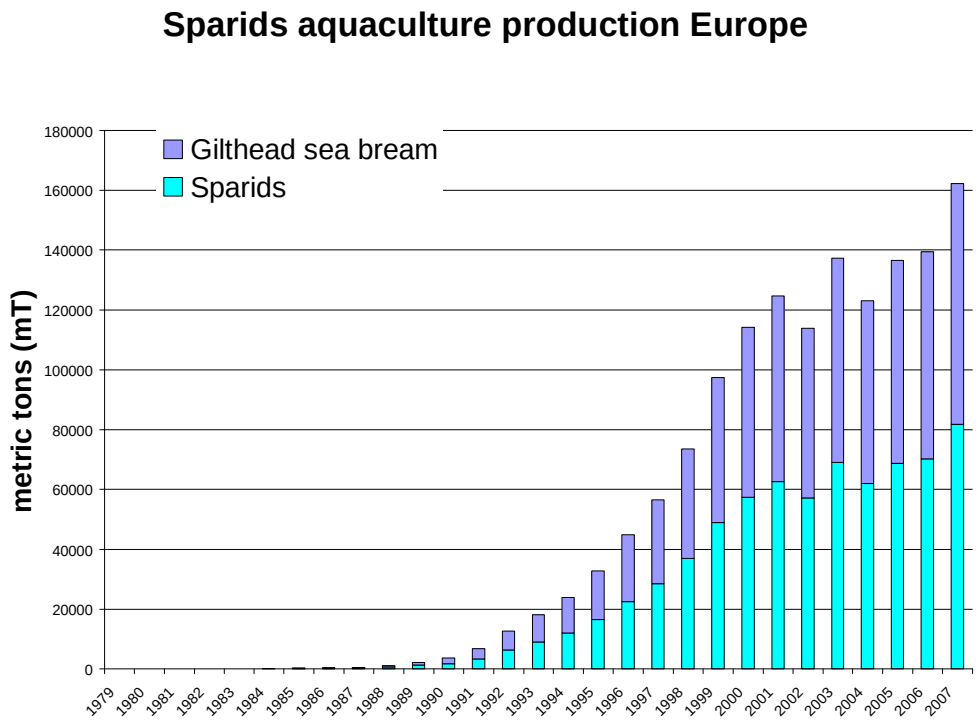


Figure 4 Aquaculture production data of Sparids and Gilthead sea bream for all countries of the European community (data elaborated from FAO FISHSTAT Plus).

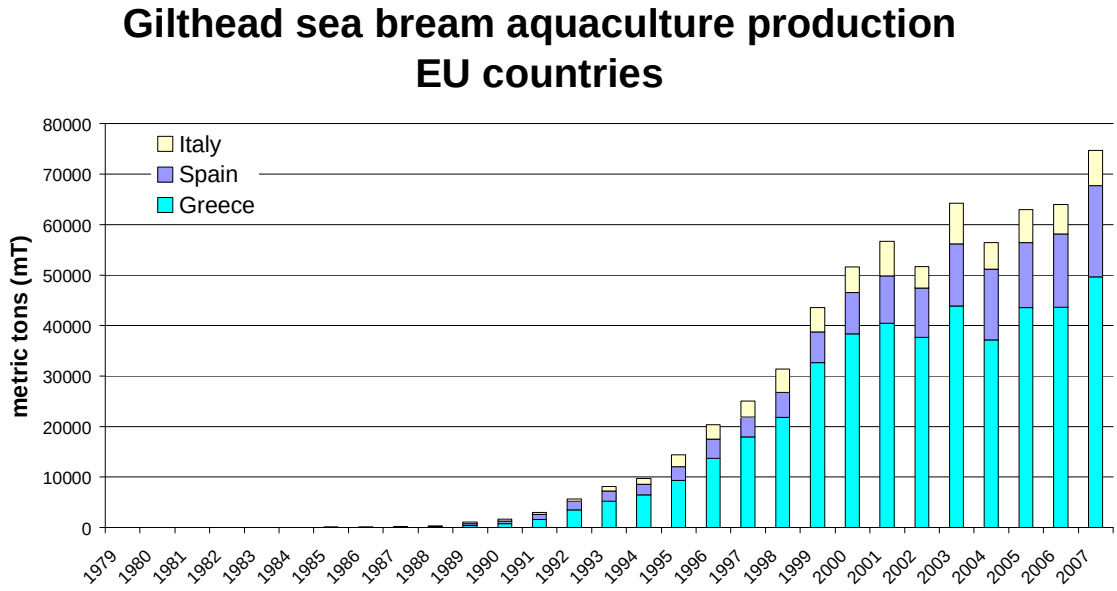


Figure 5 Aquaculture production of gilthead sea bream of the three principal European producers (data elaborated from FAO FISHSTAT Plus).

1.1.3 REARING TECHNIQUES AND PRODUCTION CONSIDERATIONS

Sea bream domestication is recent and started concomitantly with the development of reproduction and husbandry methods in the 70's, by the investigations of Barnabé and René in 1972 on reproduction and fingerlings rearing, as well as, those of Alessio and colleagues (1973) on artificial insemination, incubation and hatching of the eggs that were determinant for the development of larval rearing of this species. Rearing techniques applied to marine fish species comprises extensive, mesocosm, semi-intensive and intensive conditions based on the larval densities in the tanks, the source and quantity of food (Divanach, 1985; Van der Meeren and Nass, 1997).

Rearing of marine fish larvae implies the administration of external nutrients by feeding alive preys from 3 dah (days after hatching), before the larvae consume all yolk reserves (depending on the temperature, energy expenditure and food ingestion the yolk reserves in seabream may last until day 8). Several strains of the rotifer *Brachionus plicatilis* is the first prey fed to gilthead sebream due to the easiness of its mass production, its adequate dimensions (90-130 µm body length at the adult stage) to fit mouth size of early larvae, its planctonic habits, slow movements and the possibility to control its nutritional value for fish larvae. The nutritional value of this live prey varies notably according to its feed and environmental conditions such as light intensity and regimes, temperature, salinity, etc. For instance, high salinity induces an increase in the free amino acid pool as a response to regulate their cell volume (Rønnestad *et al.*, 1999). Rotifers are progressively replaced by brine shrimp *Artemia* sp. from 17 dah onwards, and subsequently with dry feeds of increasing dimensions (120-200 µm) until day

34 and 150-300 µm until metamorphosis stage at 40-43 dah) (Moretti *et al.*, 1999).

Nevertheless, neither rotifer nor Artemia constitute the natural preys of marine fish larvae, fact that frequently causes several problems in larval production leading to severe mortalities or malformations that impose serious constrictions to the further development of larval rearing production and controlled quality.

1.2 BONE- SKELETON DEVELOPMENT IN BONY FISHES

To address when and where abnormal development occurs under culture conditions it is essential to identify and to understand the processes involved in normal and abnormal bone formation (Fraser *et al.*, 2004) and understanding the environmental needs at different developmental larval stages (Fukuhara, 1992). Indeed, one of the major bottlenecks is the lack of precise information about bone formation during various stages of development, in order to characterize specific alterations in skeletal development associated with several factors related to rearing practices such as nutrition, abiotic elements, etc. (Lall and Lewis-McCrea, 2007).

1.2.1. GENERALITIES: functions, tissues and cells

The skeletal system has multiple functions including the support of the body structural integrity during development and locomotion. The skeleton also provides sites for muscle attachment, it protects vital organs and it serves as a mineral reservoir (Lall and Lewis-McCrea, 2007). Considering development and evolution, the skeleton can be divided into two systems: (a) the dermalskeleton to which belong teeth, scales, dermal skull bones and fin rays and (b) the endoskeleton which represents the visceral skeleton (including branchial arches), the axial and appendicular skeletons (fin endoskeleton) (Donoghue and Sansom, 2002, Hall and WItten, 2007).

Bony elements of the dermal skeleton usually develop directly from mesenchymal condensations through intramembranous bone formation. Bony elements of the endoskeleton usually have a cartilaginous precursor, which is remodelled into bone in the frame of endochondral bone formation. In addition bone can develop around cartilaginous elements (perichondral

bone) in a process that resembles intramembranous bone formation. Deviations from standard pathways exist for all skeletal systems especially in teleost fish, which show a very high diversity of skeletal tissues. Indeed, more than bone and cartilage, fish skeletal tissue is often best described as a continuous spectrum ranging from connective tissue to cartilage and to bone (Hall and Witten, 2007).

Bone is a specialized vascularised connective tissue consisting of cells and a mineralized extracellular matrix. Before being mineralized, the extracellular matrix is composed mainly by collagen type I called osteoid that subsequently becomes mineralized through the osteoblast mediated deposition of hydroxyapatite (Hall and Witten, 2007; Nordvick, 2007).

Cartilage is an avascular skeletal tissue composed of chondrocytes that are embedded in an extracellular matrix that is composed primarly by collagen type II and proteoglycans (Witten *et al.*, 2010).

Chondroid bone is an intermediate tissue that is found, for example, in the mandibular and maxillary tissue of teleost fishes and has intermediate characteristics of both bone and cartilage (it has both collagen of type I and II respectively) and may be mineralized.

Cells involved in the formation or remodelling of axial skeleton bones are principally osteoclasts, osteocytes and osteoblasts.

Osteoblasts, bone forming cells, are of mesenchymal origin. Osteoblast secretes the non-mineralised bone matrix and control matrix mineralisation. In basal osteichthyans, including basal teleosts, and mammals, some osteoblasts become embedded into the bone matrix as osteocytes that connect to each other and to osteoblasts on the bone surface via cell

processes function as strain and stress sensors. In advanced teleosts, osteoblasts never turn into osteocytes. Thus advanced teleosts, like seabream, have acellular bone, without osteocytes.

Osteoclasts, are the cells involved in the resorption of the bone tissue. Osteoclasts can be viewed as specialized multinucleated macrophage that derive from hematopoietic precursor cells that can also give rise to monocytes and macrophages (Witten and Huysseune, 2009). They are mobile cells like macrophages and migrate to the site of bone resorption. Osteoclasts in mammals and basal teleosts are typically multinucleated (Hall and Witten, 2007). Their action is controlled by hormones such as estrogen and calcitonin (osteoclasts inhibiting factors) or parathyroid hormone and parathyroid hormone-related protein (osteoclast stimulating factors).

Different from tetrapods (including mammals) fishes do not have hematopoietic tissue in their bone marrow spaces and osteoclasts in teleosts with acellular bone are typically mononucleated.

1.2.2 ACELLULAR BONE OF ADVANCED TELEOST FISH AND BONE REMODELLING

Processes that lead to the formation of bones, in fish may result in acellular (anosteocytic) bone, i.e. bones that do not contain osteocytes and cell processes in the bone matrix (advanced Teleosts) or to cellular bone (osteocytic bone) in the more basal Teleosts (Salmonids, Cyprinids, Clupeids). Acellular bone is restricted to Teleosts and it is the most widespread type of bone in this group of vertebrates (Hall and Witten, 2007), even though, in pathological situations (e.g. due to prolonged exposure to high pressures) it may be present in mammals (Hall and Witten, 2007). It has

a woven structure, contains vascular channels and has a chemical composition that matches bone consistency of other vertebrates (Witten and Willock, 1997). The formation of acellular bone is similar to dentine (tissue that forms teeth) formation, i.e. the cells are involved in the osteoid and bone matrix deposition but the cells never become entrapped (Hall and Witten, 2007; Franz-Odendaal *et al.*, 2006). With few exceptions, presence or absence of osteocytes is uniform in all the elements of teleost skeleton (Witten and Willock, 1997).

Apart from the formation of bones, the other process that is typical of hard tissues is remodelling. In general, vertebrate hard tissue structures are never permanent therefore they are subjected to continuous reshaping (cartilage), remodelling (bones) and replacement (teeth) (Witten and Huysseune, 2009). Initially it was thought that fish skeleton was metabolically inactive, due to the almost weightless conditions of life in the water, unlimited access to calcium and the often reported absence of bone resorbing cells (Witten and Huysseune, 2009), now it is known that fish skeleton is metabolically active and that it is subjected to resorption and remodelling. Remodelling of bones, during ontogeny, is triggered by the requirements of allometric growth, later it continues as an adaptation to mechanical load (Wolff's law). Remodelling and resorption are also necessary for mineral homeostasis (even though there is scant evidence that skeleton of teleosts serves as a calcium reservoir), fracture healing and replacement of teeth (Witten and Huysseune, 2009).

1.2.3 FISH VERTEBRAL COLUMN AND THE ROLE OF THE NOTOCHORD IN THE AXIAL SKELETON DEVELOPMENT

Vertebrates have vertebrae consisting of neural arches, haemal arches and a vertebral body (centrum) (Inohaya *et al.*, 2007). The number of vertebral bodies is variable and species-specific. They are interconnected by remaining, modified, or vestige notochord tissue that occupies the intervertebral spaces and act as a shock absorber (Astorga Contreras, 2005).

In Chordates, body axis is laid down during gastrulation and results in the formation of the notochord flanked by two rows of somites. The notochord is a slender rod of cells (discoid cells, i.e. chordoblasts) encased in a fibrous sheat (notochord sheat) that extend from the brain to the tail, defining the central axis of the chordate embryo. It lies beneath and parallel to the central nervous system, and dorsal to the coelom (Nordvick, 2007).

In primitive jawless fish (lampreys), the notochord and the notochordal sheat serve as the main skeletal axis throughout life.

In chondrichthyan fishes, the centra derive from the chondrification of mesenchymal cells that invade the notochord sheat at intersegmental intervals. The formation of these centra is dependent on the presence of the arches because the cells that form the centrum migrate from the arches into the notochordal sheat.

In advanced Teleosts, the formation of the vertebral body centrum is initiated by direct mineralization of the notochordal sheath. In a second step, intramembranous bone is deposited around the mineralised notochord sheath. Different from more basal bone fish and tetrapods, teleost vertebral body development does not pass through a cartilaginous stage (Inohaya *et al.*, 2007).

The formation of segmentally arranged vertebrae is intrinsically associated with two main structures, the notochord and the somites (Hall, 1975; Kaplan *et al.*, 2005; Bensimon-Brito *et al.* 2010). The differentiation of the somites into the different cellular types, is a multistage process that, at a molecular basis is initiated by the *shh* (sonic hedgehog) signalling from the notochord (Fan *et al.*, 1995) which, in turn, induces the expression of transcription factors from the *Pax* (pairbox) and *Sox* (Sry HMG box) families (Balling *et al.*, 1996; Akyiama *et al.*, 2002). Somites give rise to both muscles and vertebrae but, a single somite that gives rise to a single muscle element yet contributes to two adjacent vertebrae (Remak, 1850) in mammals and birds (resegmentation), and to more than two vertebrae in teleosts (leaky resegmentation) (Morin-Kensicki *et al.*, 2002). Recent studies on zebrafish (*Danio rerio*) (Fleming *et al.*, 2003) and Atlantic salmon (*Salmo salar*) (Grotmol *et al.*, 2005b), suggest a role of the notochord itself on the pattern of vertebral column segmentation. Ablation of the notochord in zebrafish and in amphibians causes failure in vertebral body segmentation (Fleming *et al.*, 2003), whereas disturbed sclerotome patterning has no influence on the segmentation of vertebral bodies. The authors therefore concluded that, segmentation was due to both the activity of notochord and of the somites in which, notochord may impart metameric arrangement of the centra while somites acts directly in the patterning of the arches.

Apart from its role in the pattern of notochord segmentation, notochord orchestrate a series of mechanisms like promoting the formation of the vertebral structures in a ventro-dorsal direction by repressing dorsal structures (Fleming *et al.*, 2001).

Once notochord has been segmented, bone is deposited external to the notochord and form the vertebral body.

1.3 FINFISH: MOST COMMON SKELETAL DEFORMITIES

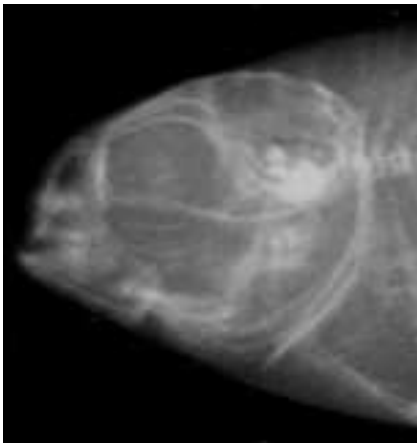
Osteological malformations are a frequent and important problem of intensive aquaculture, especially in relatively young specimens. Deformities can be a complex mixture of different bone disorders. Skeletal malformations include all the anomalies regarding deformities of the dermalskeleton. In round shaped fish, such as sea basses, sea breams or milkfish (*Chanos chanos*), the incidence of spinal malformations have been the most frequently described (scoliosis, lordosis, kyphosis, coiled vertebrae, missing or additional vertebrae or spines).

Deformities of the fin rays have been described in many Sparids, especially regarding the caudal fin complex (Sfakianakis *et al.*, 2004) or the lack of the dorsal fin (saddleback syndrome) (Koumoundourous *et al.*, 2001; Sfakianakis *et al.*, 2003). In other cases, deformities regarding the cranial region (jaw region and opercular complex) have been considered.

In the jaw region can be enumerated:

- pugheadness or the reduction of frontal skull and upper jaw bones, is the anterior-posterior compression of the ethmoid region and upper jaw (Baeverfjord *et al.*, 2009; Koumoundourous and Georgakopoulou, 2009) (Figure 6 A-C).
- crossbite or lateral shift of the inferior jaw bones, angular and/or dentary bones (Koumoundourous and Georgakopoulou, 2009) (Figure 6 B).

- sucker mouth or reduction of the upper and lower jaws (Barahona-Fernández, 1982).



A

B

C

Figure 6 A: example of pugheadness (figure from Koumoundourous and Georgakopoulou, 2009) **B:** example of crossbite (from Koumoundourous and Georgakopoulou , 2009) **C:** example of pugnose in first feeding fry (from Baeverfjord *et al.*, 2009).

Opercular anomalies are one of the most common described pathologies for sea bream (Koumoundourous *et al.*, 1997b; Galeotti *et al.*, 2000; Beraldo *et al.*, 2003; Bongiorno *et al.*, 2007). They can affect up to 80% of sea bream population (Paperna, 1978). They generally consist in the folding of the opercule inside the gill cavity and can be mono or bi lateral if they affect one or both side of the fish (Koumoundourous *et al.*, 1997b; Galeotti *et al.*, 2000; Beraldo *et al.*, 2003).

Regarding axial skeleton deformations, a wide review has been done by Witten and collaborators (2009), which define up to 20 general types of vertebral anomalies in reared Atlantic salmon based on radiographies of adult specimens. Basically axial skeleton malformations include:

1. Compressed and/or fused vertebral bodies:

These anomalies comprise two general pathologies: platyspondyly (the flattering of vertebral end plates) and vertebral fusion (ankylosis, short tail). Severe cases of both pathologies can result in a similarly shortened animal with a pathologically increased condition factor. Platyspondyly can result in vertebral fusion but also occurs without vertebral fusion (Witten *et al.*, 2005). Ankylosis can occur between two or more vertebrae and may lead to a remodelling of the vertebral body into one with a more or less regular shape but with one or more haemal and neural spines (Witten *et al.*, 2006). In this category of deformities, elongation of the vertebral bodies can be included, a pathology that is compensatory to compression of other parts of the column (Gill and Fisk, 1966; Kvellestad *et al.*, 2000; Witten *et al.*, 2009). Impairment of segmentation during embryonic development leads to congenital fusion of vertebrae (Kaplan *et al.*, 2005; Bensimon-Brito *et al.*, 2010).

2. Spine curvatures

In this category, kyphosis (dorsal curvature), lordosis (ventral curvature) and scoliosis (lateral curvature) are included.

Kyphosis (hunchback or humpback) is defined as the curvature of the upper spine. Scoliosis is a pathological condition of the spinal column in which the spine is curved from side to side and may also be rotated. Lordosis

(swayback) defines an inward curvature of a portion of the vertebral column. However, axis deviations may also be due to neuromuscular rather than a bone-related aetiology (Eissa *et al.*, 2009; Witten *et al.*, 2009; Betancor *et al.*, 2010).

3. Simmetry deviations and displacement of vertebral bodies

Vertical shift of a vertebral body within an otherwise non-deformed spine. Vertebral bodies can be shifted dorsally or ventrally. Vertebral bodies can present irregular internal malformation that may not affect the external shape (Madsen and Dalsgaard, 1999; Witten *et al.*, 2009).

4. Radiotranslucent and radiopaque vertebral bodies

In some cases it is possible to evidence vertebral bodies spaced and undersized, as the bone matrix seems translucent to x-rays. This may be due to undermineralization of the vertebral body (Helland *et al.*, 2005). This pathology may be reflected also in a biconcave vertebrae shape.

The opposite situation is represented by hyper radio dense vertebral body. In some cases (Helland *et al.*, 2006), it has been shown that increased radio density of hyper dense vertebra not only relates to increased trabecular density but also to the ectopic presence of cartilage.

5. Severe multiple malformations

In this category can be included all the complex malformations that are sums of all the precedent ones with the possible presence of vertebral fractures.

6. Anomalies of the haemal and neural arches and spines

In this category, anomalies regarding wrong direction (S-shaped, dorsoventral compression), disorganization, twisting or bifurcation of haemal and neural arches are included. Mechanical stress (Grotmol *et al.*, 2005a), nutritional factors (Fernández *et al.*, 2008) and embryonic origin (Fleming *et al.*, 2003) have been indicated as possible causative factors.

Another vertebral anomaly that have been described both in flat fishes (*Hippoglossus hippoglossus*) (Lewis and Lall, 2006) and in round fishes like *Diplodus sargus* (Saavedra *et al.*, 2009) is the presence of hypertrophic vertebrae (i.e. vertebrae that are dimensionally larger than the adjacent ones). It has been proposed that skeletal abnormalities are a result of accelerated organogenesis while the body size maintains a normal size (Gould, 1977). Anyway, this is not considered a serious abnormality, because as development proceeds, the smaller vertebrae increase growth and/or larger vertebrae decrease growth (Lewis *et al.*, 2004).

1.3.1 FACTORS AFFECTING SKELETAL DEVELOPMENT AND BONE FORMATION

Many genetic and epigenetic factors have been linked to skeletal abnormalities in several teleost fish (Lewis-McCrea and Lall, 2007). Despite wild malformed animals are rare, in captivity fish with many morphological anomalies can survive (Zambonino *et al.*, 2005). Boglione and collaborators (2001) have shown that only 4% of wild caught gilthead sea bream show body deformations in contrast to the high values reported in hatchery reared specimen. Equally, the saddleback syndrome was not encountered in natural

populations of *Dentex dentex* by Koumoundourous and co-workers (2001). In contrast Favarolo and Mazzola (2003) found that wild *Diplodus puntazzo* showed higher malformation frequency than the hatchery reared ones.

Sometimes, early development of skeletal deformities can be already detected before the vertebral column develops. In newly hatched sea bream larvae, both Santamaria and collaborators (1994) and Andrades and collaborators (1996) could evidence axial anomalies, in the first case associated also with strong alteration in the muscle bundles, disorganized connective tissue and irregularities in notochord and perinotochordal collagen sheet.

However, skeletal anomalies are often detected only late in the larval development. In *Lates calcifer* the so called LSK syndrome (lordosis – scoliosis – kyphosis syndrome) could be detected only at 38 dah (Fraser *et al.*, 2004). In *Diplodus sargus,* the age with the higher incidence of skeletal anomalies was between 23-25 dah (Saavedra *et al.*, 2009).

Genetic and environmental factors

Regarding genetic factors, mutations, hybridization and inbreeding have been considered to relate to bone malformations (Madsen and Dalsgaard, 1999, Afonso *et al.*, 2000). In hatchery reared gilthead sea bream an association between skeletal deformities and the family structure has been shown, with a heritability of 85% concerning the presence or absence of deformities. Moreover the triple column deformity LSK correlates significantly with the family structure, linked to the microsatellite SaGT26 (Astorga Contreras, 2005). In contrast, differences in the occurrence of skeletal

malformations were not found to be linked to the genetic background in Atlantic salmon smolts (Fjelldal *et al.*, 2009).

Among the main environmental factors that are known to affect skeletal development in hatchery conditions, abiotic and biotic factors have been studied. Among those hydrodynamics (Sfakianakis *et al.*, 2006), temperature, light intensity and duration (Wargelius *et al.*, 2009), pH, larval density (Roo *et al.*, 2005; Roo 2009) and general rearing conditions as abiotic factors and infections/general disease and nutritional factors as biotic factors.

Principally temperature has been extensively studied as a deformity inducing factor in different species. In particular high temperatures negatively influence the development of skeletal elements in sea bream (> 16ºC, Koumoundourous and Georgakopoulou, 2009), in sea bass (> 20℃, Sfakianakis *et al.*, 2006), in common pandora (*Pagellus erythrinus*) (> 21℃; Sfakianakis *et al.*, 2004). Polo *et al.* (1991) indicated that also low temperatures (≤ 14℃) induce higher skeletal anomalies in sea brea m larvae at the opening of the mouth.

More generally, rearing techniques have been shown to influence the performance of larvae. In *Dentex dentex*, Koumoundourous and collaborators (2001) shown that semi extensive system *versus* extensive one (in particular referred to larval density) induces a higher incidence of column and caudal deformity. Also Giménez and Estévez (2008), in the same species, noticed a higher incidence of scoliosis and vertebral fusion of the pre-ural vertebrae between semi extensive and extensive system. Roo (2009) reported for red porgy (*Pagrus pagrus*) differences in skeletal

ontogeny and frequency of cranial anomalies in relation with semi-intensive and intensive rearing system. In sea bream (Roo *et al.*, 2005) rearing system is reported to be positively correlated with opercular anomalies and incidence of lordosis.

Nutritional factors:

Among nutritional factors, several vitamin and minerals were early recognized to influence the occurrence of bone malformations (Wilson and Poe, 1973; Dedi *et al.*, 1997; Takeuchi, 1999; Takeuchi *et al.*, 1998). Nevertheless, other nutrients have been also related to skeletal malformations (Satoh *et al.*, 1989; Cahu *et al.*, 2003; Roo *et al.*, 2005, Roo, 2009; Lewis-McCrea and Lall, 2010), as well as feeding practices (Imsland *et al.*, 2006).

1) Minerals

Among the six essential macro elements, calcium and phosphorous play an important role in skeletal development. In teleost fish, unlike tetrapods, bone is not the principal site of calcium regulation. Gills provide continuous access to an unlimited calcium reservoir, and calcium regulation occurs at the gills, oral and fins epithelia (this last playing a minor role) (Witten, 1997, Lall and Lewis-McCrea, 2007). On the contrary, the concentration of phosphorous (P) is low both in freshwater and marine fishes, therefore, the diet is the main source of this mineral and its nutritional supply regulation is considered to be critical (Lall and Lewis-McCrea, 2007).

Calcium deficiencies are uncommon in fishes, while phosphorous (and phosphorous forms) deficiencies have been described in haddock (*Melanogrammus aeglefinus*) (Roy *et al.*, 2002) and halibut (*Hippoglossus hippoglossus*) (Lewis McCrea and Lall, 2010). P-deficient diet caused a delay in bone mineralization and while an excess P in the diet increased matrix deposition and resulted in accelerated mineralization. In halibut it induced a high percentage of haemal and neural spine anomalies.

2) Vitamins

Vitamin E (α- tocopherol, vit E) and vitamin C (ascorbic acid, AA, vit C) are important antioxidant components in all living organisms. The action of both molecules is linked to their reduction ability that permit to scavenge, respectively, the reactive species of oxygen (ROS) [vit C] and lipid peroxyl radicals [vit E] generally called free radicals (Sies, 1993). In this way, both vitamins protect cellular membranes from damage by free radicals. Moreover, a strong interaction between the two micronutrients exists as vit C regenerates the α- tocopherol reduced form and tocopherol free form.

Besides vit C is a co-substrate of di-oxygenases (2-ODDs), such as prolil-4-hydroxilase that catalyzes the post-traslational hydroxylation of the proline residuals in the polypeptid chains during the synthesis of collagen. Therefore vit C has an important role in the prevention of skeletal anomalies.

In channel catfish (*Ictalurus punctatus*) the quantity of vit C in the diet is inversely proportional to the incidence of broken back syndrome (Lovell and Lim, 1978), as well as in milkfish (*Chanos chanos*) cranial anomalies (in particular opercular deformities), are reduced with higher levels of vit C in live preys (Gapasin *et al.*, 1998). In agreement, restricted dietary vit C contents

have been found to cause cartilage reduction and damage that reflects in anomalies especially in structures that undergo chondral ossification (jaws and some elements of the caudal fin as epurals and the specialized neural arch) together with an over stimulation of the receptor of vit C transporter (Svct1) (Darias *et al.*, 2009).

Studies on the effects of vit E on fish skeletal development or deformity incidence are scarce and almost associated with the presence of oxidized lipids (Lall and Lewis-McCrea, 2007) or high levels of PUFAs (poliunsatured fatty acids) (Atalah, 2008; Betancor *et al.*, 2010). Indeed, tissue vitamin E contents are closely related to tissue PUFA levels (Izquierdo and Fernández-Palacios, 1997). In juvenile Atlantic halibut (*Hippoglossus hippoglossus*), it affects neither growth nor survival, but reduce the incidence of lordosis in animals fed un-oxidized lipids if supplemented with vit E. On the other side the different combination of DHA (docosahexaenoic acid) levels and appropriate levels of vit E, reduce the symptoms of skeletal muscle degeneration probably reducing the possibilities to develop skeletal malformations (Betancor *et al.*, 2010).

However, it was suggested that the effects of tocopherols on cellular oxidation may affect lipid metabolism including the alteration of fatty acids desaturation and/or elongation at least in basal teleosts (Mourente *et al.*, 2007). Indeed, in salmons isolated hepatocytes fatty acids desaturation and elongation was increased in fish fed diets deficient both in tocopherol and astaxantin (Bell *et al.*, 2000).

Apart antioxidant vitamins, other vitamins take part in bone formation with various mechanism. Vit K affects the synthesis of plasma bone proteins being a co-factor in the protein carboxylation of γ-carboxiglutamate (Gla) that is essential in the production of osteocalcin (calcium (hydroxy apatite) no collagenous binding protein). One of the effects of this vitamin is the reduction in bone mineralization and mass as detected in haddock (*Melanogrammus aeglefinus*) (Roy and Lall, 2007).

Calciferols, vit D, are important for calcium and phosphate homeostasis and protection of skeletal integrity. One of the active forms of vit D is the hormonal form (vit D3), which functions through the vit D receptor (VDR) inducing the expression of various calcium binding and transport proteins in the intestine to stimulate active calcium uptake. Darias *et al.*, 2009 showed that low levels of vit D3 produce poor mineralized larvae with major deformities such as pugheadness and light fin caudal deformities. Nevertheless they also suggest damage in bone structures due to hypervitaminosis of vit D through the delay of intestinal maturation affecting TRPV6 (transient receptor potential cation channel, subfamily V, member 6, a transcellular mediator of calcium uptake from the intestinal lumen), and, in sequence osteoblast differentiation via the BMP4 (bone morphogenic protein 4), VDR and osteocalcin expression levels. In other cases, no signs of intoxication or hypercalcemia were detected feeding Atlantic salmon with high doses of vit D (Graff *et al.*, 2002).

Vit A (retinol) is an essential morphogenic lipid-soluble micronutrient that has essential roles in vision, growth, bone development, reproduction and normal maintenance of epithelial tissue in all vertebrates (Lall and Lewis McCrea,

2007; Fernández *et al.*, 2008). Regulation of cellular differentiation and proliferation are among the important functions of vit A through the orchestration of molecular mechanisms that involve nuclear receptors (RARs, retinoic acid receptors and RXRs, retinoid X receptors) present on the cell membrane of the notochord during the segmentation process and some regulatory genes (Dedi *et al.*, 1997; Balmer and Blomhoff, 2002). In particular, the genes of the *Hox* family (homebox) and of *shh* family (sonic hedgehog) control the chondrocytes and osteoblast differentiation, activity and proliferation (Mazurais *et al.*, 2008). Hypervitaminosis A advances chondrocyte maturation while stimulating osteoclasts activity which delays the production of bone matrix resulting in a precocious mineralization and possible skeletal anomalies. Indeed, both Dedi and collaborators (1997) and Fernández *et al.* (2008) found skeletal anomalies positively correlated to hypervitaminosis A, the first in Japanese flounder (*Paralichtys olivaceus*), with vertebral compression during the larval developmental stages of notochord segmentation, the second in sea bream with the presence of supernumerary vertebrae, cranial anomalies and haemal spines defects. However, Takeuchi *et al.*, 1998, also indicated different activity and potential toxic effects of the different molecular forms of the vitamin.

Mazurais and collaborators (2008) pointed out the effects of vitamin mix (VM) on skeletal anomalies together with the expression of some genes involved in osteoblast differentiation (BMP-4) in sea bass larvae.

In particular, they found an inverse correlation between BMP-4 expression and vitamin level, and with the elevation of proliferator peroxisome activated receptors (PPAR-γ) expression. These results suggested that high levels of PPAR-γ transcripts in larvae-fed diets with a low VM content converted some

osteoblasts into adipocytes during the first two weeks of life. This loss of osteoblasts, was suggested to be likely to cause higher axial malformations.

3) Proteins – amino acids

Marine pelagic fish eggs from various latitudes contain up to 50% of the total amino acid pool as free amino acids (FAAs). The FAA pool is established during final oocyte maturation and seems to derive from the hydrolysis of a yolk protein. During yolk resorption, the FAA pool is depleted and reaches low levels at first feeding. The FAAs (free amino acids) are predominantly used as metabolic fuel, but they are also utilized for body protein synthesis. Amino acids are also important catabolic substrates after the onset of first feeding and may account for 60% or higher of the energy dissipation. Since growth is primarily an increase in body muscle mass by protein synthesis and accretion and fish larvae have very high growth rates, they have a high dietary requirement for amino acids (Rønnestad *et al.*, 1999).

Regarding the effects that singular AAs (aminoacids) have in the development of skeletal anomalies in fish, different conclusions were derived for freshwater and marine species. In salmon fries the deficiency of tryptophan was indicated to be the principal factor associated with the prevalence of scoliosis (57% more in respect to normal specimens), incidence that was recuperated if fish fed, in a second phase, with diets incorporating 0.24% of tryptophan (Akiyama *et al.*, 1986). In contrast, in the marine fish *Diplodus sargus* analyzed from15 dah on, the supplementation of tryptophan did not enhance larval growth, survival or quality showing variable percentages and type of skeletal malformations (kyphosis at trunk vertebrae

12%, scoliosis at caudal vertebrae 9%, lordosis maximum 3%) (Saavedra *et al.*, 2009). Concerning protein larvae nutrition, it has been showed that partial replacement with protein hydrolysates improved growth and survival both in freshwater and marine fishes. Moreover, in sea bream larvae it was demonstrated that exist an inverse correlation between malformation rate (spinal or jaw) (2% *vs* 13%) and hydrolysate level (58% hydrolysate level *vs* total fish meal) (Kolkovski and Tandler, 2000). Zambonino Infante and collaborators (1997) indicated that the partial substitution of native proteins with short peptides (di and tri peptides), reduces the malformation rate in sea bass larvae (from 15% of the control group with all native proteins to 6% to 3% of larvae fed hydrolysate substituted diets). Irrespective of variations in malformation rates, no effects in the activity of alkaline phosphatase were reported in the same work.

4) Lipids – fatty acids

Lipids are materials for the formation of cell membranes and sources of metabolic energy. In particular larvae rely on lipids for almost all their embryonic development (Rainuzzo *et al.*, 1997). Among the reserve lipids, triglycerids are always mobilized before phospholipids (PL) during starvation as these last components play an important structural role in the cell membranes and tend to be conserved (Sargent *et al.*, 1989). In the same way, among fatty acids, the ones of the n-3 series are metabolized after the n-6 and n-9 series, and in particular DHA probably because it is a preferred substrate for the synthesis of PE (phosphatidylethanolamine) (Koven *et al.*, 1989).

In mammals' bone metabolism, among phospholipids, phosphatidylinositol (PI), apart from its structural role, is a precursor of second messengers and is a membrane anchor for a great variety of cell surface proteins. A major function is to supply second messengers such as Ins (1, 4, 5) P3 (D-myo-inositol 1, 4, 5 triphosphate) and dyacilglycerol in response to calcium-mobilizing agonists (Berrige and Irvine, 1989).

In fishes, since the 1980's it has been showed that larvae have a higher phospholipid requirement compared to juvenile fish (Kanazawa *et al.*, 1983) and that the incorporation of phospholipids of animal (chicken egg lecithin) or vegetal origin reduce the incidence of skeletal malformation in ayu larvae (*Plecoglossus altivelis*) (Kanazawa *et al.*, 1981) and in sea bass (Cahu *et al.*, 2003). Contrasting results are reported by Fontagné–Dicharry and collaborators (2009) that indicated better grow performances for salmon fries fed diets without phospholipids. Anyway, the type of phospholipids used (phosphatidylinositol *vs* phosphatidylcholine), seems to differently influence skeletal deformities at least in freshwater fishes like *Cyprinus carpio* (Geurden *et al.*, 1998).

In other vertebrates, the fatty acid compositions of osteoblasts and osteoclasts can be altered through dietary lipids and oxidative stress, consequently affecting their function and formation of bone (Xu *et al.*, 1994; Watkins *et al.*, 2000).

In chicks, PUFAs serve as precursors for the synthesis of the phospholipids present in the matrix vesicles, but in general they serve as substrates for prostaglandine (PG) E_2 biosynthesis. This last component acts as a potent bone resorbing agent and appears to regulate cytokines and growth factors

in bone (Xu *et al.*, 1994). In humans, elevated production of prostaglandin E_2 has been associated with several osteolytic disorders (Watkins *et al.*, 2001). In mice, Kruger and Shollum (2005) showed that high levels of DHA reflect on increased adsorption of calcium, bone mineral density and increased bone strength. In fish (cod, *Gadus morhua*) it has been shown that the onset of ossification coincides with the increase expression of Osteix (a gene required for the maturation of pre-osteoblast) that may be controlled by the action of PGE_2 (Moren *et al.*, 2009), but no other studies on the effects of lipids through the action of prostaglandins on bone formation are available for fish.

Bones of some marine fish contain as high as 24-90% w/w lipid (Phleger, 1991) and with relatively higher proportions of PUFAs than found in mammals comprising cartilage, bones, matrix vescicles, osteoblast and chondrocytes (Lall and Lewis McCrea, 2007).

Marine fish are unable to synthesize n-3 PUFA such as EPA (eicosapentaenoic acid) and DHA, which are particularly important as they occur in large amounts in fish cell membranes, and as a consequence, these long chain fatty acids have to be included in the diet (Izquierdo, 1996). Requirements of EPA and DHA are species and stage dependent (Izquierdo, 2005) even though the optimal level of these essential dietary components for marine fish is known to be around 3% of dry matter for EPA and DHA (Sargent *et al.*, 1999).

In particular DHA has been indicated inversely correlated with the incidence of opercular anomalies for sea bream (Beraldo, 1998; Roo *et al.*, 2005) and

milkfish (Gapasin and Duray, 2001), and with skeletal anomalies for red porgy (Roo *et al.*, 2009).

Some authors indicated moreover, that following the fraction in which EPA and DHA are incorporated, they differently influence the occurrence of bone anomalies and the ossification process. In European sea bass Villeneuve *et al.* (2005) showed that low percentages of vertebral and cephalic deformities if these two PUFAs are present in a moderately percentage in the phospholipid fraction while if EPA and DHA are present in high concentration in the neutral lipid fraction they result teratogenic and lethal. In particular, larvae fed EPA-DHA in the neutral lipid fraction showed also an up regulation of RARs, RXRα genes that may disrupt normal larval development. In cod fed diets where high levels of EPA and DHA are in the phospholipid fraction showed a faster ossification and a higher number of fin rays in respect of a control group Kjørsvik and collaborators (2009).

Due to their poikilothermic nature, fish lipids are more highly unsaturated than those from homeotherms, particularly those adapted to coldwater environments (Raisz, 1993; Abele and Puntarulo, 2004), which would seem to predispose them to lipid peroxidation. PUFA peroxidation may produce potentially toxic compounds such as aldehydes and hydrocarbons will eventually lead to a complete loss of membrane integrity (Mourente *et al.*, 2007) as well as reduce antioxidant stores (Lewis-McCrea and Lall, 2010). In tetrapods, oxidized lipids alter bone cellular membrane components and can result in reducing osteoblastic differentiation and an increase in osteoclastic differentiation ultimately resulting in net bone loss (Lewis-McCrea and Lall, 2007).

In halibut (*Hyppoglossus hyppoglossus*) Lewis-McCrea and Lall (2007, 2010), the presence of moderately oxidized lipids induced a higher incidence of scoliosis and the presence of lordosis

1.4 OBJECTIVES

1. Evaluate possible differences in whole mount staining protocols on old stored (five years) formaldehyde sea bream larvae.

2. Evaluate skeletal development on old stored formaldheyde sea bream larvae reared in semi-natural conditions (mesocosm system) in terms of mineralization of the different skeletal elements in comparison with literature data.

3. Evaluate the axial skeletal development on old stored formaldheyde sea bream larvae of 35, 45 and 50 dah reared in semi-natural conditions (mesocosm system) in terms of relative dimensions (length and height) of the vertebral centra.

4. Evaluate the effects, in terms of growth (length and dry weight) and in terms of proximal composition of sea bream larvae reared in intensive system and fed for 25 days with rotifers enriched with different levels of DHA and vitamin E along 34 days.

5. Evaluate the effects, in terms of skeletal anomalies and in terms of skeletal mineralization, of sea bream larvae reared in intensive system and fed with rotifers enriched with different levels of DHA and vitamin E at the age of 34 dah.

2.
MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 TRIAL I: SELECTION OF STAINING PROTOCOLS FOR VISUALIZATION OF MINERALISED TISSUES AND ON SKELETAL DEVELOPMENT IN SEA BREAM LARVAE

2.1.1 FISH AND HUSBANDRY CONDITIONS

Larvae for this trial were obtained from an unpublished study conducted by Roo and coworkers in 2005. Fish eggs were obtained from a natural spawning from the gilthead seabream broodstock of the Grupo de Investigación en Acuicultura (Canary Islands, Spain). They were distributed in cylindroconical 40 m^3 tanks at a density of 7 eggs l^{-1}, provided with filtered and UV sterilized sea water, following the protocol described by Roo *et al.* (2009). Combined natural and artificial light provided a photoperiod regime of 0D: 24L (dark : light) from 2-20 days after hatching (dah) and of 12D:12L from 40-50 dah (400-1200 lux light intensity). Sea water was previously filtered by a sand filter and a UV sterilizer. Water salinity and temperature along the experiment were 37 ± 0.5‰ and 20 ± 1ºC, respectively. Average water oxygen concentration was of 6.5 ppm.

From day 4 after hatching, and until larvae reached 8.5 mm, 5 to 7 enriched rotifers ml^{-1} were added twice a day. Rotifers, daily fed with baker`s yeast (*Saccharomyces cerevisiae*) were enriched, prior to larval feeding, with DHA Protein Selco™, (Inve Aquaculture, Dendermonde, Belgium) following the manufacturer instructions. During rotifers feeding, live phytoplankton (*Nannochloropsis oculata*) was added once a day to maintain a concentration of 250 ± 100x10^3 cells ml^{-1} in the tanks.

When larvae reached 6.0 mm in total length, and until the end of larval phase at 50 dah, Artemia enriched metanauplii were added until they reached a density of 0.25 to 0.5 individual ml^{-1} three times a day. When larvae reached 7.5 mm, they were fed a starter commercial diet (Gemma Micro, Skretting, France) four times a day for 5 days and automatic feeding every hour from day 25.

2.1.2 SAMPLING TECHNIQUES

For this trial, numerous samples (267 specimens) (Figure 7) of sea bream larvae of 10, 17, 21, 28 and 31 dah stored in buffered (10%) formaldehyde were measured before staining by a profile projector (Mitutoyo, PJ-A3000, Japan), under a magnification objective of 50 X, and classified in standard length (SL) size classes of 0.2 mm. Once classified, three larvae from each age and from most numerous size class were separated and stained with the three different whole mount staining protocols described in paragraph 2.1.3.

2.1.3 WHOLE MOUNT STAINING PROTOCOLS

Different whole mount staining protocols were applied in order to evaluate the most suitable method to determine tissue mineralization in early larval stages.

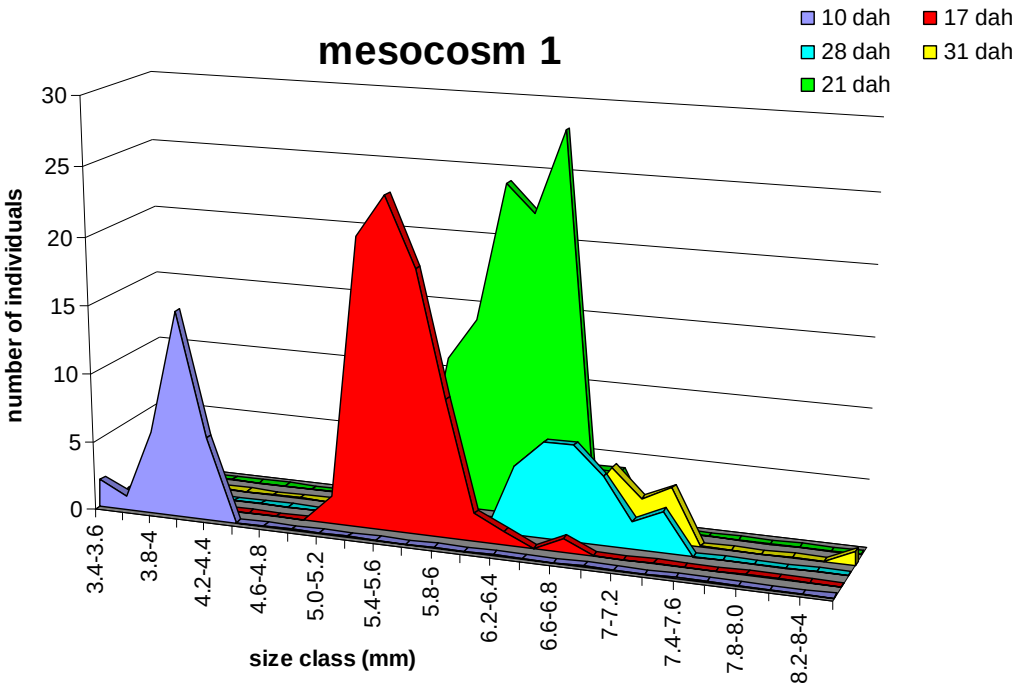


Figure 7 Classification of 0.2 mm size classes of the old stored larvae used for the whole mount staining trial (Trial I).

Three whole mount staining protocols were tested:

1. a double staining according to Taylor and Van Dyke (1985) and Dingerkus and Uhler (1977) as reported by Socorro (2006) (Table II),

2. an acid free variant of this protocol with single staining for alizarin red (Vandewalle *et al.*, 1998) (Table III),

3. a second acid free single stain alizarin red staining protocol (Walker and Kimmel, 2007) (Table IV).

Basically, the principal differences between the last two protocols consisted of using a single step of dehydratation with ethanol 50%, in leaving overnight alizarin red staining and by using different dilution of glycerol in 1% KOH instead of 0.5%.

Larval bleaching was not need due to the low level of body pigmentation in the sampled larvae.

Table II Summary of the double staining (protocol 1) (Socorro, 2006 after Taylor and Van Dyke, 1985 and Dingerkus and Uhler, 1977)

Step	Duration	Significance of the step	Solutions
Alcian Blue (8GX)	2 h	Proteoglycan (cartilage) Staining	For 100 ml: 80 ml alchol 95% 20 ml glacial acetic acid 10 mg alcian blue
Ethanol 95%	1 h	Hydratation of the sample	For 100 ml: 95 ml absolute ethanol 5 ml distilled water
Ethanol 95%	1 h		
Ethanol 95%	1 h		
Ethanol 75%	1 h		For 100 ml: 75 ml absolute ethanol 15 ml distilled water
Ethanol 40%	1 h		For 100 ml: 40 ml absolute ethanol 60 ml distilled water
Ethanol 15%	1 h		For 100 ml: 15 ml absolute ethanol 75 ml distilled water
Distilled water	1 h or all night		
Trypsine solution	1 h	Tissue digestion	90 mg pancreas porcine trypsine; 70 ml distilled water 30 ml saturated solution of $Na_2B_4O_7$
Alizarin red	1 h and a half	Calcium (bone) staining	1g l^{-1} alizarin red In a 0.5% KOH solution
Glycerin KOH 1:3	12 to 24 h	Clearing	For 100 ml: 25 ml glycerin 75 % KOH (0.5%)
Glycerin KOH 1:1	12 to 24 h	Clearing	For 100 ml: 50 ml glycerin 50 ml KOH (0.5%)
Glycerin	12 to 24 h	Clearing	For 100 ml:

KOH 3:1		75 ml glycerin 25 ml KOH (0.5%)
Pure glycerine and some grains of thymol	Clearing & storage	

Table III Single staining protocol (protocol 2) according to Vandewalle *et al.* (1998)

Step	Duration	Significance of the step	Solutions
Ethanol 95%	1 h	Hydratation of the sample	For 100 ml: 95 ml absolute ethanol 5 ml distilled water
Ethanol 95%	1 h		
Ethanol 95%	1 h		
Ethanol 75%	1 h		For 100 ml: 75 ml absolute ethanol 15 ml distilled water
Ethanol 40%	1 h		For 100 ml: 40 ml absolute ethanol 60 ml distilled water
Ethanol 15%	1 h		For 100 ml: 15 ml absolute ethanol 75 ml distilled water
Distilled water	1 h or all night		
Trypsine solution	1 h	Tissue digestion	90 mg pancreas porcine trypsine; 70 ml distilled water 30 ml saturated solution of $Na_2B_4O_7$
Alizarin red	1 h and a half	Calcium (bone) staining	1g l^{-1} alizarin red In a 0.5% KOH solution
Glycerin KOH 1:3	12 to 24 h	Clearing	For 100 ml: 25 ml glycerin 75 % KOH (0.5%)
Glycerin KOH 1:1	12 to 24 h	Clearing	For 100 ml: 50 ml glycerin 50 ml KOH (0.5%)
Glycerin	12 to 24 h	Clearing	For 100 ml:

KOH 3:1		75 ml glycerin 25 ml KOH (0.5%)
Pure glycerine and some grains of thymol	Clearing & storage	

Table IV Single staining protocol (protocol 3) according to Walker and Kimmel (2007)

Step	Duration	Significance of the step	Solutions
Ethanol 50%	10 minutes	Hydratation of the sample	For 100 ml: 50 ml absolute ethanol 50 ml distilled water
Alizarin red	Overnight	Staining bone	For 100 ml 0.5 g alizarin red 100 ml distilled water
20% glycerol 0.25% KOH	30 minutes to overnight	Clearing	For 100 ml: 20 ml glycerol 80 ml 0.25% KOH
50% glycerol 0.25% KOH	2 h to overnight	Clearing	For 100 ml: 50 ml glycerol 50 ml 0.25% KOH
50% glycerol 0.1% KOH		Clearing & Storage (4℃)	For 100 ml 50 ml glycerol 50 ml 0.1% KOH

2.1.4 OSTEOLOGICAL DEVELOPMENT OF OLD STORED BUFFERED FORMALDHEYDE LARVAE

A total number of 139 sea bream larvae bred in a semi-intensive system (mesocosm system) (see paragraph 2.1.1) were measured, stained with the best staining method previously detected and observed under a stereomicroscope (Leica DM, 2500, Germany) in order to obtain information on the general

osteological development of sea bream larvae in the first day of life. Larvae of 10, 17, 21, 28, 31, 35, 45 and 50 dah were analyzed and described.

Moreover, 5 larvae from 35, 45 and 50 dah each, from the same experiment were also considered in order to analyze the development of the skeletal elements and to evaluate vertebral dimension of this species in early life stages when reared in semi-intensive conditions (mesocosm).

The nomenclature used was the one of Faustino and Power (1998) and Koumoundourous *et al.* (2001). Description was done considering both larval age and standard length. General description of skull structures was done using terminology reported in Gregory (1933).

Vertebral body dimensions were standardized with the standard length of each larva. Measures were expressed in millimetres at the third decimal part.

Measures taken were as showed in Figure 8.

- Vertebral length at the dorsal side
- Vertebral length at ventral side
- Vertebral height cranial (anterior) side (a.h.)
- Vertebral height caudal (posterior) side (p.h.)
- Ratio a.h/p.h

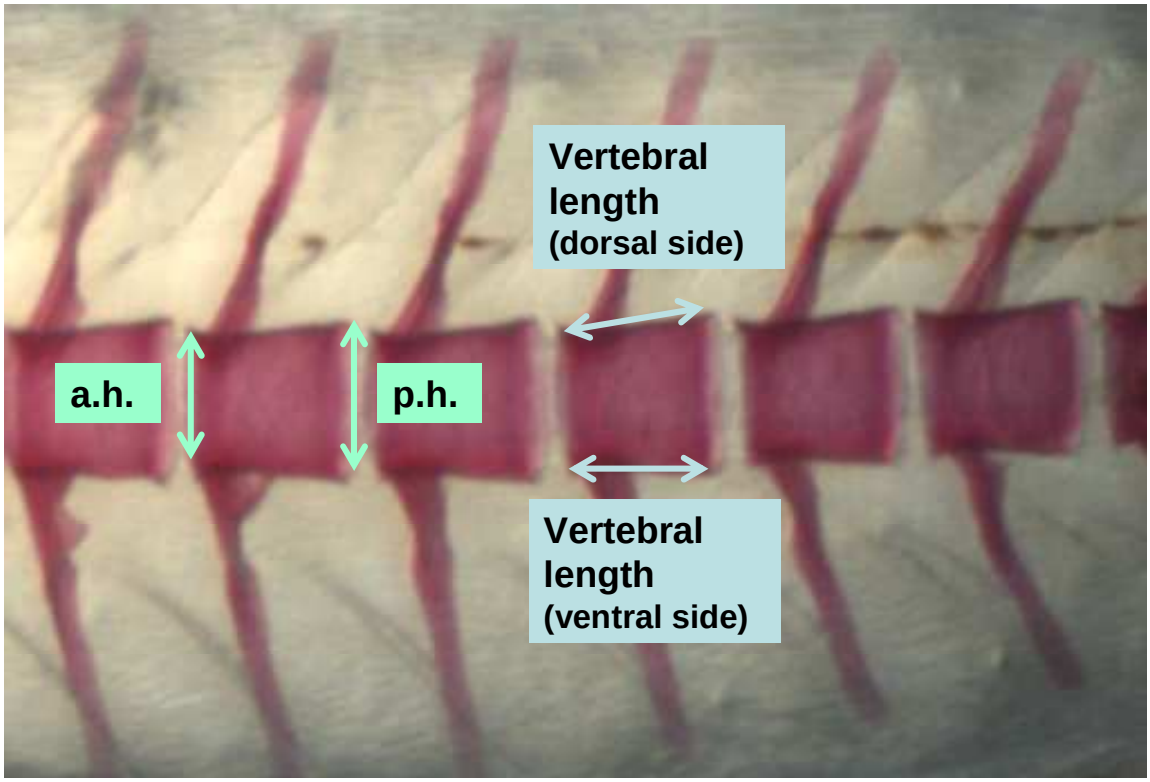


Figure 8 Measures taken in mesocosm bred larvae of 35, 45, 50 dah.

2.2 TRIAL II EFFECTS OF DIFFERENT DHA ENRICHMENT LEVELS ON EARLY MINERALIZATION AND BONE FORMATION IN THE AXIAL SKELETON OF *SPARUS AURATA*

The objective of this trial was to evaluate the effect of different levels of DHA (docosahexaenoic acid) and different levels of vitamin E during the feeding period with live preys (rotifers) on the skeletal development and vertebral formation of gilthead sea bream and the possible anomalies derived at the end of a period of 34 days.

2.2.1 EXPERIMENTAL DESIGN

The trial was conducted feeding sea bream larvae from 2 to 25 dah with differently enriched rotifers (different for their DHA levels and vitamin E content) (Table V) and therefore with commercially enriched Artemia and commercial dry food up to 34 dah. The trial was conducted in quadruplicate (a total of 20 experimental tanks) until15 dah, and in triplicate from 15 to 34 dah (15 tanks).

Growth biological parameters (length and dry weight) were measured at 3 dah (start feeding), when Artemia feeding started (15 dah) when dry feed start (20 dah) and at the end of the experiment (34 dah). A total of 120 larvae per treatment (30 x quadruplicate) were sacrificed at the beginning and at 15 dah, while a total of 90 larvae per treatment (30 larvae per triplicate) were sacrificed at 20 and 35 dah.

One tank per treatment was entirely sacrificed to obtain samples for proximal analysis on entire larvae before Artemia feeding started (15 dah).

Ingestion was checked every three days on 5 larvae per tank (20 larvae per treatment) until the end of the experiment. Swimbladder inflation was checked at 7 and 12 dah[1].

At 34 dah (end of the experimental period) 50 larvae per tank (150 per treatment) were stored in buffered (10% phosphate buffer) formalin and checked for skeletal column deformities once stained with alizarin red following the protocol of Vandewalle *et al.* (1998).

Proximate composition of eggs, rotifers, larvae, experimental enrichments and the products employed to prepare them were conducted too (crude proteins, total, neutral and polar lipids, moisture, ash).

In particular, rotifers from each feeding step were sampled, pooled and analyzed per treatment every three days (8 pools – 40 total samples).

2.2.2 ENRICHEMENT PREPARATION

The levels of DHA and vitamin E were achieved using two different commercial products: a rotifer enrichement (DHA Protein Selco™, Inve, Dendermonde, Belgium) and DHA capsules (MorDHA omega-3 I.Q™, Minami Nutrition, U.K.), differently mixed and/or manipulated in order to achieve the desired content of DHA and vitamin E. The general characteristics of the products used to prepare the different enrichments are presented in Table V, while their total lipids fatty acids profiles are presented in Table VI.

[1] Sea bream is indeed a fisoclist fish. In its larval phase larvae inflate two times the swim bladder. The first (incomplete) is around 7 dah, the second one is around 12 dah, when the connection between the esophagus and the swim bladder is closed. If larvae do not inflate swim bladder during this time, they will not be able to move along the water column, generally will sink and die.

Table V DHA and vitamin E content of the two commercial products used for the preparation of the experimental enrichments

	DHA content (% DW)*	Vitamin E content (mg kg^{-1})**
DHA Protein Selco™	3.9	7,200
MorDHA omega-3 I.Q.™	69.76	3,487

DW: dry weight basis;
* DHA: analyzed;
** vit E: Producer provided.

Table VI Total lipids fatty acids profiles of the two products used to prepare the five experimental enrichments

	DHA Protein Selco™		MorDHA omega-3 I.Q. ™	
	% TFA	**% DW**	**% TFA**	**% DW**
14:0	1.98	0.43	0.06	0.06
14:1n-7	0.07	0.01	-	-
14:1n-5	0.03	0.01	-	-
15:0	0.42	0.09	-	-
15:1n-5	< 0.01	< 0.01	-	-
16:0ISO	0.05	0.01	-	-
16:0	22.40	4.92	0.17	0.16
16:1n-7	5.55	1.22	0.33	0.31
16:1n-5	0.04	0.01	0.02	0.02
16:2n-6	0.67	0.15	0.06	0.05
16:2n-4	0.58	0.13	0.04	0.03
17:0	0.59	0.13	0.08	0.07
16:3n-3	0.12	0.03	-	-
16:3n-1	0.02	< 0.01	-	-
16:4n-3	0.13	0.03	0.12	0.11
18:0	5.11	1.12	0.13	0.12
18:1n-9	15.69	3.45	0.52	0.49
18:1n-7	2.19	0.48	0.17	0.17
18:1n-5	0.16	0.03	-	-
18:2n-9	0.06	0.01	0.02	0.02
18:2n-6	8.89	1.95	0.15	0.15
18:2n-4	0.16	0.03	-	-
18:3n-6	0.23	0.05	0.03	0.03
18:3n-4	0.07	0.02	-	-
18:3n-3	1.30	0.28	0.07	0.06
18:4n-3	0.69	0.15	0.46	0.44
18:4n-1	0.03	0.01	-	-
20:0	0.24	0.05	0.62	0.59
20:1n-9+n-7	1.63	0.36	1.05	1.00
20:1n-5	0.20	0.04	0.17	0.16
20:2n-9	0.05	0.01	0.02	0.02
20:2n-6	0.19	0.04	0.17	0.16
20:3n-9	0.04	0.01	-	-
20:3n-6	0.10	0.02	0.04	0.03
20:4n-6	1.14	0.25	0.38	0.36
20:3n-3	0.12	0.03	0.13	0.13
20:4n-3	0.49	0.11	0.62	0.59
20:5n-3	7.21	1.58	9.38	8.86
22:1n-11	0.85	0.19	5.39	5.09
22:1n-9	0.27	0.06	0.77	0.72
22:4n-6	0.15	0.03	0.39	0.37
22:5n-6	0.78	0.17	1.47	1.39
22:5n-3	1.59	0.35	3.10	2.93
22:6n-3	17.76	3.90	73.85	69.76

DHA Protein Selco™ (INVE, Dendermonde, Belgium).
MorDHA omega-3 I.Q.™ (Minami Nutrition, U. K.).
- indicates component no detected.

The three maximum desired levels of DHA in rotifers for the five different enrichments were 0.5%, 2%, 5% (in % of total lipids dry weight). The previous cited levels of DHA in rotifers were achieved after an enrichment trial feeding rotifers with the two enrichment products, which results are reported in Annex I.

Enrichement 1 (DHA low, DL) was prepared, defatting DHA Protein Selco™ (Inve, Dendermonde, Belgium) washing the product 3 times with chloroform (3:1 v/v ratio chloroform/enrichment) and by adding, to the solid derived, the same quantities of eicosapentaenoic acid (EPA) (EPA50, CRODA, U.K.) and arachidonic acid (ARA) (ARA, CRODA, U.K.) as the entire product. Also vitamin D3 (cholecalciferol, Sigma-Aldrich, Madrid, Spain) and vitamin A (retinol, Sigma-Aldrich, Madrid, Spain) were added in order to get to the same profile of the original product. Vitamin E (DL- α-tocopherol acetate, Sigma-Aldrich, Madrid, Spain) was added in order to obtain a concentration of 10687 mg kg^{-1} (vitamin E content in enrichment 3 – highest in DHA). Oleic acid was added to complete the total lipid content of the entire product.

Enrichment 2 (DHA medium, DM) was the entire DHA Protein Selco™ plus vitamin E up to reach the concentration of 10687 mg kg^{-1} (vitamin E content in enrichment 3 – highest in DHA).

Enrichment 3 (DHA high, DH) was prepared combining DHA Protein Selco™ and MorDHA omega-3 I.Q.™ (Minami Nutrition, U.K.) capsules after an enrichment trial on rotifers (Annex I).

Enrichment 4 (DHA medium plus vitamin E, DME) was prepared adding to entire DHA Protein Selco™ an aliquot of vitamin E in order to reach the concentration of 15000 mg kg^{-1} of the original product.

Enrichement 5 (DHA high plus vitamin E, DHE) was prepared as enrichment 3 adding an aliquot of vitamin E in order to reach, in the final enrichment, a concentration of 15000 mg kg^{-1} of the original product.

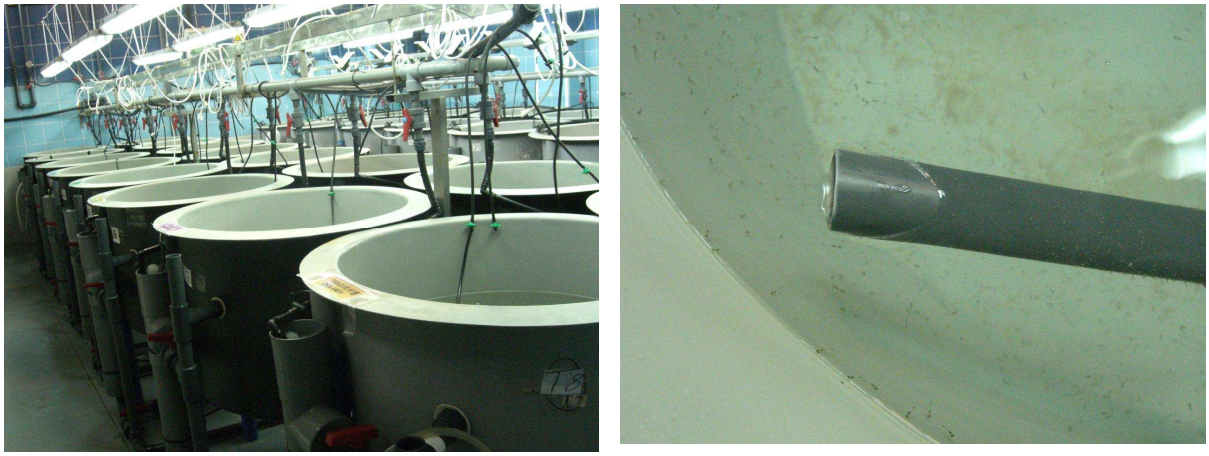
Once prepared, enrichments were stored at + 4℃ for the entire duration of the experiment.

2.2.3 EXPERIMENTAL PROCEDURES

Eggs from a natural mass spawning of sea bream broodstock of the ICCM (Instituto Canario de Ciencias Marinas, Canary Islands, Spain) were seed by weight of eggs at a concentration of 120 eggs per litre of water and incubated for 34 days post hatch in cylindro-conical 170 l tanks from the 29th January 2010 until the 5th March of the same year (Figure 9A).

Eggs showed a percentage of no-viable eggs of 2% in the floating fraction, the hatching rate was of 80.5% and survival at 3 dah of 89.06%.

Therefore, approximately 15 grams[2] were seed in each tank.



A **B**

Figure 9 A: experimental cylindro - conical tanks used in trial II. **B**: surface skimmer used to clean the surface of the tanks.

Sea bream experimental groups were 5 with four replicates until 15 dah, when one tank from each treatment was sacrificed to obtain sample for the biochemistry of larvae previous to the introduction of the live food *Artemia* sp.

[2] One gram of sea bream eggs contains on average 1285 eggs.

Larvae were fed the different enriched rotifers (*Brachionius plicatilis*) together with phytoplankton (*Nanochloropsis gaditiana*) from 3 dah until 25 dah, following Roo *et al.* (2009).

Rotifers were mass cultivated and enriched as described in Roo *et al.* (2009), but with an enrichment process that lasted 8 hours. Enrichment was added following the routine method of the research group (0.125 g enrichment per litre of water).

From 15 dah started the co-alimentation with enriched (DHA Easy Selco™, Inve, Belgium) *Artemia* sp. Instar I (A_0, nauplius until day 24) following the routine method of the research group (0.6 g per L of water). From 24 dah, co-alimentation with dry feed (Gemma Micra, Skettring, France) and with enriched *Artemia* sp. Instar II (A_1, metanauplius) started. A diagrammatic representation of the feeding sequence is reported in Figure 10.

Light regime was fixed at 12D: 12L (dark: light) and was achieved through fluorescent lamps remotely controlled, the air flow to the tanks was maintained on 170 ml min^{-1}, salinity was checked at the beginning of the experiment (38 ppm) with a refractometer. Routine procedures implied checking temperature and oxygen daily at h: 12:00 and 18:00 with a portable oxymeter (Oxyguard Polaris, Denmark), pH weekly with a portable pHmeter (HM Digital Inc., USA) when live preys were present, and twice a week when dry feed started. Average values of the physical parameters along the entire experiment are showed in Table VII.

Tank surface was cleaned with a skimmer (Figure 9B) after every feeding step starting from 6 dah until Artemia started to be added. Therefore, the whole tanks were siphoned half an hour after every feeding administration until the end of the experiment when Artemia started to be added.

A schematic diagram of the routine procedures is represented in Figure 10.

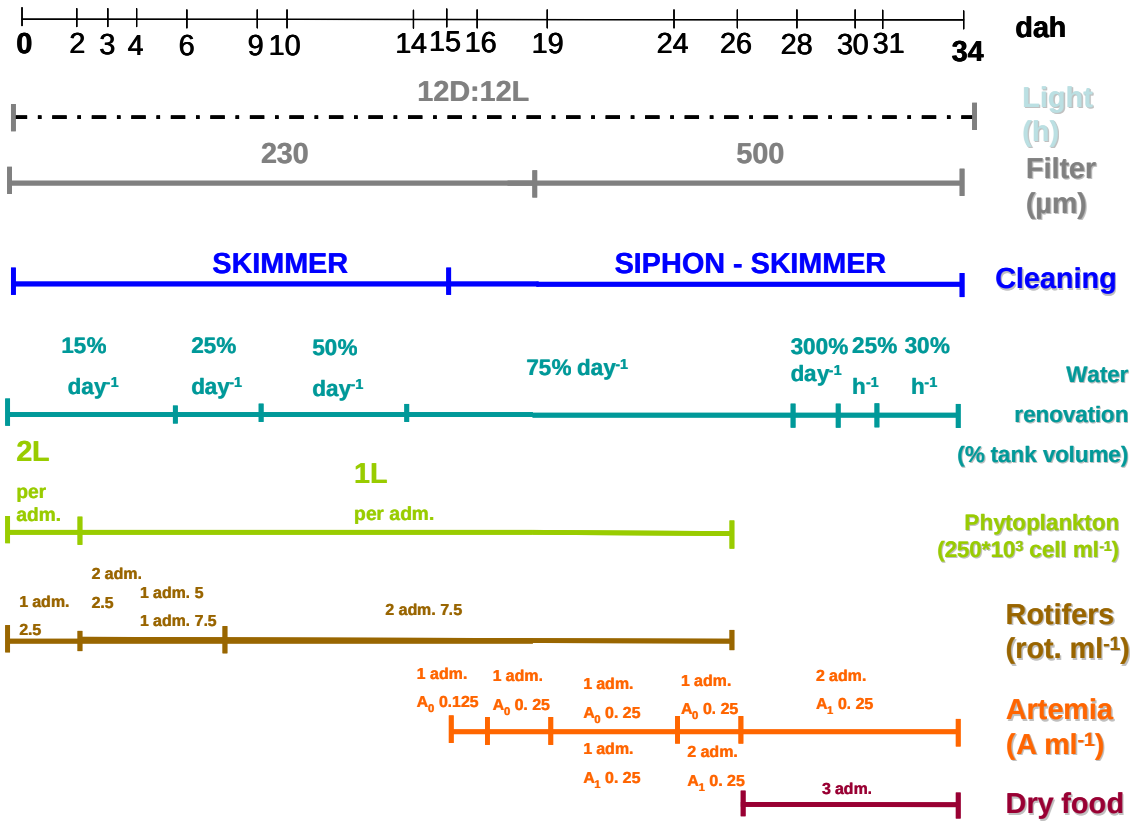


Figure 10 Scheme representation of the feeding sequence and cultivation routine during the experiment. D:L means hours of dark and hours of light. Adm.: indicates the number of food administration.

Table VII Average values (± s.d.) of water quality parameters along the 35 days of experimentation

Temperature (℃)	***Dissolved oxygen (ppm)***	***pH***	***Salinity (ppm)***
20.66 ± 0.65	5.87 ± 0.31	8.26 ± 0.08	38

2.2.4 SAMPLING PROCEDURES

2.2.4.1 BIOMETRIC MEASURES AND DRY WEIGHT

Biometric measures and dry weight determination were done on 30 larvae per tank at 3, 15, 20 and 34 dah. Larvae were directly caught from the rearing tanks with a non-sterile 3 ml plastic pipette and placed onto a clean crystal slide. Measures were taken in a profile projector (Mitutoyo, PJ-A3000, Japan). Standard length (SL) was chosen in order to avoid errors, if larvae, during manipulation lose the caudal fin. Standard length was measured as described in Faustino and Power (1998). Results were expressed in millimetres up to the second decimal part.

The same larvae used for length measurements, were used for dry weight determination (105ºC for 24 h and 1h until constant weight was reached) (Atalah, 2008).

2.2.4.2 SURVIVAL RATES DETERMINATION

Survival rate was established for the 5 different treatment groups without triplicate at 15 dah and on three tanks per treatment at 34 dah.

To establish the survived larvae, larvae from each tank were manually counted as shown in Figure 11 (Atalah, 2008; Roo, 2009).

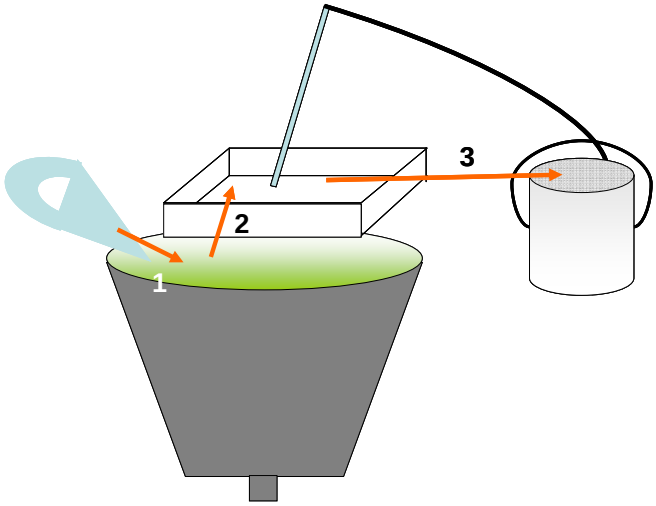


Figure 11 Sampling technique to determine survival rates. 1- Larvae are caught from the tank with a cup, 2- they are transferred into a tray , 3- they are counted while transferred with a pipette into a bucket with water.

2.2.4.3 LARVAL HANDLING FOR WHOLE MOUNT STAIN

Larvae sampled for deformities at 34 dah, were fixed and stored in buffered (10% phosphate) formaldehyde after a light sedation with 10% clove oil solution.

2.2.5 ACTIVITY TEST

To evaluate the potential effects of the live prey enrichments on sea bream larvae, an activity test was carried out. Fifteen larvae per tank at 20 dah (five day after introduction of Artemia and after 20 days alimentation with rotifers) were submitted to an acute stress handling them out of the water for 60 seconds and returning them to a bucket with aerated fresh water. Survival rate was then determined 24 hours later, manually counting surviving larvae (Izquierdo *et al.*, 1989). Results were expressed as percentage of survived larvae.

2.2.6 TREATMENT OF LARVAE FOR WHOLE MOUNT STAIN, CLASSIFICATION OF SKELETAL ANOMALIES AND EVALUATION OF THE EFFECTS OF THE DIET ON SKELETAL MINERALIZATION

Fifty specimens from each tank of 34 dah were randomly chosen and measured for their standard length under a profile projector (Mitutoyo, PJ 3000, Japan) three weeks after formaldehyde storage. They were separated in 32 size classes of 0.2 mm SL starting from 4.285 mm up to 10.685 mm and therefore stained with alizarin red following Vandewalle *et al.* (1998). Staining procedures were accomplished in one week time, in order to avoid errors due to staining. The contemporary evaluation of skeletal anomalies and development was done in two weeks time.

The different regions of the axial column were divided according to Boglione and collaborators (2001).

Vertebrae were numerated from one to twenty four using Roman numerals in caudal direction.

Axial skeleton anomalies (kyphosis, lordosis, scoliosis, vertebral fusions, neural and haemal spines anomalies), maxillary – mandibular and opercular anomalies were considered. Anomalies affecting the caudal fin complex were not considered due to the high natural variability of the skeletal structures in this region (Koumoundourous *et al.*, 1997a; Grünbaum and Cloutier, 2009; Bensimon-Brito *et al.*, 2010).

The presence of additional vertebral bodies, the presence of urinary calculus and anomalies different from the ones described by Boglione *et al.* (2001) were analyzed separately.

The effects of the different enriched rotifers on the axial skeleton mineralization were evaluated considering the total number of completely mineralized vertebral bodies, in each size class per treatment.

2.2.7 ANALYTICAL PROCEDURES (PROXIMATE COMPOSITION)

All the biological material for proximal analysis was stored at -80° C until processing. Each sample was analyzed in triplicate.

Rotifers from each feeding step were filtered with a 500 µm mesh, dried at best and stored in plastic bags at -20° C up to three days. Therefore they were pooled together in eight different pools and stored at -80° C until analysis. Larvae collected for biochemical analysis were caught before the first feeding in the morning and directly stored at -80° C.

Enrichments were stored at + 4º C until analysis.

Total lipids extraction was based on the method described by Folch and collaborators (1957) using an extraction mix of chloroform: methanol (2:1 v/v) with BHT (Butylated hydroxytoluene, an industrial antioxidant).

Separation of neutral and polar lipids was conducted on larvae samples of 15 and 34 dah.

The procedure consists of selectively separate neutral and polar lipids from a sample of total lipids through adsorption chromatography on silica cartridges (Sep-

pak; Waters S.A., Massachussets, USA). Thirty ml of chloroform and 20 ml of chloroform/methanol (49: 1, v/v) were used as solvent for neutral lipid, followed by a 30 ml methanol wash to obtain the polar fractions according to Juaneda and Rocquelin (1985).

For the determination of fatty acids, total lipids were trans-esterifed with sulfidric acid: absolute methanol 1% (Christie, 1989). If not directly metilated, the extracted lipids were stored in chloroform in an atmosphere of nitrogen at -20℃. Separation and identification of the fatty acids was realized with gas chromatography (GC) (GC Termo Finnigan Fucus GC, Milan, Italia) under the conditions reported in Izquierdo *et al.* (1990).

Determination of crude proteins was accomplished though the technique of Kjieldall (A.O.A.C., 1995) considering the total nitrogen present in the samples.

Moisture was always evaluated contemporary to the extraction of total lipids in order to obtain the percentage of total lipids on a dry weight basis (A.O.A.C., 1995).

Vitamin E content was analyzed only on the five different enrichments due to the high quantity of sample necessary for α- tocopherol determination. Samples were analyzed by the Laboratorio de Diagnóstico General (Barcelona, Spain) by HPLC (high pressure liquid chromatography).

2.2.8 MEASUREMENT OF THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS) OF LARVAE

The measurement of TBARS was carried out on 15 dah larvae. The method was adapted from that of Burk *et al.* (1980) as reported in Tocher *et al.* (2002). Values were expressed as nanomoles of malonaldheyde (M) per gram of sample.

2.2.9 STATISTICAL ANALYSIS

Statistical differences were checked with one-way ANOVA in the case of more than two samples or with a Student t-test for two samples mean comparisons, once the conditions for the application of parametric tests (homogeneity of the variance with Levene's test, data normality with Shapiro-Wilk test) were confirmed using calculus prepared sheets with Microsoft Excel™ 2003. In case of statistical differences among the treatment groups, a Tukey post-hoc test was applied in order to evaluate inter-group differences. Significance level was fixed at 95%. 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

3. RESULTS

3.1 TRIAL I: DIFFERENT WHOLE MOUNT STAINING PROTOCOLS AND VISUALISATION OF SKELETAL DEVELOPMENT IN LARVAE STORED LONG-TERM IN FORMALDHEYDE

3.1.1 STAININGS PROTOCOLS

The most numerous size class of larvae used for the staining trial had a standard length ranging between 4.0-4.2 mm for 10 dah larvae, 5.4-5.6 mm for 17 dah larvae, 6.4-6.6 mm for 21 dah larvae and 28 dah, 6.8-7.0 mm for 31 dah larvae. The ranges of standard length for each age were between 3.4 and 4.4 mm for 10 dah larvae, from 5.0 up to 6.8 mm for 17 dah larvae, from 5.2 to 7 mm for 21 dah larvae, from 5.8 to 7.4 mm for 28 dah and from 6.2 up to 8.6 mm for 31 dah larvae. At 21 dah larvae showed a precocious flexion of the notochord. In all test tubes pH of formaldehyde was 6.7.

Considering all larval length and ages, the whole mount staining protocol proposed by Walker and Kimmel (2007) showed a completely nonspecific staining in the entire larvae, therefore this protocol was not used for the following trials (Figure 12). Both double staining Taylor and Van Dyke (1985) combined with the one of Dingerkus and Uhler (1977) and the single alizarin red staining (Vandewalle *et al.*, 1998) successfully stained the entire larvae (Figure 12).

In larvae of 21 dah (6.4-6.6 mm of standard length), both single alizarin staining and double staining revealed the same skeletal structures but the older age (28 dah) showed differences between double staining and

single alizarin staining. Thus, in double staining larvae, the vertebral elements were not distinguishable, while, in single stained larvae, up to 7 vertebral bodies (from vertebra II to vertebra VIII) and 8 neural spines were visible.

Cranial structures, as well as the urostyle and four hypurals were almost equally stained by both techniques. In the same way, larvae from 31 dah (6.8 - 7.0 mm) showed differences at level of the axial skeletal staining between double staining and single alizarin staining. Also in this case, double staining did not reveal the mineralization of vertebral bodies, while in single stained alizarin red larvae, up to 14 vertebral bodies (vertebra (v.) II up to v. XV) and 16 neural spines were distinguishable.

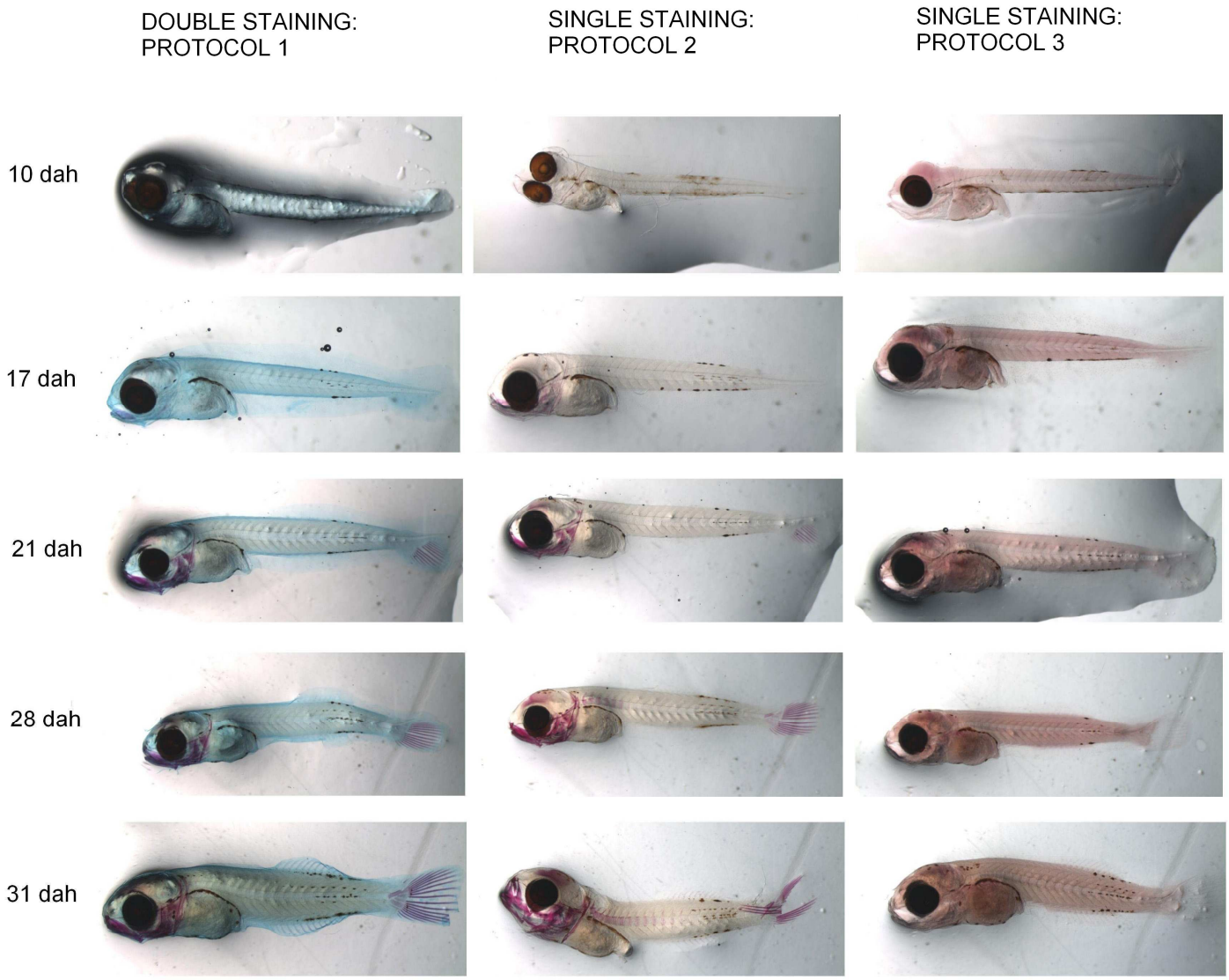
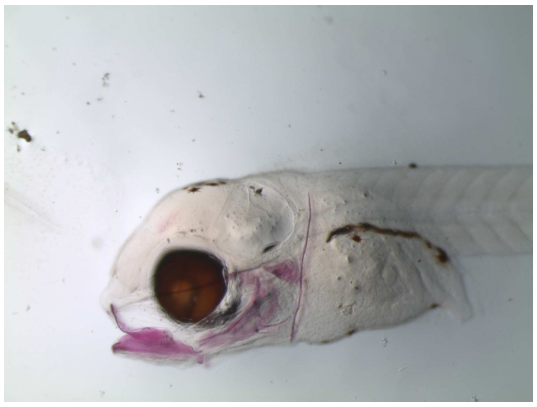


Figure 12 Resuming graphical table for the three different staining protocols checked in trial I.

3.1.2 OSTEOLOGICAL DEVELOPMENT OF LARVAE STORED LONG-TERM IN FORMALDHEYDE



A

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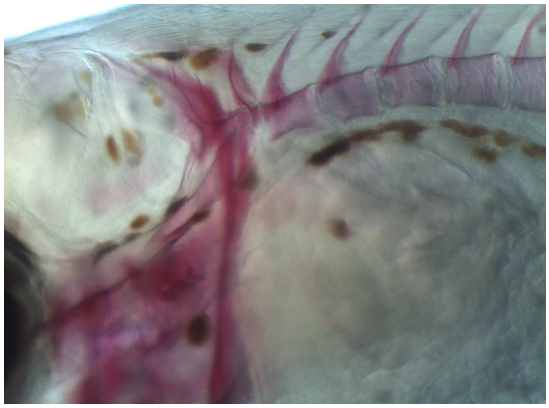

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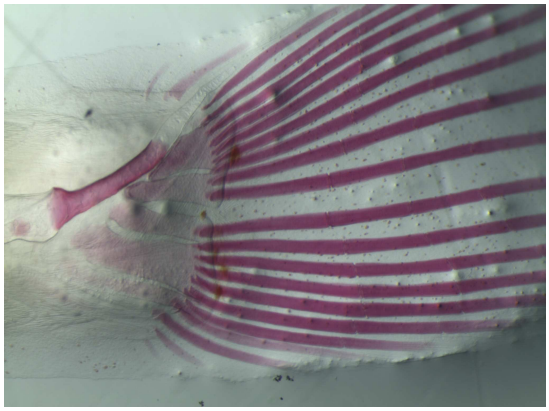

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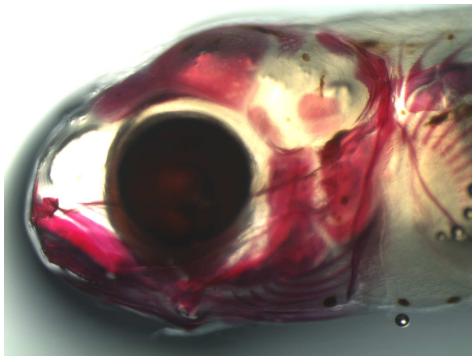

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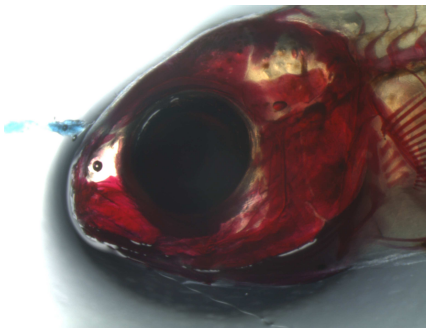

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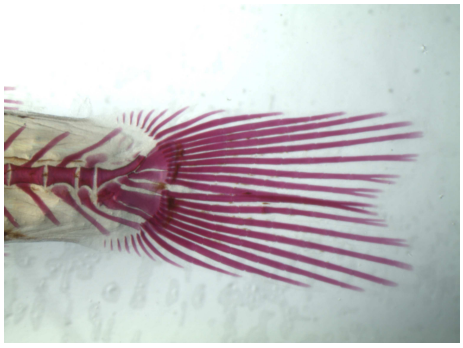

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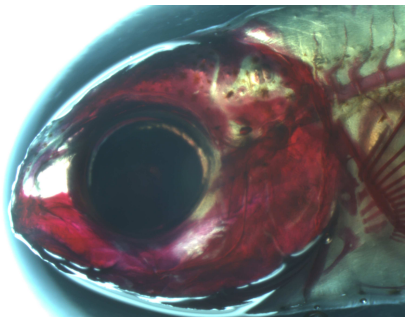

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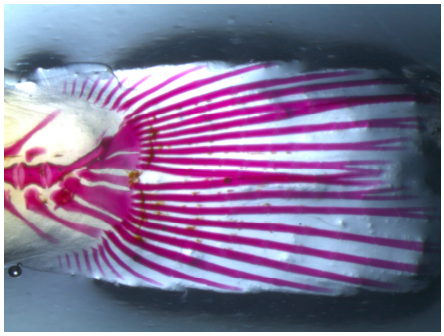
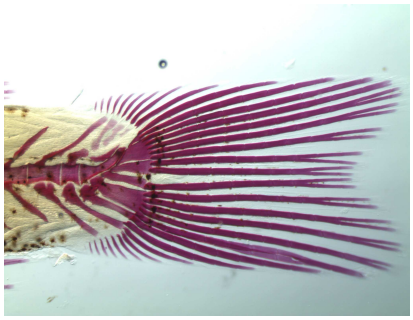


P

Q

R



S

Figure 13 Various stages of *S.aurata* osteological development. Alizarin red single staining. **A** – head region (17 dah 5 mm SL); **B** – whole larvae 21 dah 6.2 - 6.4mm SL; **C** – cranial and prehaemal region (28 dah 6.4-6.6 mm SL); **D** – cranial and prehaemal region (31 dah 7.2-7.4 mm SL); **E** – entire column (31 dah 7.2-7.4 mm SL); **F** – caudal region (31 dah 7.2-7.4 mm SL); **G** – entire larva (35 dah 8.09 mm SL) **H** – head region (35 dah 8.09 mm SL); **I** – entire larva (45 dah 10.9 mm SL); **L** – head region (45 dah 10.9 mm SL); **M** – caudal region (45 dah 10.9 mm SL); **N** – entire larva (45 dah 12.16 mm SL); **O** – head region (45 dah 12.16 mm SL); **P** – caudal region (45 dah 12.16 mm SL); **Q** – entire larva (50 dah 12.57 mm SL); **R** – head region (50 dah 12.57 mm SL); **S** – caudal region (50 dah 12.57 mm).

In early larval stages sea bream larvae had a straight notochord for the whole of its length. At 10 dah (SL between 3.4 - 4.4 mm) specimens were principally represented by 4.0 to 4.4 mm size class (Figure 7). Notochord was still not flexed, nor any axial skeleton elements could be recognized and the only mineralized structures were the chleitrum and the pre-maxilla.

At 17 dah larvae had a standard length between 5.0 mm and 6.6 mm with the majority of the specimens in the size class between 5.2 and 5.4 mm. Around 5 mm of standard length notochord was still not flexed and no axial skeleton elements could be recognized (Figure 12). The principal differences with the previous day/length considered were at cranial level. Indeed, new cranial elements started to mineralize, such as the trabecula (that will develop into the parasphenoid bone), the preoperculum and subopercular, some opercular spines and two or

three brachiostegals. Dentary and articular bones could be recognized, even though they showed a weak mineralization. A slight and little zone of mineralization was the parietal bone (Figure 13 A). Even though the larva did not show any signs of flexion, in the caudal complex, 7 up to 11 rays of the caudal complex were weakly mineralized in their central part.

Between 5.8 – 6 mm of SL (both larvae of 17 and 21 dah), larvae started to flex the notochord and a more extended zone of the parietal bone is mineralized. The complex of the hyoid arch started to be defined with symplectic bone and the hyomandibular. From 7 to 11 elements of the principal caudal rays (PCR) were mineralized (Figure 13 B).

At 28 dah larvae showed a standard length between 5.8 and 7.4 mm with the majority with a standard length between 6.6 and 6.8 mm. The same size classes were presented in larvae of 31 dah plus a single individual between 8.4 and 8.6 mm with the majority of the individuals presenting a standard length between 7.2 and 7.4 mm.

From 5.8 mm the notochord was flexed, and up to 17 rays of PCR, the urostyle and four of the five hypurals were defined and mineralized. Also one ray of the dorsal secondary caudal rays (SCR) could be occasionally seen in specimen starting from 6.8 mm of SL.

The cranial structures already present at 6 mm SL belonging to the mandibular arch (maxilla, pre maxilla, mandibula, angular and articular) were more strongly mineralized, and mineralization of the hyomandibula and the symplectic bone (hyoid arch complex) could be recognized for

the first time. Mineralized teeth were found in one specimen of this size class.

At the vertebral column, a variable number (from two up to nine) of mineralized neural spines became visible starting from number II up to number VIII (SL between 6.2 to 6.4 mm). In general the last three spines were only mineralized in their central part. Visibly mineralised vertebral centra at this stage correspond to v. II up to v. XII. Generally from v. II to v. V a strong mineralization can be detected, while following vertebral centra only showed a weak but complete mineralization of the notochord sheath which can be seen at v. VI up to v. IX. Mineralization could be only seen in the ventral portion of the notochord for v. X up to XIII. In the larger size class (between 6.4-6.6 mm SL), the same pattern of mineralization of the vertebral column elements could be identified (Figure 13 C). The only differences with the previous size class consisted on a recognisable weak mineralization of the first four haemal spines (from I to IV) in the central part.

Larvae between 6.6-6.8 mm SL did not showed significant differences with the previous size class but now almost all individuals showed mineralized teeth and a more extended mineralization of the frontal bone. The opercular complex showed strong mineralization of opercular, interopercular and subopercular bones. The caudal complex occasionally showed mineralization of the central part of the V^{th} hypural.

Axial skeletal elements did not show evident variations in comparison with the previous size class, but a slight mineralization of the neural spine on v. I became visible.

Specimens belonging to size class between 7.0-7.2 mm SL, showed mineralization of the first 13 neural spines in cranial to caudal direction with the following four spines (from the XIVth to the XVIIIth) being mineralized only in the central portion of the spine. Almost eight haemal spines were slightly mineralized, even though the last three only in the central part of its structure. More or less 14 vertebral bodies are distinguishable with decreasing degree of mineralization in cranial to caudal direction. Vertebra I started to mineralize beginning from the dorsal side of the notochord in opposition to the majority of the other vertebral bodies that started their mineralization from the ventral side.

In the caudal complex four of the five hypurals were mineralized and the parhypural presented ossification close to the caudal rays. Hypural III and IV were well defined and completely mineralised. Sixteen fin rays of the PCR and one of both dorsal and ventral SCR were mineralized.

The cranium showed a wide zone of mineralization in the frontal region and a strong mineralization of the post temporal bone. Also strongly mineralized are the bones of the pelvic girdle (cleithrum, postcleithrum, supracleithrum).

Specimens between 7.2-7.4 mm showed up to 18 mineralized neural spines, with a decreasing degree of mineralization in cranial to caudal direction. Generally, the last three neural spines were mineralized only in the central part of the structure. Haemal spines were generally less

mineralized but up to the eight elements could be distinguished. All vertebral centra could be visualized with alizarin red staining, with different degrees of mineralization. Vertebra I only showed mineralization at its dorsal part (Figure 13 D). Vertebrae v. II to v. XVII showed sharp borders of the vertebral body and a homogenous slight mineralization of the notochord sheath. Vertebrae v. XVIII to v. XX showed a complete mineralization along the notochord but with blurred borders, while v. XXI-XXII-XXIII showed an incomplete mineralization along the notochord height and were not accompanied by neural spines (Figure 13 E).

In the caudal complex level the parhypural started to mineralize towards the urostyle. Seventeen PCR are completely mineralized and three and two SCR were mineralized at the dorsal and ventral side of the caudal complex respectively (Figure 13 F).

In the cranium, frontal and parietal bones were more widely mineralized, as well as the opercular complex, the pelvic girdle and the mandibular arch with clearly visible teeth.

Larvae of 35 dah presented an average standard length of 8.33 mm (± 0.65 mm). Considering also specimens of the same age but of 8 mm SL, 20 of the 23 vertebral bodies were well developed starting from v. I up to v. XX plus the urostyle which were completely mineralized. Vertebral bodies of v. XXI XXII were not completely defined and only a complete first mineralization of the notochord sheath could be observed. Vertebra XXIII presented only slight mineralization of the nothochord sheath at the haemal side (almost 1/3 of the nothochord height) (Figure 13 G).

The associated neural spines were well defined up to v. XIX, while the neural spine XX, although completely mineralized showed weaker mineralization.

All parapophysis were precisely distinguishable as well as all haemal spines with the same pattern than the neural ones. Pleural ribs were still not distinguishable.

The caudal complex presented all 17 caudal rays of the PCR completely mineralized as well as three respectively in the dorsal and in the ventral SCR. Four of the five hypurals were well mineralized, while the parahypural showed only mineralization in its most distal part. The fifth hypural was not mineralized neither all epural elements.

In the cranium (Figure 13 H), the entire opercular complex, as well as the elements of the mandibular arch were completely mineralized. Slight, but complete mineralization could be detected at the parietal, frontal and supraoccipital bones while slighter and less extended mineralized zone was found in the sphenotic and pterotic bones. Hyomandibular and quadrate bones showed a strong mineralization, while no signs of mineralization could be detected at the metapterygoid bone. In the shoulder girdle the suprachleitrum is now mineralized.

At 45 dah examined larvae presented an average standard length of 11.30 mm (± 0.93 mm). The smallest specimen analyzed had a standard length of 10.9 mm (Figure 13 I). At this length, in the vertebral column all neural and haemal spines and the pectoral, anal and dorsal fin elements were mineralized. Pleural ribs were also mineralized.

Dorsal fin elements were completely mineralized (proximal and distal radial and all the relative rays), and only the predorsals were still not mineralized. The proximal radials of the anal fin are almost completely mineralized, even though the proximal radials that sustain the three hard spines were only slight mineralized.

The caudal complex showed mineralization of all the hypurals. The hypural II and the hypural I started to fuse with the parahypural. Slight signs of the three epurals and of the uroneural I could be identified. Secondary caudal rays (SCR) were 8 in the dorsal side and 6 in the ventral part, 17 principal fin rays were present (Figure 13 M).

The entire opercular complex, the hyoid arch and the mandibular complex were mineralised. In the post-cranial region, the pectoral girdle was completely mineralised. (Figure 13 L).

The paleoquadrate complex was almost complete even though entopterigoid and the bones close to the circumorbital series were not completely mineralized. In the olfactory zone nasal and lacrimal bones were mineralized but not up to the orbital region. In the occipital region, the pterotic bone was not completely mineralized.

At 12.16 mm principal differences occurred principally in the fin skeleton and in the head skeleton. In the caudal complex, a stronger definition of the uroneural I and the presence of an additional ray in the ventral SCR could be recognized (Figure 13 P). In the dorsal fin an ossified predorsal could be observed. In the anal fin, the proximal radials of the first two spines were highly ossified (Figure 13 N). In the cranium, the occipital bones were almost completely ossified as well as the bones of the

paleoquadrate. Now also the mineralization of the entopterygoid was clearly complete (Figure 13 O).

At 50 dah examined larvae presented an average standard length of 12.22 mm (± 0.61 mm) (Figure 13 Q). As well as the previous stage, principal differences regarded to increased mineralization of the fin skeletal structures and the cranium.

The examined specimens displayed caudal fins with 9 SCR both at the ventral and the dorsal side. Now the uroneural was perfectly ossified and the three epurals were clearly but less mineralised (Figure 13 S). The anal and the dorsal fin skeleton were perfectly mineralized as well as the three pre-dorsals.

The occipital region shows an almost completely mineralised cranium, the frontal bone was almost connected to the nasal and the slightly less mineralized palatine can be identified (Figure 13 R).

3.1.2.1 EVALUATION OF VERTEBRAL DIMENSION OF LARVAE STORED LONG-TERM IN FORMALDHEYDE

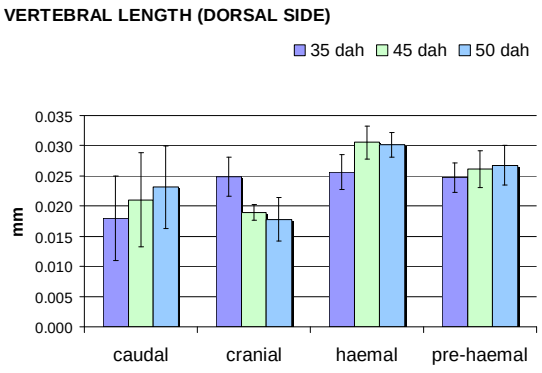
Values of the vertebral dimension of sea bream larvae of 35, 45 and 50 dah are reported in Table VIII and in Figure 14 A,B,C,D,E.

In mesocosm sea bream larvae analyzed in this study, since 35 dah, haemal and pre haemal regions account for the majority of total vertebral length both considering vertebral length at neural (dorsal) and haemal (ventral) side (~ 30% and 27% respectively in all regions

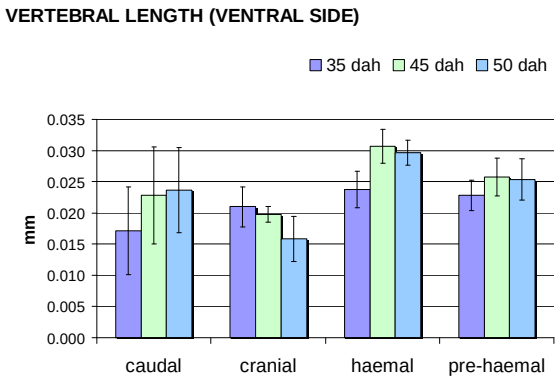
considered. Vertebrae from the cranial region and caudal region, on the contrary, account on average for 18% and 21% of the total vertebral length respectively (Figure 14 A – B).

As well as for length, if considering relative vertebral height of each region, vertebral centra from haemal and pre- haemal region account for the majority of total vertebral height at all ages considered (Figure 14 C – D). In particular the posterior height of these vertebrae was always higher than the anterior one at all age considered. This consideration is also corroborated by the a.h./p.h. ratios that, for both regions is always less than the unity (Figure 14 E). Anyway, haemal vertebrae, almost account in the same proportion to the total vertebral height along ages (a.h. = 29%, p.h. = 31%), while a slight decrease in both a.h. (from 26% at 35 dah to 24% at 50 dah) and p.h. (from 29% at 35 dah to 25% at 50 dah) can be detected for pre – haemal vertebral centra with increasing age.

Both caudal and cranial centra, on the contrary, increased homogenously mineralization around the notochord as suggested by the a.h./p.h. ratio close to one for both regions. Anyway, caudal centra, progressively with age, represented an increasing percentage of total vertebral height (from 22% at 35 dah to 28% at 50 dah), while cranial centra represented a decreasing percentages (from 22% to 17%), probably due to the development of the following vertebral bodies.



A



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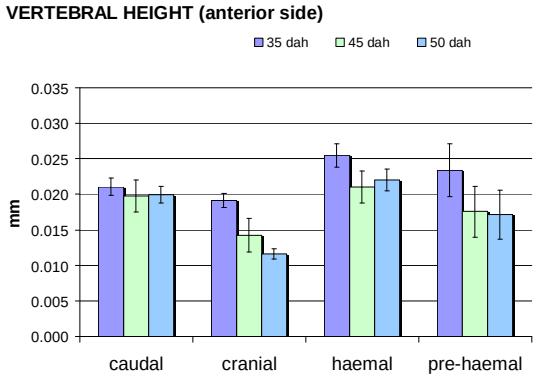

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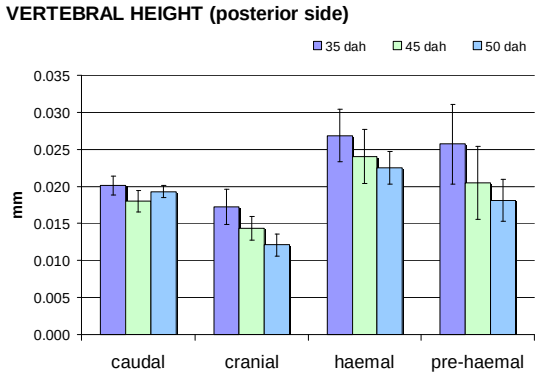

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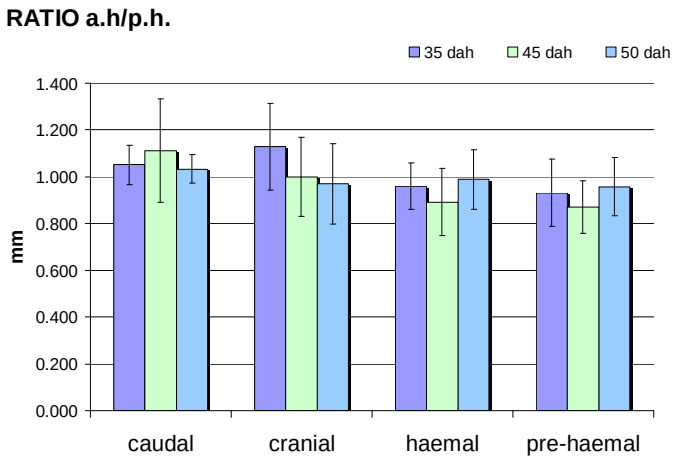


E

Figure 14 Graphical representation of the different vertebral centra measures taken in mesocosm bred larvae stored for an extensive period of time in buffered formaldheyde. **A**: vertebral length at the neural side; **B**: vertebral length at the haemal side; **C**: vertebral height at the neural side; **D**: vertebral height at the haemal side; **E**: ratio a.h / p.h. All data are presented as mean ± s.d.

Table VIII Average values (± s.d.) of vertebral centra dimension in mesocosm bred larvae of 35, 45, 50 dah

age (dah)	region	VERTEBRAL LENGTH (dorsal side) (mm)	VERTEBRAL LENGTH (ventral side) (mm)	POSTERIOR HEIGHT (p.h.) (mm)	ANTERIOR HEIGHT (a.h.) (mm)	RATIO a.h./ p.h.
35 dah	caudal	0.018 ±0.007	0.017 ± 0.006	0.020 ± 0.001	0.021 ± 0.001	1.05 ± 0.08
	cranial	0.025 ± 0.003	0.021 ± 0.002	0.017 ± 0.002	0.019 ± 0.001	1.13 ± 0.18
	haemal	0.026 ± 0.003	0.024 ± 0.003	0.027 ± 0.004	0.025 ± 0.002	0.96 ± 0.10
	pre-haemal	0.025 ± 0.002	0.023 ± 0.003	0.026 ± 0.005	0.023 ± 0.004	0.93 ± 0.14
45 dah	caudal	0.021 ± 0.008	0.023 ± 0.008	0.018 ± 0.001	0.020 ± 0.002	1.11 ± 0.22
	cranial	0.019 ± 0.001	0.02 ± 0.003	0.014 ± 0.002	0.014 ± 0.002	1.00 ± 0.17
	haemal	0.031 ± 0.003	0.031 ± 0.003	0.024 ± 0.004	0.021 ± 0.002	0.89 ± 0.14
	pre-haemal	0.026 ± 0.003	0.026 ± 0.003	0.020 ± 0.005	0.018 ± 0.004	0.87 ± 0.11
50 dah	caudal	0.023 ± 0.007	0.024 ± 0.008	0.019 ± 0.001	0.02 ± 0.001	1.03 ± 0.06
	cranial	0.018 ± 0.004	0.016 ± 0.002	0.012 ± 0.001	0.012 ± 0.001	0.97 ± 0.17
	haemal	0.030 ± 0.002	0.03 ± 0.002	0.023 ± 0.002	0.022 ± 0.002	0.99 ± 0.13
	pre-haemal	0.027 ± 0.003	0.025 ± 0.003	0.018 ± 0.003	0.017 ± 0.003	0.96 ± 0.12

3.2 TRIAL II: EFFECTS OF DIFFERENT DIETARY DHA LEVELS ON BONE FORMATION AND EARLY MINERALIZATION OF THE AXIAL SKELETON OF *SPARUS AURATA*

3.2.1 PROXIMATE COMPOSITION OF THE EXPERIMENTAL ENRICHMENTS FOR ROTIFERS

The five enrichments for rotifers showed an average content of proteins of 29% (DW) with a lesser content in the enrichments richest in DHA (Table IX). The average value of ashes was of 7.5%, with lowest values belonging to enrichments richest in DHA. Total lipids were on average 23.4% (DW) with the lowest values represented by DL enrichment and the highest by DH-DHE.

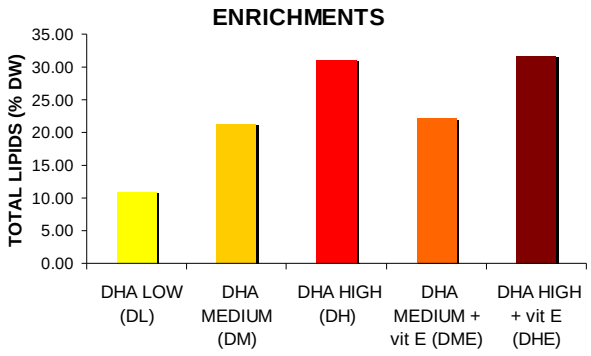
As expected, DH – DHE and DM – DME showed comparable values of DHA between them with an average value of 13.3% DW and 3.1% DW respectively, while DL enrichment was the one that presented less DHA (0.17% DW) (Table X). Enrichments reflected the attended values of fatty acids established at the beginning of the experiment, even if slightly lower for the DME and DM enrichment in respect of the DHA values reported by the producer (3.9% TFA DW).

Addition of vit E to the enrichment products significantly increased vit E contents. Thus, analyzed average α-tocopherol contents were 6,288 mg kg^{-1} for enrichments without extra addition of vit E and 11,758 mg kg^{-1} in enrichments DME and DHE. Elevation of DHA contents in enrichments DH and DHE, in comparison to DM and DME,

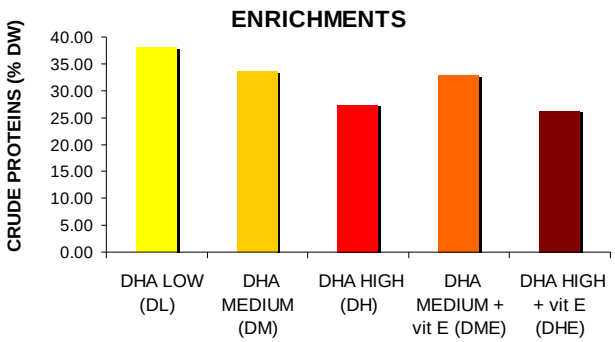
respectively, markedly reduced α-tocopherol contents in the enrichment.

Table IX Proximate composition of the five experimental enrichments

Enrichment	***DHA LOW (DL)***	***DHA MEDIUM (DM)***	***DHA HIGH (DH)***	***DHA MEDIUM + vit E (DME)***	***DHA HIGH + vit E (DHE)***
% lipids (dw)	10.93	21.36	31.11	22.12	31.71
% ash (dw)	8.29	8.25	6.42	8.26	6.40
% proteins (dw)	34.23	30.66	25.62	30.46	24.28



A



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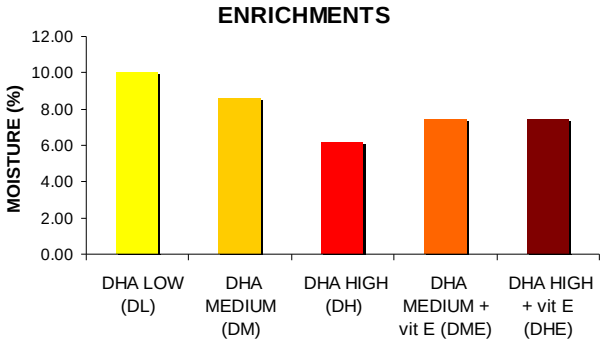
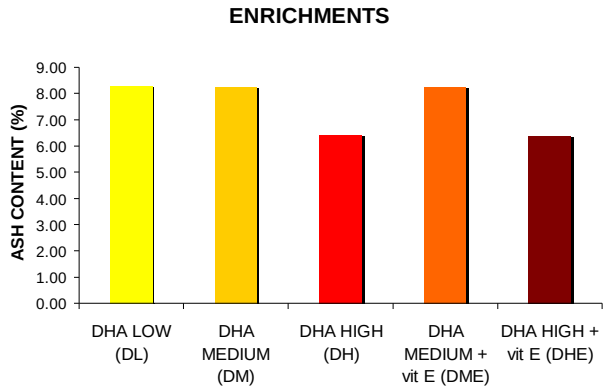
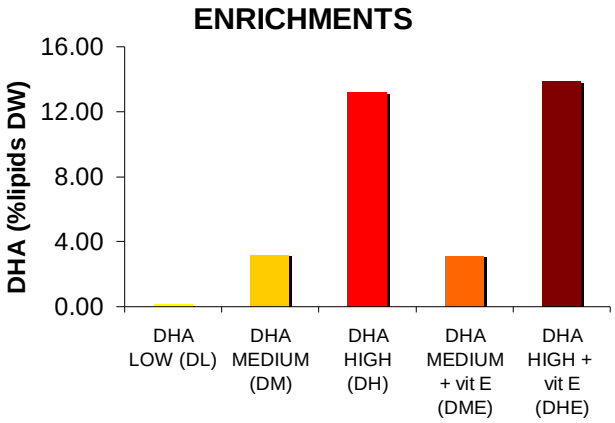


C



D



E

Figure 15 Proximate composition of the five experimental enrichments. **A**: total lipids content; **B**: crude proteins; **C**: moisture; **D**: ash content; **E**: DHA content. All data are expressed as percentage of dry weight.

Table X Total lipids fatty acids profiles (% DW) of the five experimental enrichments of trial II

Enrichment	*DHA LOW (DL)*	*DHA MEDIUM (DM)*	*DHA HIGH (DH)*	*DHA MEDIUM + vit E (DME)*	*DHA HIGH + vit E (DHE)*
14:0	0.16	0.41	0.34	0.45	0.34
14:1n-7	0.04	0.01	0.01	0.01	0.01
14:1n-5	0.02	0.01	0.01	0.01	0.01
15:0	0.03	0.10	0.07	0.11	0.07
16:0ISO	0.02	0.01	0.01	0.01	0.01
16:0	1.14	6.19	3.85	6.96	3.61
16:1n-7	0.76	1.09	1.07	1.05	1.03
16:1n-5	0.02	0.04	0.04	0.04	0.04
16:2n-6	0.01	0.12	0.11	0.12	0.11
16:2n-4	0.03	0.15	0.11	0.16	0.11
17:0	0.14	0.11	0.11	0.11	0.10
16:3n-4	0.01	0.03	0.03	0.05	0.04
16:3n-3	--	0.01	0.01	--	--
16:3n-1	0.01	0.01	--	--	0.01
16:4n-3	< 0.01	0.02	0.04	0.02	0.04
18:0	0.14	1.38	0.88	1.53	0.86
18:1n-9		3.23	2.71	3.16	2.70
18:1n-7	--	0.35	0.35	0.30	0.35
18:1n-9+n-7	6.27	--	--	--	--
18:1n-5	0.08	0.03	0.03	0.04	0.04
18:2n-9	0.03	0.01	0.02	0.03	0.03
18:2n-6	0.38	1.60	1.43	1.63	1.42
18:2n-4	0.04	0.02	0.03	0.03	0.03
18:3n-6	0.01	0.04	0.04	0.04	0.05
18:3n-4	0.02	0.01	0.01	0.01	0.03
18:3n-3	0.04	0.24	0.20	0.24	0.22
18:4n-3	0.09	0.14	0.18	0.14	0.19
18:4n-1	0.01	0.01	0.01	0.01	0.01
20:0	0.01	0.07	0.14	0.08	0.14
20:1n-9+n-7	0.08	0.31	0.42	0.32	0.43
20:1n-5	0.03	0.03	0.05	0.03	0.06
20:2n-9	0.01	0.01	< 0.01	0.01	0.01
20:2n-6	0.01	0.04	0.06	0.04	0.06
20:3n-9	0.01	0.02	0.01	0.01	0.01
20:3n-6	--	0.01	--	--	--
20:4n-6	0.14	0.22	0.28	0.21	0.26
20:3n-3	< 0.01	0.02	0.04	0.02	0.04
20:4n-3	0.03	0.08	0.15	0.08	0.16
20:5n-3	0.93	1.29	2.43	1.24	2.50
22:1n-11	0.01	0.22	1.11	0.23	1.17
22:1n-9	< 0.01	0.02	0.08	0.02	0.08
22:4n-6	< 0.01	0.03	0.12	0.04	0.03
22:5n-6	< 0.01	0.14	0.32	0.14	0.33
22:5n-3	0.03	0.27	1.08	0.26	1.14
22:6n-3	0.17	3.20	13.15	3.14	13.84
15:1n-5	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
16:4n-1	--	< 0.01	--	--	--
18:3n-1	< 0.01	--	--	--	--
Total saturated	0.37	8.27	5.39	9.24	5.13
Total monounsatured	8.45	5.33	5.87	5.21	5.92
Total n-3	1.26	5.29	17.28	2.22	18.13
Total n-6	0.25	2.21	2.36	2.22	2.27
Total n-9	6.74	0.35	3.23	3.53	3.25
Total n-3HUFA	1.16	4.87	16.85	4.75	17.69
DHA/22:5 n-6	37.94	22.95	41.43	23.21	42.39
EPA/ARA	6.76	5.92	8.68	5.87	9.46
DHA/EPA	0.18	2.47	5.42	2.53	5.53
DHA/ARA	1.21	14.65	47.03	14.86	52.32
oleic acid/DHA	37.64	1.01	0.21	1.01	0.19
oleic acid/n-3HUFA	5.42	0.66	0.16	0.67	0.15
n-3/n-6	5.01	2.40	7.32	1.00	7.99
ARA/EPA	0.15	0.17	0.12	0.17	0.11
ARA/DHA	0.83	0.07	0.02	0.07	0.02

-- indicates component not detected.

3.2.2 ROTIFERS COMPOSITION

No statistical differences have been found among groups regarding crude protein content ($P > 0.05$) and ash content ($P > 0.05$) (Table XI, Figure 16).

Significant lower lipid content was found in rotifers fed DL enrichment ($P < 0.05$), whereas addition of either DHA or vit E, markedly increased the lipid content of the rotifers (Table XI, Figure 16).

Rotifers from DM and DME groups showed the highest percentages of saturated fatty acids principally due to higher concentrations of myristic acid (14:0), palmitic acid (16:0) and stearic acid (18:0) (Table XII). On the contrary, DL group presented the lowest concentration of this fatty acid group due to the lowest content of 16:0 and 18:0. Rotifers fed DL enrichment, showed the highest percentage of monounsatured fatty acids among groups followed by DM-DME groups and DH-DHE groups. Differences were principally related to a higher content in oleic acid (18:1n-9) in DL group in respect to the other groups. The latter group showed also the lowest content of total fatty acids of the n-3 and n-6 series, principally due to a lower content of all the n-3 PUFAs and lower levels of linoleic acid (18:2n-6) and docosapentanoic acid (22:5n-6). The treatment groups enriched with vit E (DME and DHE) showed a slightly higher content of total n-3 fatty acids, principally due to a higher content to a higher content of EPA. Indeed, higher values of total n-3 HUFA could also be detected. Due to the highest content of oleic acid and the lowest content in total n-3 and total n-3 HUFAs, rotifers fed DL

enrichment were also the ones presenting the highest ratios oleic acid/total n-3, oleic acid/total n-3 HUFA.

Regarding the three principal HUFAs (EPA, ARA and DHA), DL rotifers showed the lowest concentration of DHA ($P < 0.05$) among the groups. For the same fatty acid, DM and DME groups showed significant lower contents in respect to DH and DHE groups. DL group showed also a significant lower concentration of EPA in respect to DH, DME and DHE groups, while its ARA content was significantly lower only in respect to DME group ($P < 0.05$).

Increase in DHA content in the enrichment products markedly enhanced DHA uptake in the rotifers ($R= 0.96$, $P<0.05$) (Figure 17).

Table XI Proximate composition (mean ± s.d.) of the different enriched rotifers. (n = 8 per treatment)

Enriched Rotifers	DHA LOW (DL)	DHA MEDIUM (DM)	DHA HIGH (DH)	DHA MEDIUM + vit E (DME)	DHA HIGH + vit E (DHE)
% lipids (dw)	14.71 ± 2.56[a]	18.87 ± 3.33[ab]	19.33 ± 2.80[b]	21.03 ± 2.97[b]	19.43 ± 4.09[b]
% ash (dw)	2.03 ± 0.38	2.00 ± 0.35	1.79 ± 0.64	1.88 ± 0.28	1.96 ± 0.50
% proteins (dw)	40.92 ± 8.24	42.82 ± 7.71	52.11 ± 9.06	47.59 ± 7.93	53. 89 ± 9.84

Different letters indicate significant differences among treatments ($P < 0.05$)

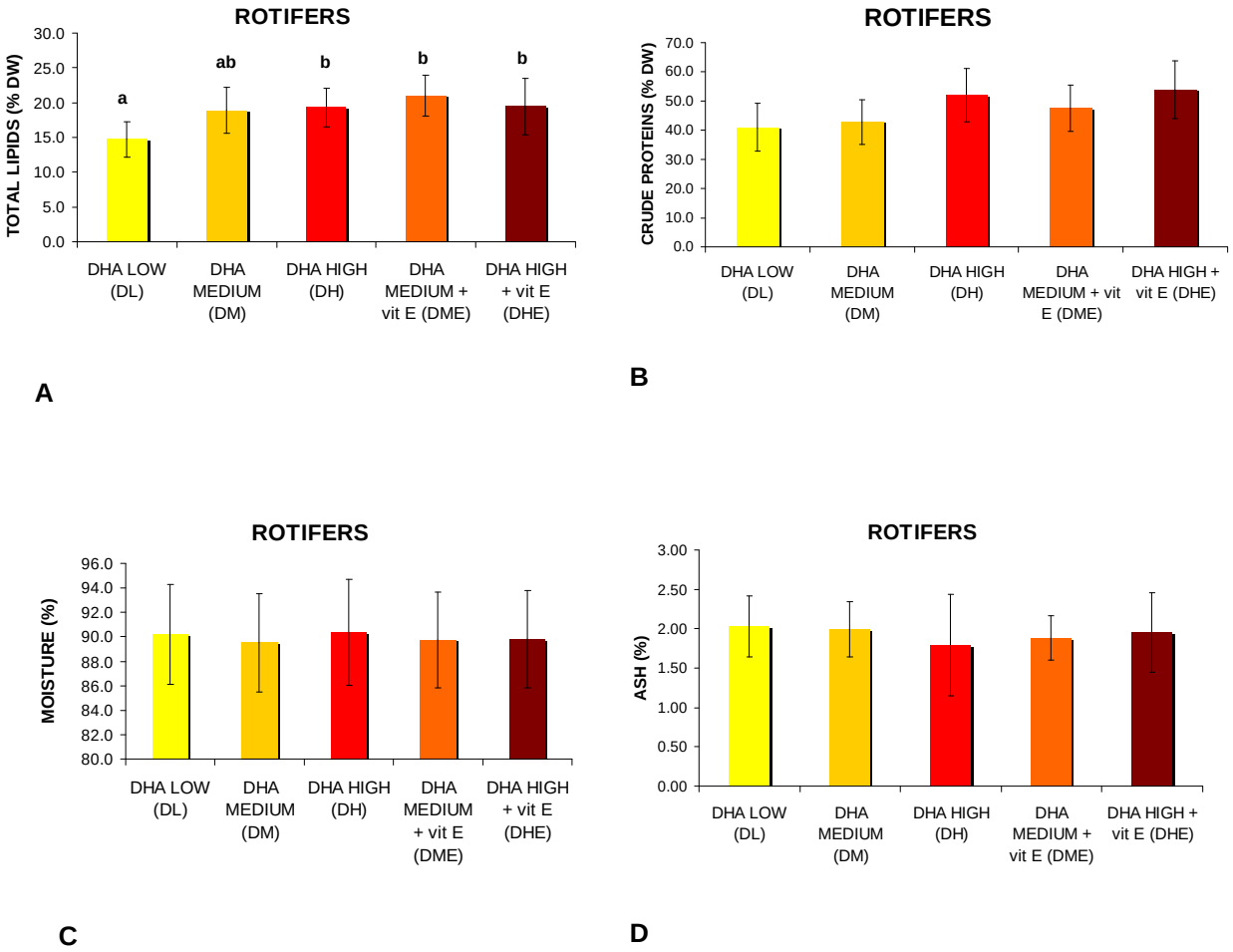


Figure 16 Proximate composition (mean ± s.d.) of the rotifers used. **A**: total lipids content; **B**: crude proteins; **C**: moisture; **D**: ash content; **E**: DHA content. All data are expressed as percentage of dry weight. Different letters indicate statistical differences among treatments.

Table XII Total lipids fatty acids profiles (mean ± s.d.) of enriched rotifers offered to the larvae (n = 8 per treatment)

Enriched Rotifers	DHA LOW (DL)	DHA MEDIUM (DM)	DHA HIGH (DH)	DHA MEDIUM + vit E (DME)	DHA HIGH + vit E (DHE)
14:0	0.23 ± 0.05	0.34 ± 0.07	0.24 ± 0.03	0.37 ± 0.06	0.24 ± 0.05
14:1n-7	0.04 ± 0.02	0.07 ± 0.06	0.05 ± 0.02	0.04 ± 0.06	0.06 ± 0.08
14:1n-5	0.07 ± 0.05	0.05 ± 0.04	0.08 ± 0.07	0.04 ± 0.02	0.08 ± 0.06
15:0	0.06 ± 0.01	0.09 ± 0.02	0.07 ± 0.01	0.09 ± 0.02	0.06 ± 0.02
16:0ISO	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.03 ± 0.01	0.04 ± 0.01
16:0	1.69 ± 0.32	4.32 ± 1.11	2.76 ± 0.47	4.76 ± 0.80	2.60 ± 0.87
16:1n-7	1.66 ± 0.35	1.70 ± 0.28	1.61 ± 0.26	1.80 ± 0.25	1.68 ± 0.23
16:1n-5	0.08 ± 0.03	0.08 ± 0.02	0.08 ± 0.03	0.09 ± 0.02	0.09 ± 0.03
16:2n-6	0.01 ± 0.00	0.09 ± 0.02	0.06 ± 0.01	0.10 ± 0.02	0.08 ± 0.06
16:2n-4	0.07 ± 0.02	0.11 ± 0.02	0.10 ± 0.03	0.13 ± 0.04	0.11 ± 0.06
17:0	0.18 ± 0.03	0.14 ± 0.02	0.12 ± 0.02	0.15 ± 0.02	0.13 ± 0.05
16:3n-4	0.01	0.01	0.01± 0.00	0.01 ± 0.00	0.02 ± 0.00
16:3n-3	--	0.01	0.01± 0.00	0.01± 0.00	0.02 ± 0.01
16:3n-1	0.03 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.01 ± 0.00	--
16:4n-3	0.11 ± 0.02	0.10 ± 0.03	0.09 ± 0.05	0.10 ± 0.04	0.07 ± 0.05
16:4n-1	--	<0.01	0.01	0.02	--
18:0	0.43 ± 0.11	0.79 ± 0.15	0.59 ± 0.26	0.89 ± 0.15	0.62 ± 0.16
18:1n-9	5.99 ± 1.06	3.15 ± 0.47	2.31 ± 0.26	3.37 ± 0.47	2.37 ±0 .39
18:1n-7	0.60 ± 0.16	0.57 ± 0.13	0.53 ± 0.17	0.60 ± 0.10	0.50 ± 0.21
18:1n-5	0.14 ± 0.03	0.09 ± 0.02	0.09 ± 0.01	0.10 ± 0.02	0.08 ± 0.01
18:2n-9	0.28 ± 0.07	0.22 ± 0.07	0.24 ± 0.07	0.25 ± 0.06	0.25 ± 0.02
18:2n-6	0.55 ± 0.12	1.49 ± 0.32	1.10 ± 0.21	1.64 ± 0.25	1.09 ± 0.28
18:2n-4	0.10 ± 0.02	0.06 ± 0.02	0.06 ± 0.03	0.06 ± 0.02	0.06 ± 0.02
18:3n-6	0.02 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
18:3n-4	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
18:3n-3	0.13 ± 0.02	0.27 ± 0.09	0.22 ± 0.04	0.31 ± 0.06	0.21 ± 0.04
18:4n-3	0.07 ± 0.03	0.10 ± 0.03	0.09 ± 0.02	0.11 ± 0.02	0.09 ± 0.02
18:4n-1	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
20:0	0.01 ± 0.00	0.03 ± 0.01	0.06 ± 0.01	0.05 ± 0.04	0.05 ± 0.02
20:1n-9+n-7	0.27 ± 0.05	0.38 ± 0.06	0.37 ± 0.04	0.41 ± 0.06	0.38 ± 0.08
20:1n-5	0.06 ± 0.02	0.08 ± 0.01	0,08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
20:2n-9	0.05 ± 0.02	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
20:2n-6	0.06 ± 0.02	0.07 ± 0.01	0.08 ± 0.02	0.08 ± 0.01	0.10 ± 0.05
20:3n-9	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
20:3n-6	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.038 ± 0.005
20:4n-6	0.20 ± 0.04^{a}	0.24 ± 0.05^{ab}	0.22 ± 0.04^{ab}	0.27 ± 0.04^{b}	0.21 ± 0.04^{ab}
20:3n-3	0.01 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.005	0.03 ± 0.01
20:4n-3	0.08 ± 0.01	0.11 ± 0.02	0.13 ± 0.02	0.12 ± 0.02	0.13 ± 0.02
20:5n-3	0.90 ± 0.16^{a}	1.15 ± 0.24^{ab}	1.33 ± 0.22^{b}	1.30 ± 0.19^{b}	1.36 ± 0.26^{b}
22:1n-11	0.06 ± 0.03	0.10 ± 0.02	0.21 ± 0.16	0.12 ± 0.03	0.29 ± 0.21
22:1n-9	0.03 ± 0.02	0.05 ± 0.03	0.10 ± 0.04	0.09 ± 0.04	0.07 ± 0.04
22:4n-6	<0.01	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.03	0.03 ± 0.01
22:5n-6	0.02 ± 0.01	0.11 ± 0.02	0.14 ± 0.03	0.12 ± 0.02	0.14 ± 0.03
22:5n-3	0.14 ± 0.03	0.30 ± 0.06	0.58 ± 0.07	0.35 ± 0.05	0.59 ± 0.12
22:6n-3	0.25 ± 0.06^{a}	2.39 ± 0.53^{b}	5.24 ± 0.93^{c}	2.75 ± 0.43^{b}	5.27 ± 1.25^{c}
15:1n-5	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Total saturated	2.64 ± 0.51	5.60 ± 1.31	3.87 ± 0.74	6.34 ± 1.05	3.75 ± 1.16
Total monounsaturated	9.00 ± 1.69	6.29 ± 0.87	5.52 ± 0.69	6.74 ± 0.89	5.68 ± 0.90
Total n-3	1.68 ± 0.31	4.38 ± 0.90	7.71 ± 1.23	5.06 ± 0.73	7.76 ± 1.65
Total n-6	0.90 ± 0.20	2.07 ± 0.42	1.68 ± 0.3	2.32 ± 0.33	1.70 ± 0 .42
Total n-9	6.64 ± 1.20	3.86 ± 0.53	2.87 ± 0.38	4.19 ± 0.61	1.10 ± 1.32
Total n-3HUFA	1.38 ± 0.26	3.91 ± 0.80	7.31 ± 1.20	4.17 ± 0.96	7.37 ± 1.64
DHA/22:5 n-6	14.01 ± 2.92	21.77 ± 0.66	37.76 ± 1.95	22.14 ± 0.67	37. 11 ±3.63
EPA/ARA	4.61 ± 0.26	4.78 ± 0.47	6.12 ± 0.56	4.87 ± 0.14	6.44 ± 0.28
DHA/EPA	0.27 ± 0.03	2.06 ± 0.14	3.95 ± 0.36	2.12 ± 0.12	3.86 ± 0.31
DHA/ARA	1.25 ± 0.13	9.87 ± 1.05	24.22 ± 3.74	10.30 ± 0.75	24.86 ± 2.81
oleic acid/DHA	24.82 ± 4.35	1.36 ± 0.17	0.45 ± 0.08	1.23 ± 0.10	0.46 ± 0.08
oleic acid/n-3HUFA	4.37 ± 0.40	0.81 ± 0.09	0.32 ± 0.05	0.87 ± 0.39	0.33 ± 0.05
n-3/n-6	1.89 ± 0.13	2.12 ± 0.08	4.61 ± 0.33	2.18 ± 0.08	4.63 ± 0.49
ARA/EPA	0.22 ± 0.01	0.21 ± 0.02	0.16 ± 0.02	0.21 ± 0.01	0.16 ± 0.01
ARA/DHA	0.81 ± 0.09	0.10 ± 0.01	0.04 ± 0.01	0.10 ± 0.01	0.041 ± 0.004

Different letters indicate statistical differences (P<0.05) among treatments.

– indicates component no detected.

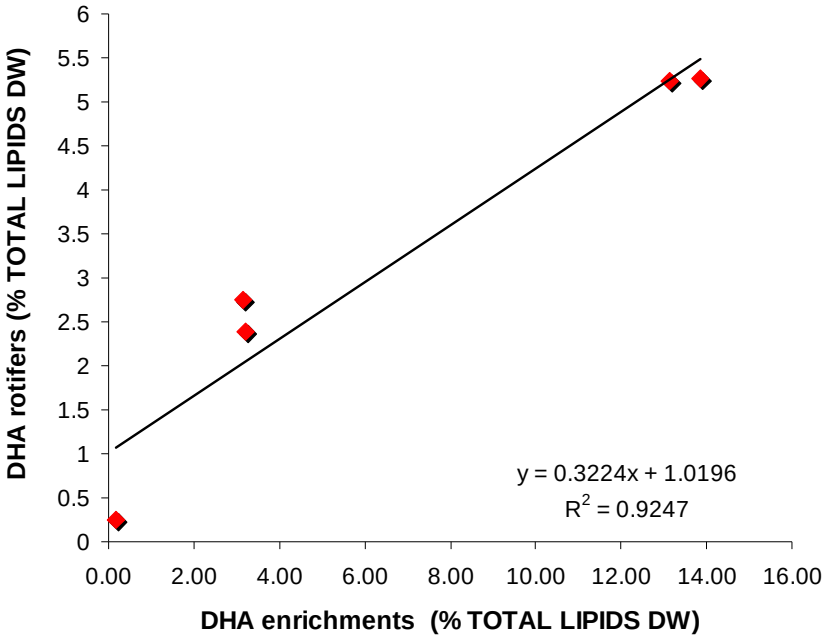


Figure 17 Effect of DHA in the enrichments on its incorporation into rotifers.

3.2.3 BIOLOGICAL PERFORMANCE

A 100% of the examined larvae, showed swim bladder inflation both at 7 and 12 dah, and all the larvae showed signs of ingestion due to the presence of rotifers in the digestive starting from 3 dah.

In all sampling points standard length did not show statistical differences among treatments ($P > 0.05$), even though from 20 dah, the highest lengths were achieved by DH group followed by DM and DHE groups, and finally by DME and DL group (Table XIII, Figure 18).

Also average values of dry weight (Table XIV; Figure 19), did not show any statistical differences between treatments at each sampling point ($P > 0.05$). A trend in dry weight could be detected starting from 20 dah when the highest values were achieved by DM, DHE and DH groups (0.43, 0.31 and 0.30 mg respectively) and the lowest for DL and DME groups with 0.22 and 0.26 mg respectively. Anyway at 34 dah DME group recovered some weight, while larvae from DL group still did not reach 1 mg of weight, unrespectfully to 14 days of Artemia and 9 days of dry feed alimentation.

Table XIII Average standard length (mm) of larvae from the five different treatments at each sampling point. All data are presented as mean ± s.d.

standard length (mm)	TREATMENTS				
dah	**DHA low (DL)**	**DHA medium (DM)**	**DHA high (DH)**	**DHA medium + vit E (DME)**	**DHA high + vit E (DHE)**
3	**3.09 ± 0.30**	**3.13 ± 0.32**	**3.11 ± 0.33**	**3.10 ± 0.30**	**3.13 ± 0.29**
15	**4.78 ± 0.50**	**4.97 ± 0.74**	**5.02 ± 0.61**	**4.69 ± 0.68**	**5.15 ± 0.50**
20	**5.74 ± 0.53**	**6.06 ± 0.74**	**6.13 ± 0.41**	**5.89 ± 0.46**	**6.03 ± 0.46**
34	**7.28 ± 0.61**	**7.72 ± 0.21**	**7.70 ± 1.08**	**7.31 ± 1.95**	**7.38 ± 1.10**

Table XIV Average dry weight (mg) of larvae from the five different treatments at each sampling point. All data are presented as mean ± s.d.

weight (mg)	TREATMENTS				
dah	**DHA low (DL)**	**DHA medium (DM)**	**DHA high (DH)**	**DHA medium + vit E (DME)**	**DHA high + vit E (DHE)**
3	**0.07 ± 0.01**	**0.06 ± 0.01**	**0.08 ± 0.04**	**0.06 ± 0.02**	**0.08 ± 0.02**
15	**0.12 ± 0.01**	**0.15 ± 0.02**	**0.15 ± 0.01**	**0.15 ± 0.02**	**0.16 ± 0.03**
20	**0.22 ± 0.02**	**0.43 ± 0.31**	**0.30 ± 0.05**	**0.26 ± 0.02**	**0.31 ± 0.10**
34	**0.92 ± 0.24**	**1.20 ± 0.27**	**1.22 ± 0.32**	**1.13 ± 0.67**	**1.16 ± 0.25**

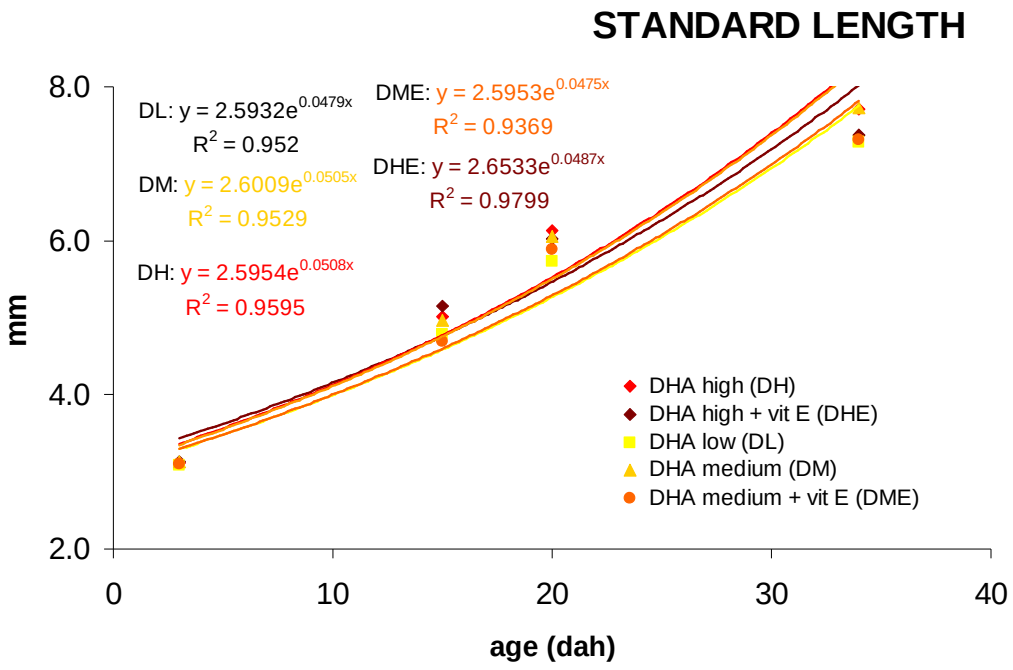


Figure 18 Standard length (mean ± s.d.) of larvae fed rotifers with different DHA and vit E contents.

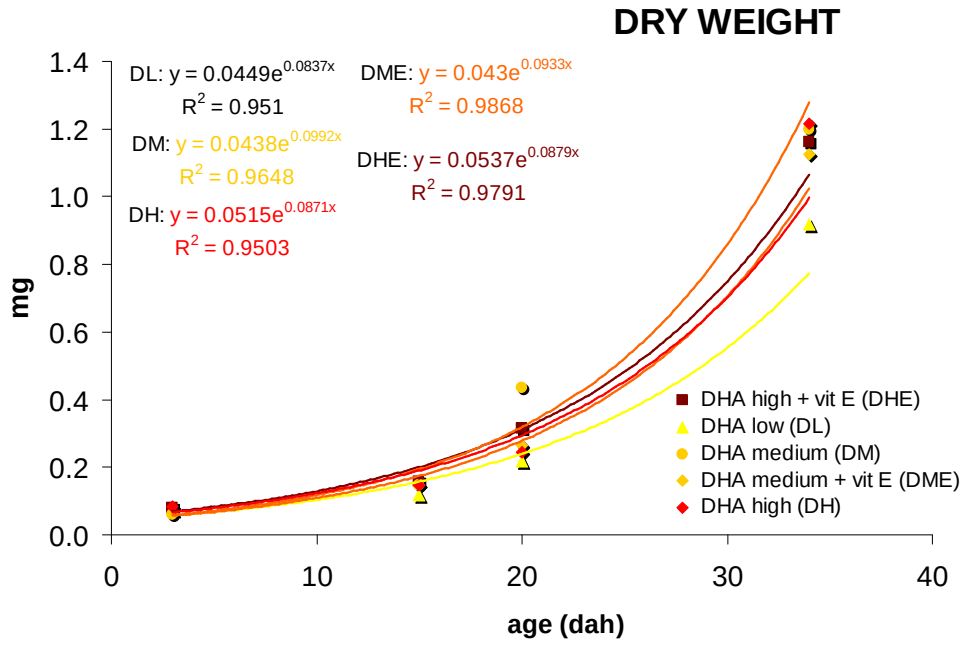


Figure 19 Dry weight of sea bream larvae (mean ± s.d.) fed rotifers with different DHA and vit E contents.

Survival of 34 dah larvae, did not show any statistical difference among the groups (P>0.05) with very variable percentages (Figure 20). In the same way, even pooling larvae fed with high DHA percentages (DH and DHE groups) and medium DHA (DM and DME groups), no significant difference could be detected in survival between larvae fed the two different levels of DHA (P>0.05).

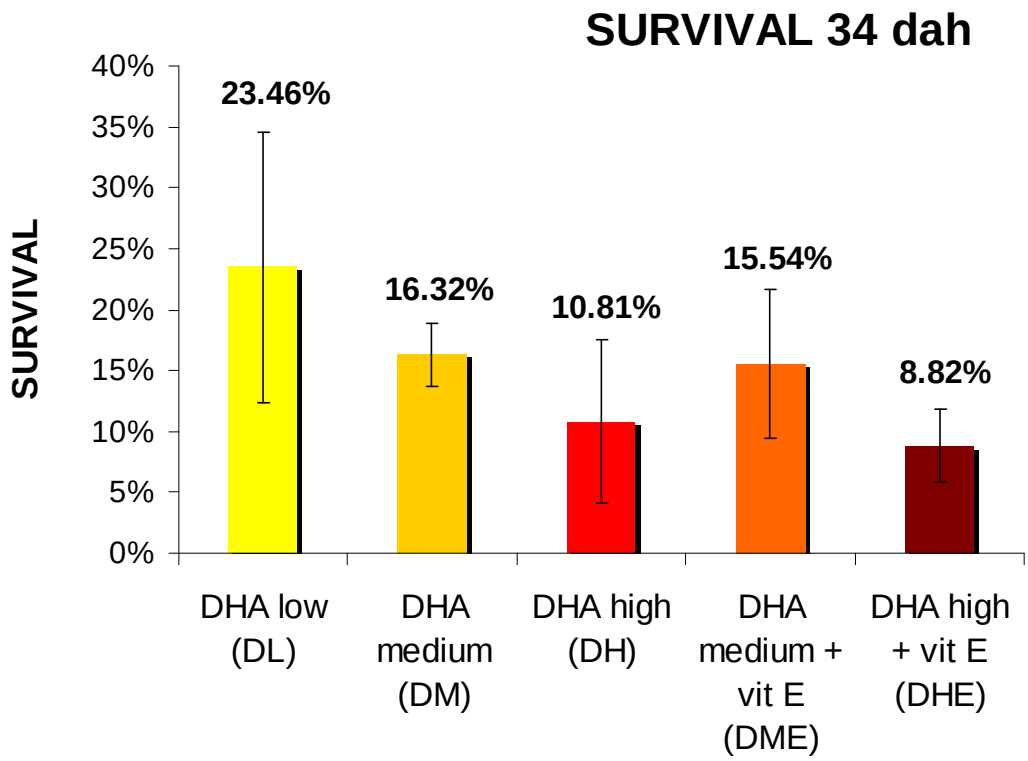


Figure 20 Survival of 34 dah larvae fed rotifers with different DHA and vit E contents followed by Artemia and weaning diet.

Larvae submitted to exposure to air from the five different treatments did not show any significant difference in survival linked to their diets (P > 0.05) which was on average 77% (Figure 21).

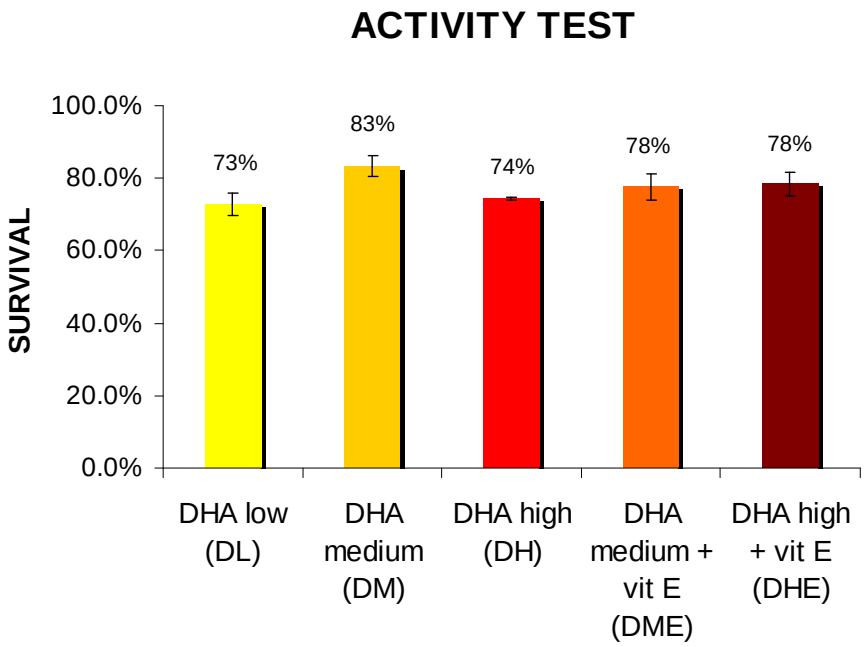


Figure 21 Survival after activity test of 20 dah larvae fed rotifers with different DHA and vit E contents followed by Artemia and weaning diet.

3.2.4 SKELETAL ANOMALIES IN 34 dah LARVAE

The most representative size classes in the experiment were the ones that covered length from 7.685 to 8.285 mm, even though at least 3 different peaks were present in length distribution (Figure 22).

Five size classes were not represented (4.485 to 5.485 mm) and only one specimen falls into SL class between 4.285 and 4.485 mm.

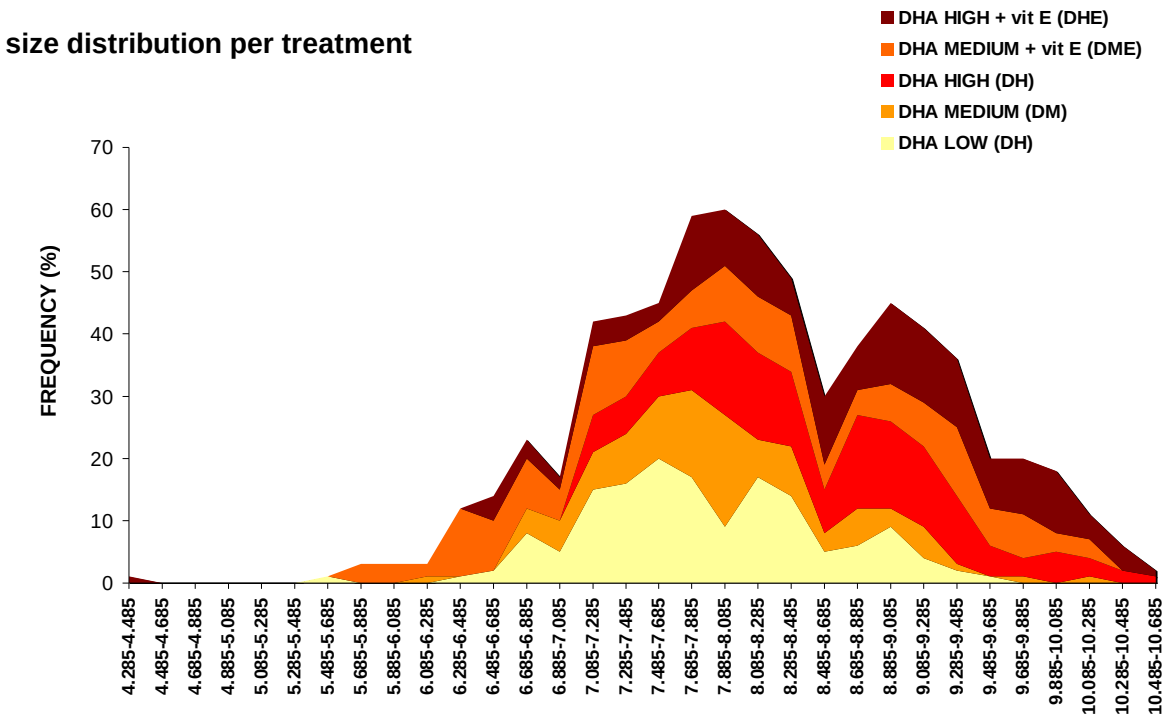


Figure 22 Size distribution of larvae evaluated for skeletal deformities and skeletal mineralization.

Generally low percentages of skeletal anomalies were detected along the study (from 5 to 10%). No significant differences considering the incidence of total anomalies were found among groups ($P > 0.05$) (Figure 23).

Deformities of the neural or haemal processes (2.2%) (curled (Figure 33), wrong directed (Figure 35), bifurcated (Figure 36)), were, on average, the most represented (3.5%), followed by lordosis (2.1%) (Figure 32, 38), prognatism of both maxilla (Figure 27) and mandibula (Figure 31) (1.8%), lack of the pre-maxilla (Figure 30), maxillary contraction (Figure 29). Minor incidence was represented by kyphosis (0.9% on average), vertebral fusions (0.8%) or other anomalies (0.3%).

No opercular anomalies were detected in larvae from every treatment except for a single individual fed DH treatment which showed S-shaped brachistegals (Figure 28).

Larvae fed DL treatment showed higher percentages of lordosis (5%) in respect of the other anomalies considered, generally showing no acute signs. Equally, centre of lordosis could be located at cranial-pre-haemal vertebrae (II-III) (Figure 32) or at haemal vertebrae (generally between v. XX-XXI). In this group, kyphosis accounted for a 2% of total anomalies, followed by malformation of neural spines (Figure 33), fusions (at caudal vertebrae) (Figure 39) and maxillary prognatism (1%).

Larvae from DM and DME treatments, both showed higher percentages of anomalies regarding neural and haemal spines, with a 2% and 3% respectively (Figure 35) in respect of other anomalies. Curled neural processes could be detected principally at the cranial (II) or the very first pre-haemal vertebrae (III-IV). In DME group bifurcation of haemal and neural spines was also detected concomitant of a double fusion of haemal vertebrae (Figure 34). The presence of an additional haemal spine was concomitant to fusion of the caudal vertebrae, while an additional neural spine was present independently from the presence of an additional vertebra.

Both in DM and DME larvae, other anomalies consisted in no acute lordosis at haemal vertebrae (1%) in DM group, or very first pre haemal vertebrae in DME group (2%) and defects of the upper jaw (1% in both groups) (Figure 27). In DME group, moreover, a 1% of the individuals showed slight kyphosis at haemal region (v. XIX – XX) and a single individual showed a double fusion with connected anomalies of the relative spines (Figure 34).

Larvae from DH group showed the highest percentages of jaws deformities in respect of the other groups (4%) (Figures 29, 30, 31) followed by deformations of the neural spines (3%) (Figure 36) that affected both cranial (v. II), pre-haemal (v. V) or haemal vertebrae. Fusions (at caudal vertebrae) and kyphosis, represented the 0.7% of total anomalies, while lordosis at haemal vertebrae (generally with the center located at v. XV or XVI) accounted for a 1% of total anomalies.

Also larvae from DHE group showed principally jaw deformities (2%), followed by fusions at the caudal vertebrae, presence of additional haemal spines, lordosis both in the very first vertebrae (v. II – III) or haemal ones (v. XX – XXI) (Figure 38). Kyphosis accounted for 0.7% of total anomalies, and only a specimen showed the lack of a caudal vertebra.

The presence of an additional vertebra (Figure 24), as well as the presence of calculi (Figure 25) was analyzed separately from the previous anomalies. No significant differences ($P > 0.05$) were found among groups, for both disorders probably due to a high dispertion in the experimental groups.

A third disorder, never described, was the presence of “narrowed vertebrae” (Figure 26). This situation was principally detected in DM larvae that presented this condition in a 40% of the larvae with significant differences with the other groups ($F=103.41 > F_{crit}= 3.63$).

Anyway, the same anomaly was observed in zebrafish and it seems, as well as hypertrophic vertebrae, to be not a pathological condition which can be recuperated with allometric growth (Witten, pers. obs.).

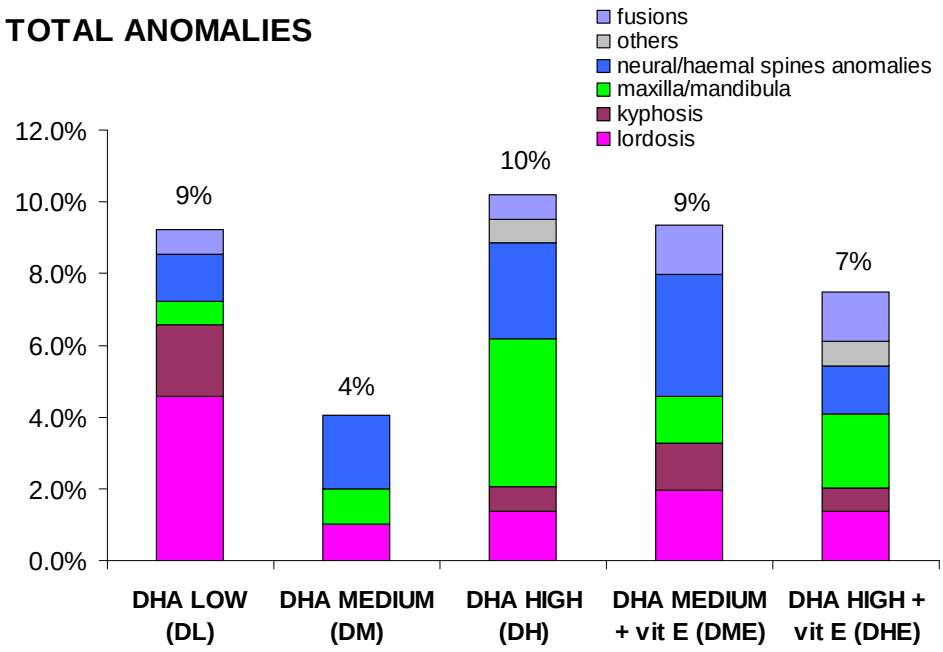


Figure 23 Total anomalies (not considering the presence of additional vertebrae or calculi) of 34 dah larvae from each treatment group. Percentages at the top of the column indicate the total anomalies percentages.

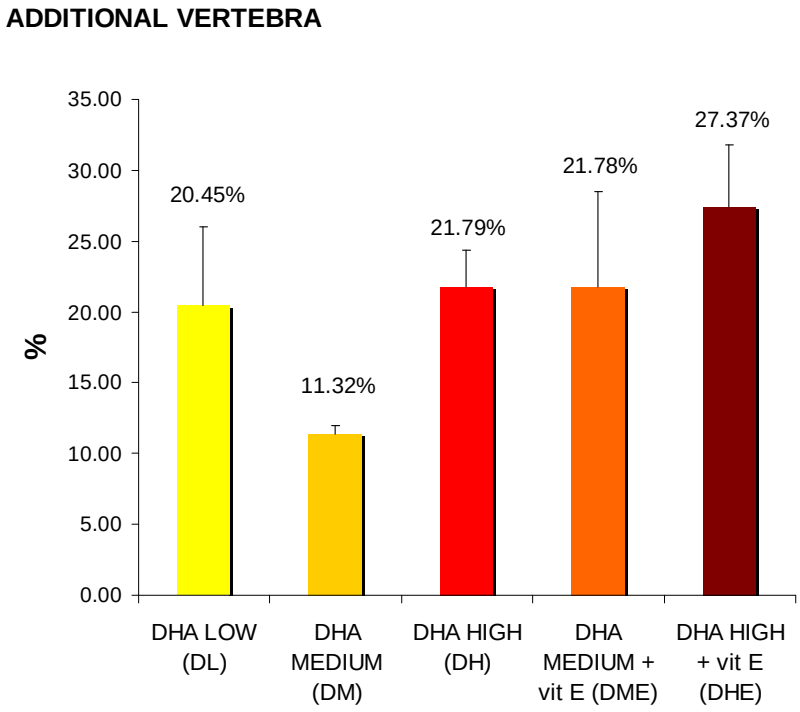


Figure 24 Occurrence of an additional vertebra in 34 dah larvae fed different enriched rotifers.

PRESENCE OF CALCULI

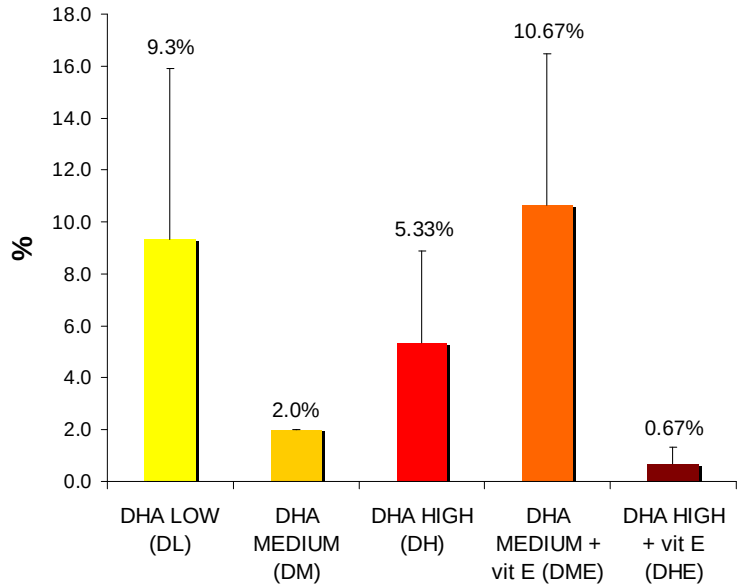


Figure 25 Presence of calculi in 34 dah larvae fed different enriched rotifers.

NARROWED VERTEBRAE

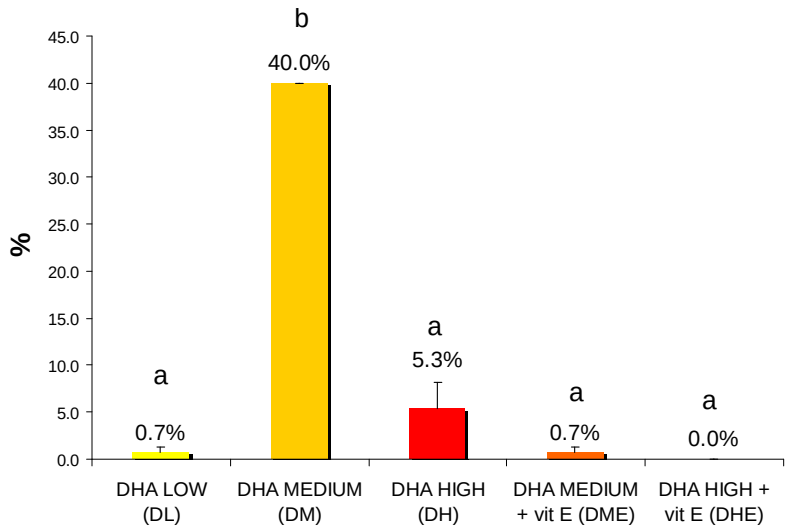


Figure 26 Percentages of "narrowed" vertebrae in 34 dah larvae fed different enriched rotifers. Different letters indicate significant differences among groups.

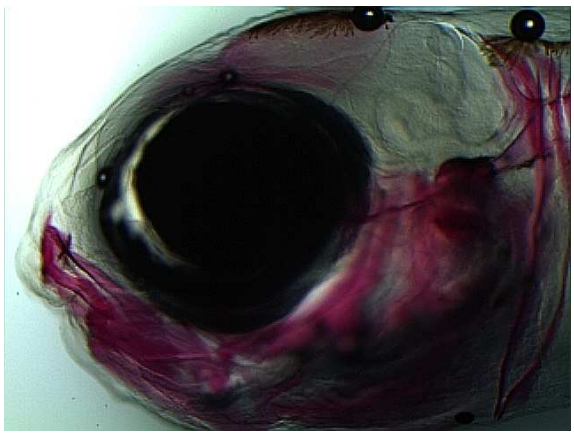


Figure 27 Maxillary prognatism. SL: 6.085-6.285 mm. Treatment: DME. Alizarin red staining. Magnification 5X.

Figure 28 Brachiostegals anomaly. SL: 8.085-8.285 mm. Treatment: DH. Alizarin red staining. Magnification 10X.

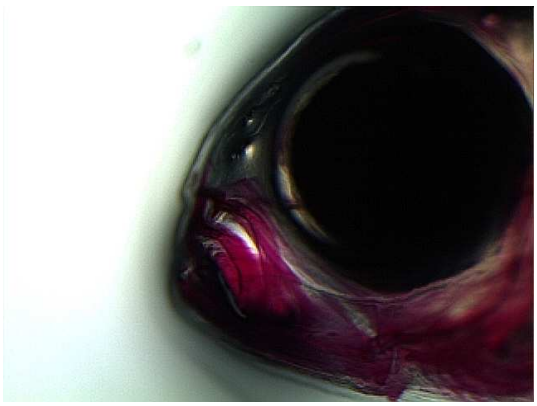


Figure 29 Pre maxilla shortening. SL: 9.685-9.885 mm. Treatment: DH. Alizarin red staining. Magnification 10X.

Figure 30 Absence of pre-maxilla. SL: 7.485-7.685 mm. Treatment: DH. Alizarin red staining. Magnification 10X.

Figure 31 Mandibular prognatism. SL: 8.485-8.685 mm. Treatment: DH. Alizarin red staining. Magnification 10X.

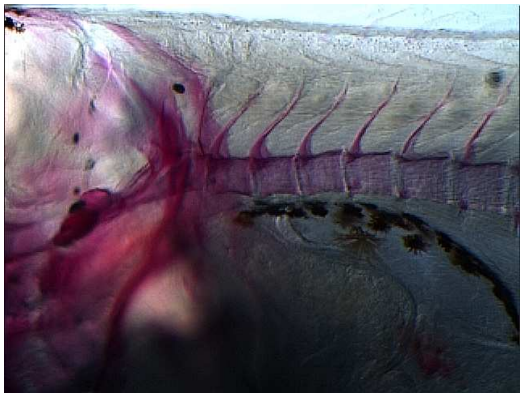


Figure 32 Precocious signs of pre-haemal lordosis localized at v. II-III. SL: 8.485-8.685 mm. Treatment: DL. Alizarin red staining. Magnification 10X.

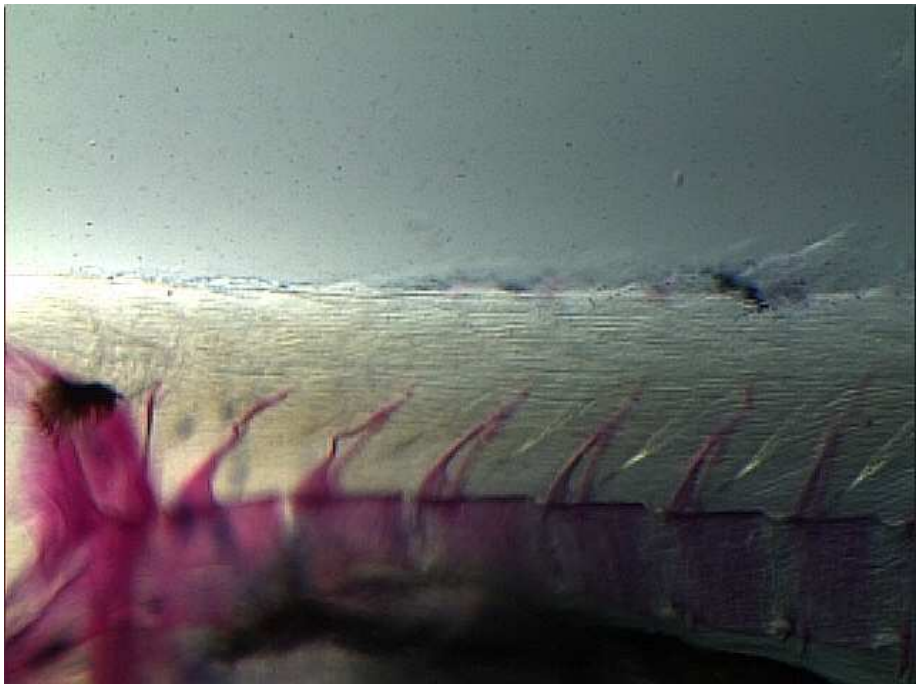


Figure 33 Curled neural processes of v. II and III. SL: 8.285-8.485 mm. Treatment: DL. Alizarin red staining. Magnification 10X.

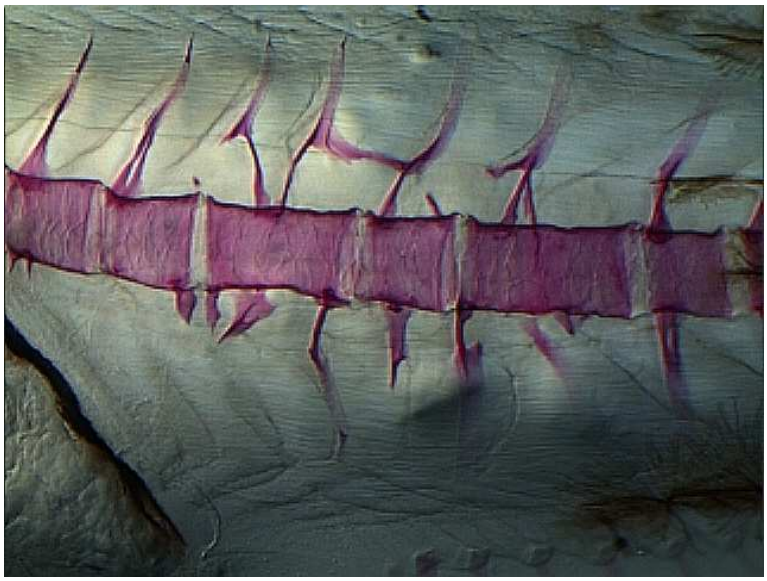


Figure 34 Double fusion between v. X-XI and XII-XIV. Anomalies of neural processes: lack of the second branch of v. XII, fusion between the left branch of v. XI and the left branch of v. XII. Anomalies of hemal processes. SL: 8.285-8.485 mm. Treatment: DME. Alizarin red staining. Magnification 10X.

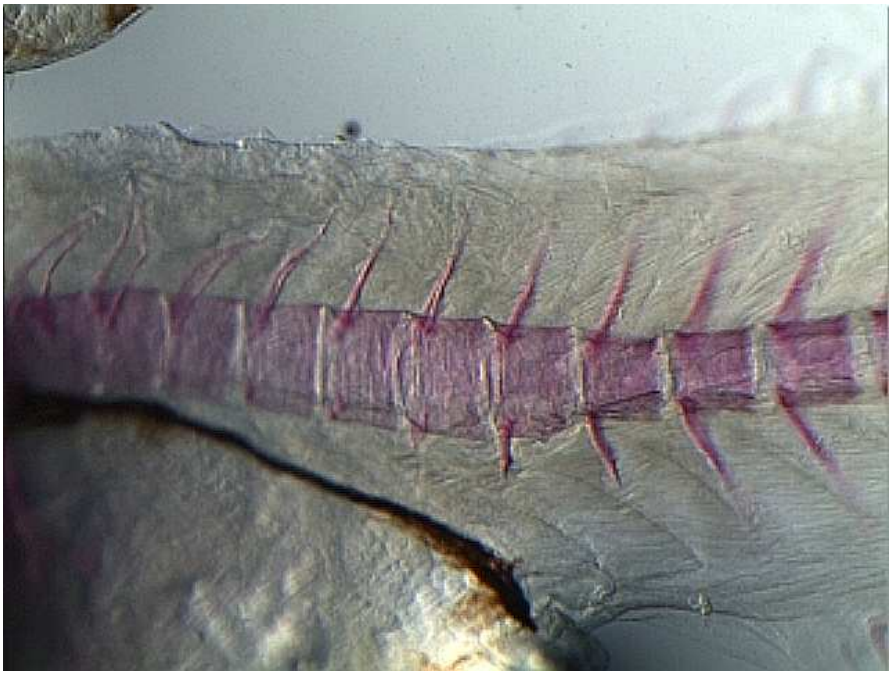


Figure 35 Abnormal direction of neural process of v. V. SL: 7.885-8.085 mm. Treatment: DM. Alizarin red staining. Magnification 10X.

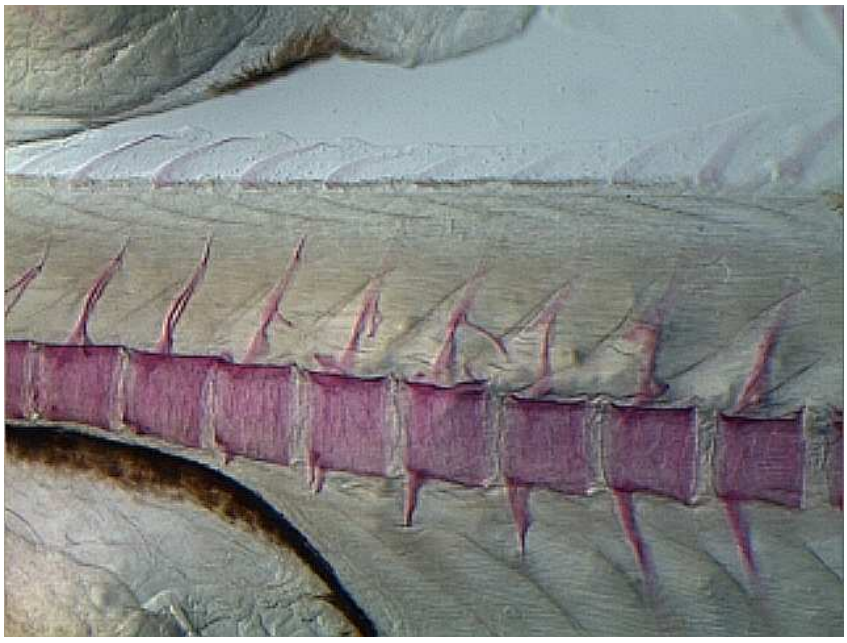


Figure 36 Bifurcated neural processes of v. IX to XIII. SL: 7.685-7.885 mm. Treatment: DH. Alizarin red staining. Magnification 10X.

Figure 37 Vertebral “narrowing” of v. X up to v. XIV. SL: 7.685-7.885 mm. Treatment: DM. Alizarin red staining. Magnification 5X.

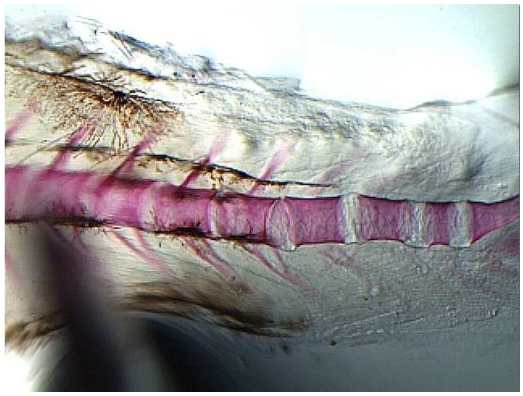


Figure 38 Precocious signs of lordosis in the haemal region localized between v. XX-XXI. SL: 6.685-6.885 mm. Treatment: DHE. Alizarin red staining. Magnification 10X.

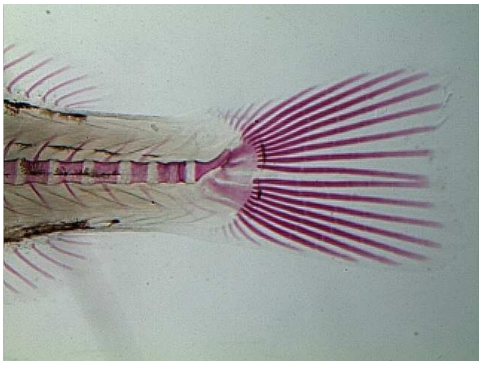


Figure 39 Vertebral fusion at the two last axial column centra between v. XXII-XXIII. SL: 9.085-9.285 mm. Treatment: DL. Alizarin red staining. Magnification 10X.

3.2.5 EFFECT OF THE DIET ON SKELETAL MINERALIZATION

The effects of the different treatments on skeletal mineralization were analyzed considering the average number of mineralized vertebral centra per each size class, and the average percentage of individuals with a complete mineralized vertebral column inside each size class per treatment.

Considering the number of mineralized centra (Table XV), the increase of the number with standard length was explained in all cases by a logarithmic equation of the type y= aln(x)+b (Figure 40). At 9.485-9.685 mm SL all groups showed yet a total number of 22 complete vertebral centra (excluding the urostyle that is already well mineralized at 5.885 mm). Larvae from DL group showed a significant lower number of mineralized centra than the groups DH DME and DHE ($F=6.11 > F_{crit}=2.54$, $T=2.36$). For a fixed size class, DHE group always showed a higher number of mineralized centra, while similar values could be reported for DM, DME and DH groups. In particular from 7.085 mm SL up to 8.085 mm SL, strong differences can be evidenced among the groups as larvae from DHE group already showed a total number of 20 complete vertebral centra, while the others have variable number of 10 to 20 (DL) from 14 to 20 (DM), from 17 to 20 (DH) and from 16 to 19 (DME).

Table XV Average number of mineralized vertebrae. Yellow zone refers to data subjected to statistical analysis as they are the size classes represented in all groups

AVERAGE NUMBER OF MINERALIZED VERTEBRAE (num.)						
size class	*SL (mm)*	**DHA LOW (DL)**	**DHA MEDIUM (DM)**	**DHA HIGH (DH)**	**DHA MEDIUM + vit E (DME)**	**DHA HIGH + vit E (DHE)**
1	**4.285-4.485**					9.0
7	**5.485-5.685**					
8	**5.685-5.885**				8.67	
9	**5.885-6.085**				7.67	
10	**6.085-6.285**		5.00		11.50	
11	**6.285-6.485**	3.00			14.55	
12	**6.485-6.685**	8.50			11.50	19.25
13	**6.685-6.885**	9.57	11.75		12.25	20.33
14	**6.885-7.085**	5.40	15.80		14.20	19.50
15	**7.085-7.285**	10.00	14.33	17.20	16.55	20.00
16	**7.285-7.485**	11.94	17.50	17.20	17.67	20.25
17	**7.485-7.685**	15.40	18.80	17.17	19.20	20.33
18	**7.685-7.885**	15.59	19.71	18.10	19.83	20.00
19	**7.885-8.085**	15.33	19.11	19.00	19.89	20.00
20	**8.085-8.285**	15.71	19.17	19.58	19.44	19.90
21	**8.285-8.485**	18.86	19.25	19.92	20.44	20.67
22	**8.485-8.685**	18.80	20.40	20.00	20.00	21.36
23	**8.685-8.885**	20.67	20.50	20.13	20.50	21.00
24	**8.885-9.085**	18.22	20.00	21.13	21.17	21.42
25	**9.085-9.285**	20.00	19.50	21.50	21.86	20.92
26	**9.285-9.485**	21.00	21.00	21.67	21.73	21.91
27	**9.485-9.685**	22.00		21.60	21.67	22.25
28	**9.685-9.885**			21.00	21.86	22.00
29	**9.885-10.085**			21.75	22.33	22.20
30	**10.085-10.285**		22.00	22.00	22.00	22.50
31	**10.285-10.485**			22.00		23.00
32	**10.485-10.685**			22.00		22.00

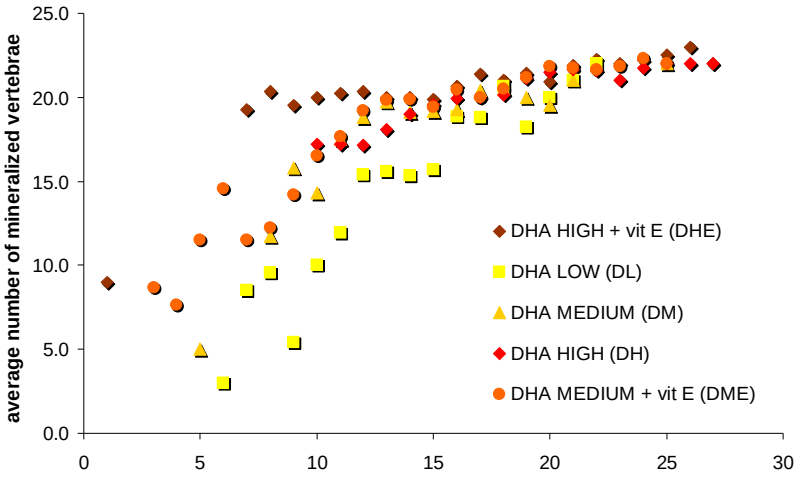


Figure 40 Graphical representation of the average number of vertebrae for each size class and treatment.

No significant differences were found in terms of number of individuals with completely mineralized vertebral column (v. c.) among treatments ($F=2.09 < F_{crit}=2.60$), probably due to the high variability within every treatment group (Table XVI).

However, DL group showed a delay in presenting all the specimens with a complete column (at 9.085 mm SL). DHE treatment, on the contrary, was the group that showed more individuals with a complete v. c. for a fixed SL in respect to the other groups and at lower SL (8.085 – 8.285 mm).

DH group even though at 7.285-7.485 SL showed a lower percentage of individuals with complete vertebral column in respect of DM, DME and DHE groups, presented the 100% of individuals with complete v.c. in a more restricted length range (7.885-8.685 mm).

DM group showed a more gradual increase along the length range considered and present all the individuals with complete v.c. starting from 8.485 – 8.685 mm.

DME group in the size range 7.285 – 7.485 mm sharply increased the percentage of larvae with completely mineralized, in the same way seen in DH group.

Table XVI Percentages of specimens per treatment that showed completely mineralized vertebral column for each size class considered

AXIAL COLUMN MINERALIZATION (%)					
SL (mm)	**DHA LOW (DL)**	**DHA MEDIUM (DM)**	**DHA HIGH (DH)**	**DHA MEDIUM + vit E (DME)**	**DHA HIGH + vit E (DHE)**
4.285-4.485					0
5.485-5.685	0				
5.685-5.885				0	
5.885-6.085				0	
6.085-6.285		0		0	
6.285-6.485	0			0	
6.485-6.685	0			0	50
6.685-6.885	0	25		0	66.6
6.885-7.085	0	20		0	50
7.085-7.285	0	16.6	0	0	50
7.285-7.485	0	50	0	22.2	75
7.485-7.685	0	80	0	80	100
7.685-7.885	6.6	78.5	30	66	91.66
7.885-8.085	12.5	83	58.3	100	88.8
8.085-8.285	25	100	89.47	88.8	100
8.285-8.485	29.4	75	91.66	88.8	100
8.485-8.685	55.5	100	100	75	100
8.685-8.885	88.2	100	93.3	100	100
8.885-9.085	85.7	100	100	100	100
9.085-9.285	100	100	100	100	100
9.285-9.485	100	100	100	100	100
9.485-9.685	100		100	100	100
9.685-9.885			100	100	100
9.885-10.085			100	100	100
10.085-10.285		100	100	100	100
10.285-10.485			100		100
10.485-10.685			100		100

3.2.6 PROXIMATE COMPOSITION OF EGGS AND LARVAE

3.2.6.1 EGGS

Gilthead Seabream eggs used in this study contained 22.4% of total lipids, 40% of proteins and 1.6% of ash (Table XVII).

The main fatty acids in gilthead sea bream eggs (Table XVIII) were 16:0 (palmitic acid) (3.9% DW), 22:6n-3 (DHA, 3.85% DW), 18:1n-9 (oleic acid) (2.9% DW), 18:2n-6 (linoleic acid) (2.56% DW) and 20:5n-3 (EPA, 1.47% DW). Arachidonic acid represented 0.19% DW of total lipids.

Table XVII Proximate composition of sea bream eggs used in trial II

SEA BREAM EGGS	
% lipids (dw)	22.37
% ash (dw)	1.59
% proteins (dw)	39.88

Table XVIII Total lipids fatty acids profile of sea bream eggs from the mass spawning used in trial II

Sea bream eggs	*% DW*
14:0	0.70
14:1n-5	0.02
15:0	0.07
15:1n-5	0.01
16:0ISO	0.08
16:0	3.89
16:1n-7	1.41
16:1n-5	0.05
16:2n-6	0.12
16:2n-4	0.08
17:0	0.12
16:4n-3	1.31
16:4n-1	0.05
18:0	0.88
18:1n-9	2.99
18:1n-7	0.43
18:1n-5	0.03
18:2n-9	0.03
18:2n-6	2.56
18:2n-4	0.06
18:3n-6	0.05
18:3n-4	0.03
18:3n-3	0.22
18:4n-3	0.56
18:4n-1	0.04
20:0	0.03
20:1n-9+n-7	0.07
20:1n-5	0.11
20:2n-9	0.01
20:2n-6	0.05
20:3n-6	0.03
20:4n-6	0.19
20:3n-3	0.01
20:4n-3	0.10
20:5n-3	1.47
22:1n-11	0.01
22:1n-9	0.01
22:4n-6	0.02
22:5n-6	0.04
22:5n-3	0.57
22:6n-3	3.85
14:1n-7	< 0.01
16:3n-4	--
16:3n-3	--
16:3n-1	--
18:1n-9+n-7	--
18:3n-1	--
20:3n-9	--
Total saturated	5.77
Total monounsaturat	5.14
Total n-3	8.09
Total n-6	3.06
Total n-9	3.12
Total n-3HUFA	6.00
DHA/22:5 n-6	103.31
EPA/ARA	7.65
DHA/EPA	2.61
DHA/ARA	19.94
oleic acid/DHA	0.78
oleic acid/n-3HUFA	0.50
n-3/n-6	2.64
ARA/EPA	0.13
ARA/DHA	0.05

-- indicates component no detected.

% DW: percentage dry weight basis.

3.2.6.2 LARVAE

Larvae of 15 dah showed a lipids content variable between 12.6 and 15.6 % in dry weight, while proteins and ash account for 76% and 1.7% respectively in dry weight (Table XIX, Figure 41).

The total lipids fatty acid profiles of 15 dah larvae (Table XX) showed a comparable level of saturated fatty acids among all treatment groups (~ 4% DW). In particular in DL group palmitic acid (16:0), myristic acid (14:0) and heptadecanoic acid (17:0) in this order, accounted for the majority of this class. Monounsatured fatty acids were higher in DL group in comparison to the other treatments, principally due to a higher percentage of oleic acid (18:1n-9).

As expected, total n-3 HUFA showed a lower content in DL group principally due to lower values of DHA. On the contrary, EPA content was higher in DL group in respect to the other treatments.

DHA content varied between 0.97% (total lipids DW) in DL larvae increasing following the DHA content in the enriched rotifers with ~ 2.5% and ~ 3.3% in DM/DME and DH/DHE larvae respectively. An increase in DHA content in the rotifers products markedly enhanced DHA uptake in the larvae irrespective to the content of vit E in the enrichments (R= 0.96) (Figure 42). Due to the higher content in the oleic acid and low content in DHA also the proportion of oleic acid/DHA and oleic acid/n-3 HUFA were altered in DL group that showed higher values.

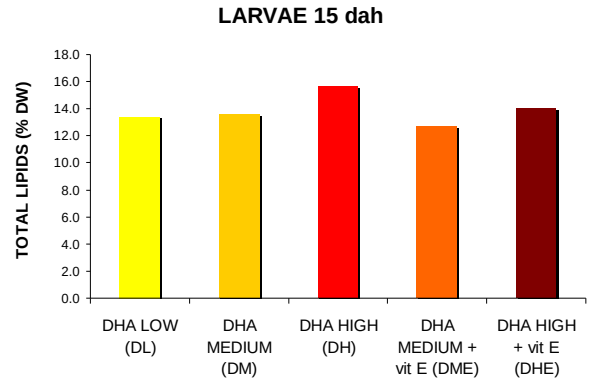
Table XIX Proximate composition of 15 dah larvae fed different levels of DHA and vit E

larvae 15 dah	**DHA LOW (DL)**	**DHA MEDIUM (DM)**	**DHA HIGH (DH)**	**DHA MEDIUM + vit E (DME)**	**DHA HIGH + vit E (DHE)**
% lipids (dw)	13.39	13.53	15.63	12.69	14.03
% ash (dw)	1.16	1.02	0.90	0.72	0.37
% proteins (dw)	73.86	77.38	75.85	78.39	77.90

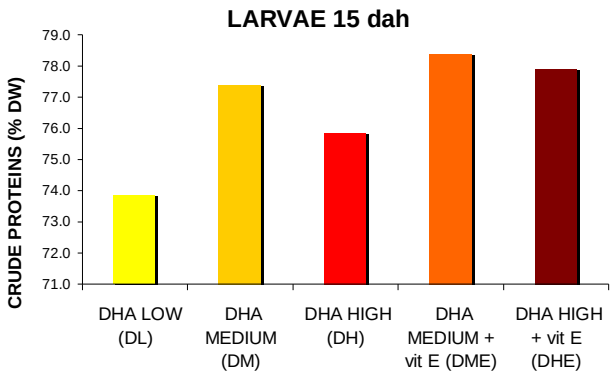
Table XX Total lipids fatty acids profiles of 15 dah larvae

Larvae 15 dah Total lipids	DHA LOW (DL)	DHA MEDIUM (DM)	DHA HIGH (DH)	DHA MEDIUM + vit E (DME)	DHA HIGH + vit E (DHE)
14:0	0.20	0.12	0.13	0.12	0.13
14:1n-7	0.02	0.04	0.04	--	--
14:1n-5	0.05	0.01	0.01	0.01	0.02
15:0	0.01	0.05	0.07	0.06	0.06
15:1n-5	0.01	0.01	0.01	0.01	0.01
16:0ISO	0.03	0.02	0.02	0.02	0.02
16:0	2.43	2.28	2.37	2.30	2.41
16:1n-7	0.97	0.81	0.97	0.88	0.81
16:1n-5	0.10	0.11	0.11	0.12	0.10
16:2n-6	0.03	0.05	0.05	0.05	0.03
16:2n-4	0.13	0.12	0.07	0.13	0.12
17:0	0.14	0.10	0.11	0.12	0.11
16:3n-4	--	0.01	0.01	0.01	--
16:3n-1	0.01	0.00	0.01	0.01	0.00
16:4n-3	0.15	0.15	0.04	0.16	0.17
16:4n-1	0.04	0.03	--	0.04	--
18:0	1.18	1.11	1.29	1.26	1.17
18:1n-9	2.09	1.66	1.76	1.83	1.35
18:1n-7	0.75	0.65	0.63	0.70	0.58
18:1n-5	0.09	0.06	0.07	0.08	0.06
18:2n-9	0.03	0.16	0.20	0.19	0.17
18:2n-6	0.53	0.57	0.58	0.57	0.44
18:2n-4	0.01	0.01	0.01	0.01	0.01
18:3n-6	0.02	0.02	0.02	0.02	0.02
18:3n-4	0.00	--	0.02	--	--
18:3n-3	0.07	0.05	0.08	0.05	0.05
18:4n-3	0.02	0.02	0.02	0.02	0.03
18:4n-1	0.01	0.01	0.01	0.01	0.01
20:0	0.02	0.02	0.03	0.02	0.03
20:1n-9+n-7	0.17	0.17	0.19	0.19	0.17
20:1n-5	0.07	0.07	0.09	0.09	0.08
20:2n-9	0.05	0.01	0.05	0.01	0.01
20:2n-6	0.07	0.08	0.08	0.09	0.06
20:3n-6	0.07	0.06	0.06	0.06	0.06
20:3n-9	0.02	--	0.02	--	--
20:4n-6	0.55	0.48	0.51	0.50	0.48
20:3n-3	0.01	0.02	0.02	0.02	0.02
20:4n-3	0.09	0.09	0.07	0.09	0.07
20:5n-3	1.47	1.14	1.31	1.08	1.22
22:1n-11	0.04	0.02	0.03	0.02	0.02
22:1n-9	0.03	0.02	0.06	0.02	0.03
22:4n-6	0.04	0.04	0.05	0.05	0.04
22:5n-6	0.03	0.09	0.09	0.09	0.06
22:5n-3	0.55	0.50	0.63	0.49	0.66
22:6n-3	0.97	2.49	3.62	2.47	3.12
16:3n-3	--	--	--	--	--
Total saturated	4.02	3.72	4.02	3.89	3.93
Total monounsatured	4.41	3.56	3.63	3.94	3.24
Total n-3	3.34	4.46	5.80	4.37	5.35
Total n-6	1.34	1.38	1.44	1.43	1.20
Total n-9	2.39	2.02	2.28	2.24	1.73
Total n-3HUFA	3.09	4.24	5.66	4.15	5.09
DHA/22:5 n-6	28.13	28.32	38.76	27.53	55.98
EPA/ARA	2.65	2.36	2.59	2.18	2.52
DHA/EPA	0.66	2.18	2.76	2.28	2.56
DHA/ARA	1.76	5.16	7.15	4.96	6.44
oleic acid/DHA	2.15	0.67	0.49	0.74	0.43
oleic acid/n-3HUFA	0.68	0.39	0.31	0.44	0.26
n-3/n-6	2.50	3.24	4.03	3.07	4.46
ARA/EPA	0.38	0.42	0.39	0.46	0.40
ARA/DHA	0.57	0.19	0.14	0.20	0.16

– indicates component no detected.



A



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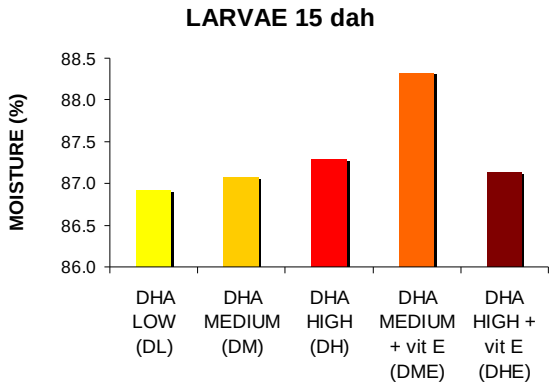

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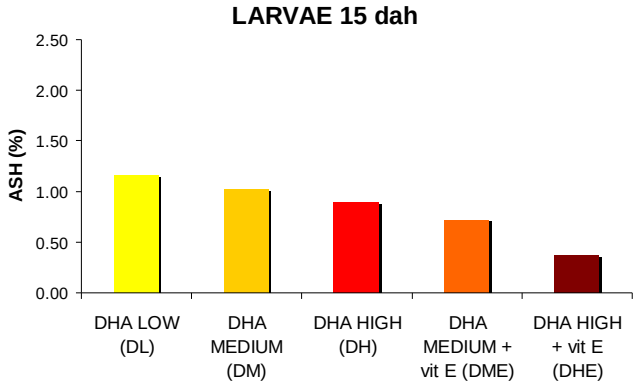

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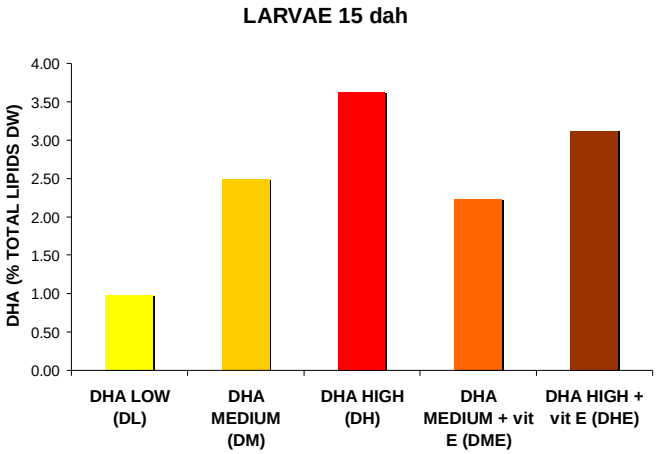


E

Figure 41 Graphical representation of the proximal composition of 15 dah larvae. **A**: total lipids content; **B**: crude proteins; **C**: moisture; **D**: ash content; **E**: DHA content. All data are expressed as percentage of dry weight.

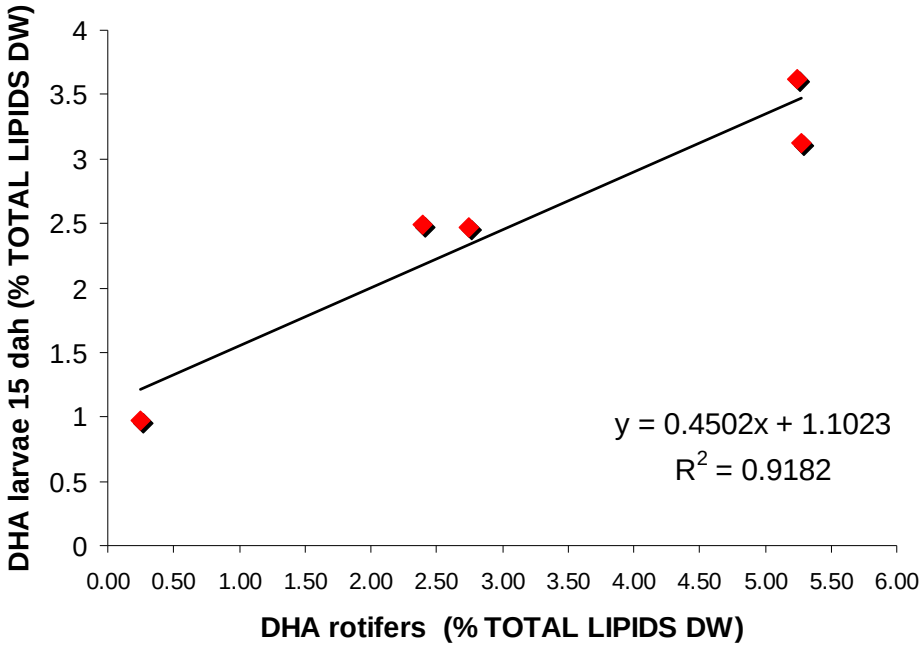


Figure 42 Effect of DHA content in the rotifers on its incorporation into the 15 dah larvae.

Total lipids of 15 dah larvae showed an average content of ~ 61% and of ~ 38% of neutral and polar lipids respectively (% total lipids DW) (Figure 43). Generally, neutral lipids represented a higher percentage than polar lipids in the total lipids content.

In the neutral lipids fraction (Table XXI), larvae from DM group showed a higher content of total saturated in respect to the other treatments, principally due to a higher content of palmitic acid (16:0), and stearic acid (18:0) while larvae from DL group showed the lowest concentration of these two fatty acids in this fraction. An opposite situation could be detected in the polar fraction (Table XXII), where, DL larvae showed the highest concentration of saturated fatty acids, principally due to 16:0 and 18:0. DM group showed the lowest level of total n-3 and total n-3 HUFA in

the neutral fraction, basically due to a lower presence of EPA. In all groups, EPA was preferentially stored in the neutral lipid fraction. DL group presented the lowest concentration of monounsatured fatty acids in the neutral fraction principally because of low concentration of 22:1n-11 and 20:1n-9+n-7, while it was the group with higher relative percentages, in both fractions, of ARA.

DHA was present in higher percentages in the neutral fraction, with an average content of 66 % of total lipids DW, while showed an average percentage of 34 % in the polar fraction (Tables XXI, XXII, Figure 44). In all groups, DHA was preferencially stored in the neutral lipid fraction rather than in the polar in particular, in the groups supplemented with vit E.

Table XXI Fatty acids profiles of the lipids neutral fraction of 15 dah larvae

LARVAE 15 dah Neutral Lipids	**DHA LOW (DL)**	**DHA MEDIUM (DM)**	**DHA HIGH (DH)**	**DHA MEDIUM + vit E (DME)**	**DHA HIGH + vit E (DHE)**
14:0	0.08	0.13	0.148	0.103	0.14
14:1n-5	0.01	0.014	0.03	0.02	0.03
15:0	0.03	0.09	0.07	0.03	0.07
16:0ISO	0.01	0.01	0.01	0.01	0.01
16:0	0.74	2.55	1.45	0.94	1.36
16:1n-7	0.44	0.44	0.69	0.54	0.60
16:1n-5	0.03	0.04	0.07	0.05	0.06
16:2n-6	0.01	0.04	0.04	0.04	0.03
16:2n-4	0.06	0.10	0.09	0.07	0.10
17:0	0.06	0.05	0.07	0.06	0.07
16:3n-3	0.02	0.02	0.03	0.02	0.03
16:3n-1	0.07	0.03	0.06	0.06	0.06
16:4n-3	0.03	0.01	0.01	0.02	0.01
16:4n-1	0.01	< 0.01	0.01	0.01	< 0.01
18:0	0.63	0.91	0.84	0.74	0.82
18:1n-9	0.87	1.19	1.03	0.94	0.86
18:1n-7	0.35	0.30	0.44	0.43	0.39
18:1n-5	0.04	0.04	0.05	0.05	0.05
18:2n-9	0.11	0.03	0.13	0.12	0.12
18:2n-6	0.22	0.46	0.38	0.37	0.33
18:2n-4	< 0.01	0.01	0.02	0.03	0.02
18:3n-6	0.01	0.01	0.02	0.01	0.02
18:3n-4	0.02	0.02	0.03	0.03	0.02
18:3n-3	0.03	0.05	0.06	0.06	0.05
18:4n-3	0.03	0.03	0.03	0.03	0.03
18:4n-1	0.01	< 0.01	0.01	0.01	0.01
20:0	0.019	0.04	0.03	0.02	0.01
20:1n-9+n-7	0.10	0.18	0.151	0.13	0.14
20:1n-5	0.05	0.03	0.06	0.06	0.06
20:2n-9	0.03	0.02	0.03	0.03	0.03
20:2n-6	0.04	0.03	0.05	0.05	0.04
20:3n-6	0.04	0.02	0.03	0.04	0.04
20:3n-9	0.02	< 0.01	0.01	0.02	0.01
20:4n-6	0.36	0.19	0.34	0.34	0.35
20:3n-3	0.01	0.01	0.015	0.01	0.02
20:4n-3	0.04	0.02	0.05	0.05	0.05
20:5n-3	0.94	0.44	0.88	0.71	0.90
22:1n-11	0.01	0.04	0.04	< 0.01	0.04
22:1n-9	0.03	< 0.01	0.05	0.03	0.07
22:4n-6	0.03	0.01	0.01	0.01	0.04
22:5n-6	0.02	0.04	0.05	0.05	0.03
22:5n-3	0.40	0.18	0.39	0.34	0.46
22:6n-3	0.93	1.09	2.33	1.66	1.97
14:1n-7	< 0.01	0.01	< 0.01	< 0.01	< 0.01
15:1n-5	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
16:3n-4	--	--	--	--	--
Total saturated	1.56	3.78	2.61	1.91	2.48
Total monounsaturated	1.93	2.27	2.61	2.25	2.28
Total n-3	2.42	1.85	3.79	2.89	3.51
Total n-6	0.74	0.79	0.93	0.92	0.88
Total n-9	1.16	1.42	1.40	1.27	1.23
Total n-3HUFA	2.32	1.75	3.67	2.77	3.39
DHA/22:5 n-6	43.69	27.56	51.04	30.36	61.46
EPA/ARA	2.62	2.34	2.57	2.08	2.58
DHA/EPA	0.98	2.45	2.65	2.34	2.19
DHA/ARA	2.58	5.73	6.81	4.88	5.65
oleic acid/DHA	0.94	1.09	0.44	0.57	0.44
oleic acid/n-3HUFA	0.38	0.68	0.28	0.34	0.25
n-3/n-6	3.25	2.36	4.10	3.14	4.00
ARA/EPA	0.38	0.43	0.39	0.48	0.39
ARA/DHA	0.39	0.17	0.15	0.20	0.18

– indicates component no detected.

Table XXII Fatty acids profiles of the lipids polar fraction of 15 dah larvae

LARVAE 15 dah ***Polar Lipids***	**DHA LOW (DL)**	**DHA MEDIUM (DM)**	**DHA HIGH (DH)**	**DHA MEDIUM + vit E (DME)**	**DHA HIGH + vit E (DHE)**
14:0	0.06	0.05	0.02	0.03	0.05
14:1n-5	0.02	0.02	0.01	0.01	0.01
15:0	0.05	0.04	0.04	0.03	0.04
16:0ISO	0.01	0.01	0.01	0.01	0.01
16:0	1.68	1.44	1.21	0.88	1.10
16:1n-7	0.45	0.34	0.27	0.23	0.25
16:1n-5	0.05	0.04	0.04	0.03	0.03
16:2n-6	0.01	0.02	0.02	0.02	0.01
16:2n-4	0.07	0.06	0.06	0.04	0.05
17:0	0.08	0.05	0.04	0.04	0.04
16:3n-3	0.01	0.01	0.01	< 0.01	0.01
16:3n-1	0.07	0.07	0.11	0.06	0.11
16:4n-3	0.01	0.01	0.02	0.01	0.02
16:4n-1	0.01	0.01	0.01	0.01	0.01
18:0	0.59	0.50	0.45	0.32	0.39
18:1n-9	1.12	0.83	0.62	0.60	0.58
18:1n-7	0.33	0.29	0.24	0.20	0.22
18:1n-5	0.04	0.03	0.02	0.02	0.01
18:2n-9	0.07	0.06	0.04	0.04	0.04
18:2n-6	0.21	0.23	0.16	0.18	0.14
18:2n-4	0.01	0.01	0.01	0.01	< 0.01
18:3n-6	0.01	0.01	0.01	< 0.01	< 0.01
18:3n-4	< 0.01	0.01	0.01	0.01	0.01
18:3n-3	0.03	0.02	0.01	0.02	0.01
18:4n-3	--	0.01	0.01	0.01	0.01
20:0	0.01	0.01	0.01	0.01	0.01
20:1n-9+n-7	0.06	0.05	0.04	0.04	0.04
20:1n-5	0.02	0.02	0.02	0.02	0.02
20:2n-9	0.02	--	0.01	--	0.01
20:2n-6	0.03	0.03	0.03	0.02	0.02
20:3n-6	0.03	0.02	0.02	0.02	0.02
20:3n-9	0.01	--	0.01	0.01	0.01
20:4n-6	0.19	0.16	0.13	0.11	0.14
20:4n-3	0.02	0.02	0.01	0.01	0.01
20:5n-3	0.61	0.45	0.36	0.29	0.38
22:4n-6	0.02	0.01	0.01	0.01	0.01
22:5n-6	0.01	0.04	0.03	0.02	0.03
22:5n-3	0.17	0.15	0.17	0.11	0.20
22:6n-3	0.46	0.80	1.28	0.60	1.01
14:1n-7	< 0.01	--	--	--	< 0.01
15:1n-5	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
16:3n-4	--	< 0.01	--	< 0.01	--
18:4n-1	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
20:3n-3	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
22:1n-11	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
22:1n-9	< 0.01	< 0.01	0.01	< 0.01	< 0.01
Total saturated	2.49	2.10	1.77	1.31	1.65
Total monounsaturated	2.08	1.60	1.25	1.13	1.15
Total n-3	1.31	1.46	1.88	1.04	1.64
Total n-6	0.49	0.53	0.41	0.38	0.38
Total n-9	1.28	0.94	0.73	0.69	0.67
Total n-3HUFA	1.26	1.41	1.83	1.01	1.59
DHA/22:5 n-6	59.52	19.44	36.69	27.79	33.73
EPA/ARA	3.30	2.80	2.75	2.65	2.68
DHA/EPA	0.75	1.79	3.56	2.10	2.67
DHA/ARA	2.48	5.01	9.76	5.58	7.17
oleic acid/DHA	2.42	1.04	0.48	1.00	0.57
oleic acid/n-3HUFA	0.88	0.59	0.34	0.60	0.36
n-3/n-6	2.66	2.76	4.55	2.77	4.30
ARA/EPA	0.30	0.36	0.36	0.38	0.37
ARA/DHA	0.40	0.20	0.10	0.18	0.14

– indicates component no detected.

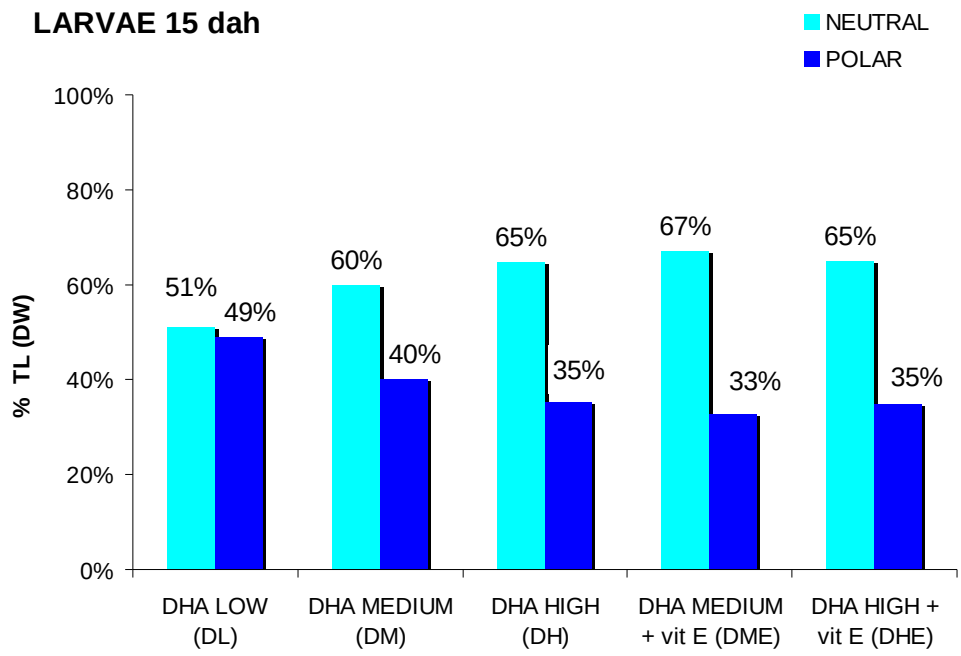


Figure 43 Neutral and polar lipids contents (%) of 15 dah larvae.

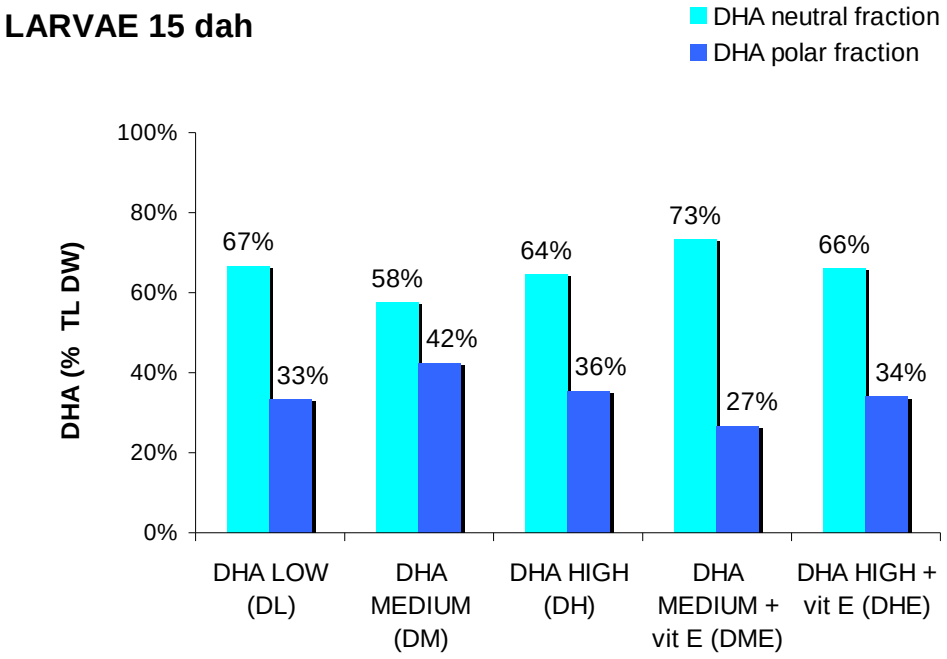


Figure 44 DHA percentages in 15 dah larvae fed rotifers with different DHA and vit E contents.

Larvae of 34 dah did not show any difference regarding total lipid (15.4% DW), crude protein (74.4% DW) and ash content (1.71%) (Table XXIII, Figure 45) ($P > 0.05$).

Larvae from DH group showed in total lipids, the highest content in saturated fatty acids in respect to the other treatments (Table XXIV), principally due to higher contents of palmitic acid and stearic acid.

Equally this group was the one to show larvae with the highest content in total n-3 fatty acids as well as n-3 HUFAs, principally due to a high amount of DHA. In DL and DM groups a high percentage of the n-3 HUFAs (31% and 25% respectively) was represented by EPA. In DL group a contribution of 9% to n-3 HUFAs concentration was ascribable to 22:5n-3. DHE group, on the contrary, was the group that presented the lowest levels of both EPA and 22:5n-3.

The principal HUFAs did not show any difference among the treatments (Table XXIV) ($P > 0.05$), except for ARA that was significantly higher in larvae from DL group in respect to DHE group the showed the lowest content of this fatty acid.

No significant correlation was found between DHA content in rotifers and DHA of larvae at this age ($R= 0.67$, $P > 0.05$) (data not shown).

Table XXIII Proximate composition (mean ± s.d.) of 34 dah larvae fed different levels of DHA and vit E (n=3 per treatment)

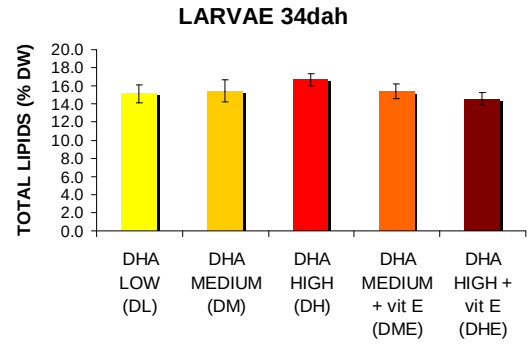
Larvae 34 dah	**DHA LOW (DL)**	**DHA MEDIUM (DM)**	**DHA HIGH (DH)**	**DHA MEDIUM + vit E (DME)**	**DHA HIGH + vit E (DHE)**
% lipids (dw)	14.99 ± 0.99	15.47 ± 1.26	16.69 ± 0.67	15.39 ± 0.84	14.54 ± 0.75
% ash (dw)	1.48 ± 0.04	1.86 ± 0.04	1.64 ± 0.38	1.99 ± 0.15	1.56 ± 0.04
% proteins (dw)	72.28 ± 3.14	75.36 ± 2.86	77.92 ± 6.56	75.01 ± 1.80	71.48 ± 2.88

Table XXIV Total lipids fatty acids profiles (mean ± s.d.) of 34 dah larvae (n=3 per treatment)

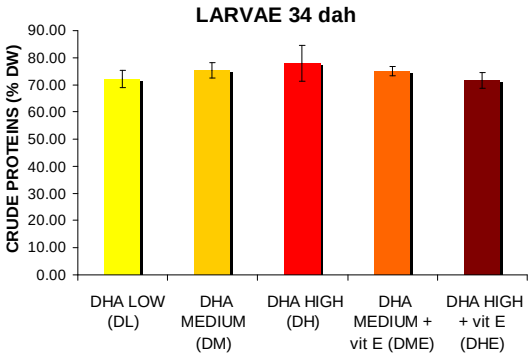
Larvae 34 dah Total lipids	DHA LOW (DL)	DHA MEDIUM (DM)	DHA HIGH (DH)	DHA MEDIUM + vit E (DME)	DHA HIGH + vit E (DHE)
14:0	0.09 ± 0.02	0.11 ± 0.01	0.09 ± 0.06	0.08 ± 0.00	0.07 ± 0.01
14:1n-7	0.06 ± 0.02	--	0.06	0.06	0.06 ± 0.01
14:1n-5	0.03 ± 0.03	0.04 ± 0.02	0.05 ± 0.04	0.05 ± 0.04	0.03 ± 0.02
15:0	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.003	0.03 ± 0.00	0.03 ± 0.00
15:1n-5	0.02 ± 0.00	0.01 ± 0.06	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.00
16:0ISO	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.01
16:0	2.22 ± 0.29	2.42 ± 0.00	2.53 ± 0.13	2.26 ± 0.06	2.02 ± 0.02
16:1n-7	0.44 ± 0.02	0.42 ± 0.05	0.47 ± 0.09	0.43 ± 0.08	0.41 ± 0.05
16:1n-5	0.11 ± 0.01	0.01 ± 0.001	0.12 ± 0.02	0.12 ± 0.01	0.12 ± 0.02
16:2n-6	0.05 ± 0.00	0.05 ± 0.01	0.05 ± 0.02	0.06 ± 0.01	0.05 ± 0.00
16:2n-4	0.10 ± 0.00	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.00	0.10 ± 0.00
17:0	0.09 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.00
16:3n-4	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.02	0.04 ± 0.01	0.03 ± 0.01
16:3n-3	--	--	0.03	--	--
16:3n-1	0.01 ± 0.00	0.01 ± 0.004	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
16:4n-3	0.09 ± 0.02	0.08 ± 0.01	0.08 ± 0.02	0.091 ± 0.005	0.07 ± 0.03
16:4n-1	0.05 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.008	0.04 ± 0.02
18:0	1.22 ± 0.08	1.23 ± 0.04	1.34 ± 0.01	1.26 ± 0.01	1.17 ± 0.03
18:1n-9	2.65 ± 0.19	2.39 ± 0.32	2.59 ± 0.00	2.65 ± 0.22	2.53 ± 0.15
18:1n-7	0.72 ± 0.06	0.62 ± 0.06	1.62 ± 1.38	0.75 ± 0.05	0.77 ± 0.08
18:1n-5	0.04 ± 0.01	0.04 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.00
18:2n-9	0.08 ± 0.01	0.06 ± 0.00	0.06 ± 0.02	0.07 ± 0.02	0.07 ± 0.02
18:2n-6	1.61 ± 0.45	1.97 ± 0.06	1.98 ± 0.48	1.53 ± 0.03	1.39 ± 0.10
18:2n-4	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.02
18:3n-6	0.05 ± 0.00	0.05 ± 0.01	0.06 ± 0.02	0.05 ± 0.01	0.06 ± 0.01
18:3n-4	< 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
18:3n-3	1.50 ± 0.42	1.20 ± 0.35	1.49 ± 0.48	1.55 ± 0.26	1.47 ± 0.27
18:4n-3	0.24 ± 0.07	0.22 ± 0.06	0.26 ± 0.07	0.25 ± 0.06	0.23 ± 0.04
18:4n-1	< 0.01	0.01 ± 0.00	< 0.01	< 0.01	< 0.01
20:0	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
20:1n-9+n-7	0.18 ± 0.02	0.21 ± 0.00	0.25 ± 0.08	0.19 ± 0.01	0.20 ± 0.03
20:1n-5	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
20:2n-9	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
20:2n-6	0.07 ± 0.01	0.08 ± 0.00	0.09 ± 0.02	0.08± 0.00	0.08 ± 0.01
20:3n-6	0.05 ± 0.00	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
20:4n-6	0.34 ± 0.03[a]	0.30 ± 0.01[ab]	0.29 ± 0.01[ab]	0.32 ± 0.01[ab]	0.26 ± 0.03[b]
20:3n-3	0.12 ± 0.03	0.096 ± 0.019	0.13 ± 0.02	0.13 ± 0.01	0.13 ± 0.01
20:4n-3	0.14 ± 0.02	0.12 ± 0.02	0.135 ± 0.004	0.14 ± 0.02	0.13 ± 0.02
20:5n-3	0.83 ± 0.18	0.79 ± 0.09	0.78 ± 0.13	0.72 ± 0.1	0.63 ± 0.11
22:1n-11	0.04 ± 0.01	0.07 ± 0.06	0.11 ± 0.04	0.07 ± 0.00	0.07 ± 0.01
22:1n-9	0.08 ± 0.04	0.09 ± 0.05	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
22:4n-6	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
22:5n-6	0.04 ± 0.01	0.06 ± 0.00	0.04 ± 0.01	0.05 ± 0.00	0.04 ± 0.00
22:5n-3	0.25 ± 0.06	0.23 ± 0.01	0.22 ± 0.04	0.21 ± 0.02	0.19 ± 0.04
22:6n-3	1.32 ± 0.39	1.97 ± 0.08	2.04 ± 0.41	1.63 ± 0.14	1.70 ± 0.43
20:3n-9	< 0.01	--	--	--	--
Total saturated	3.72 ± 0.38	3.94 ± 0.06	4.15 ± 0.11	3.79 ± 0.05	3.44 ± 0.01
Total monounsaturated	4.37 ± 0.26	4.02 ± 0.48	4.47 ± 0.29	4.42 ± 0.34	4.30 ± 0.22
Total n-3	4.46 ± 0.4	4.69 ± 0.61	5.13 ± 0.25	4.72 ± 0.50	4.54 ± 0.50
Total n-6	2.24 ± 0.51	2.54 ± 0.10	2.58 ± 0.50	2.14 ± 0.03	1.92 ± 0.16
Total n-9	3.01 ± 0.14	2.76 ± 0.28	2.11 ± 1.51	2.98 ± 0.24	2.86 ± 0.18
Total n-3HUFA	2.65 ± 0.59	3.19 ± 0.22	3.30 ± 0.54	2.83 ± 0.27	2.78 ± 0.60
DHA/22:5 n-6	31.99 ± 1.84	35.37 ± 0.10	45.88 ± 6.75	32.62 ± 2.65	42. 57 ± 5.79
EPA/ARA	2.44 ± 0.28	2.63 ± 0.18	2.66 ± 0.43	2.23 ± 0.22	2.36 ± 0.15
DHA/EPA	1.57 ± 0.13	2.51 ± 0.18	2.60 ± 0.24	2.28 ± 0.22	2.73 ± 0.24
DHA/ARA	3.84 ± 0.73	6.58 ± 0.03	6.90 ± 1.27	5.05 ± 0.30	6.47 ± 0.93
oleic acid/DHA	2.14 ± 0.65	1.21 ± 0.11	0.76 ± 0.66	1.64 ± 0.18	1.53 ± 0.42
oleic acid/n-3HUFA	1.04 ± 0.26	0.75 ± 0.05	0.48 ± 0.41	0.94 ± 0.08	0.93 ± 0.22
n-3/n-6	2.04 ± 0.34	1.84 ± 0.17	2.04 ± 0.42	2.20 ± 0.23	2.23 ± 0.24
ARA/EPA	0.41 ± 0.05	0.38 ± 0.03	0.38 ± 0.06	0.45 ± 0.04	0.43 ± 0.03
ARA/DHA	0.27 ± 0.05	0.150 ± 0.001	0.15 ± 0.03	0.20 ± 0.01	0.16 ± 0.02

– indicates component no detected.

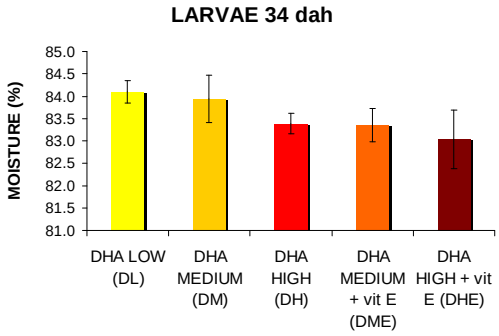
Different letters indicate statistical differences among treatments ($P < 0.05$).



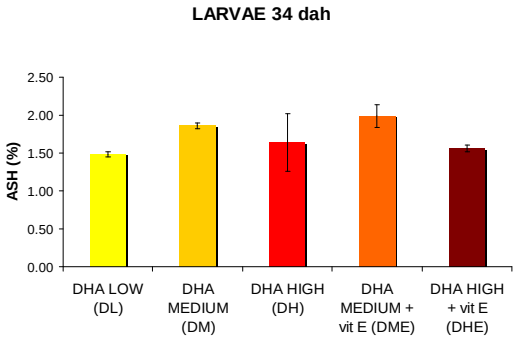
A



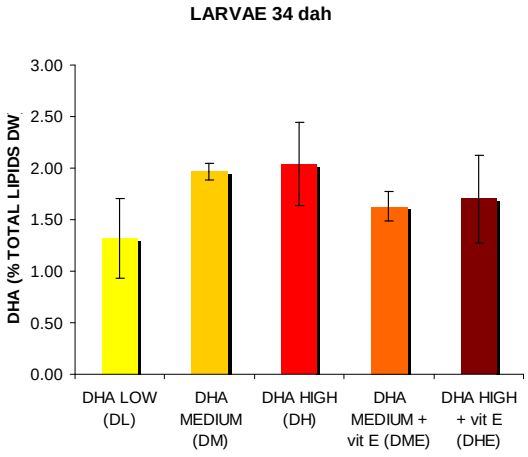
B



C



D



E

Figure 45 Graphical representation of the proximal composition of 34 dah larvae (mean ± s.d.). **A**: total lipids content; **B**: crude proteins; **C**: moisture; **D**: ash content; **E**: DHA content. All data are expressed as percentage of dry weight.

Total lipids of 34 dah larvae showed almost an inverse situation than 15 dah larvae where, the ~ 48% was constituted by neutral lipids and of ~ 52% of polar lipids (% total lipids DW) (Figure 46). No differences were found among the fish groups regarding the quantity of both neutral and polar fraction ($P > 0.05$).

In the polar fraction (Table XVI), DM group showed the highest content of saturated fatty acids, principally due to a higher amount of palmitic acid in respect to the other treatment groups. Larvae both from DL and DM groups, showed also the highest content in monounsatured fatty acids in this fraction principally due to a higher amount of oleic acid in respect to the other treatment groups. These groups were also the ones in which EPA and ARA were principally stored in the polar fraction. In 34 dah larvae, DHA was present for an average of 39 % in the neutral lipid fraction and for a 61 % in the polar fraction (Table XV, Table XVI, Figure 47).

DHA concentration in the polar fraction *versus* neutral fraction did not show any statistical difference among treatments, except for DM group where, DHA in the polar fraction was statistically higher than in the neutral one ($P < 0.05$). Anyway, a tendency to store this particular fatty acid in the polar fraction could be noticed in all groups.

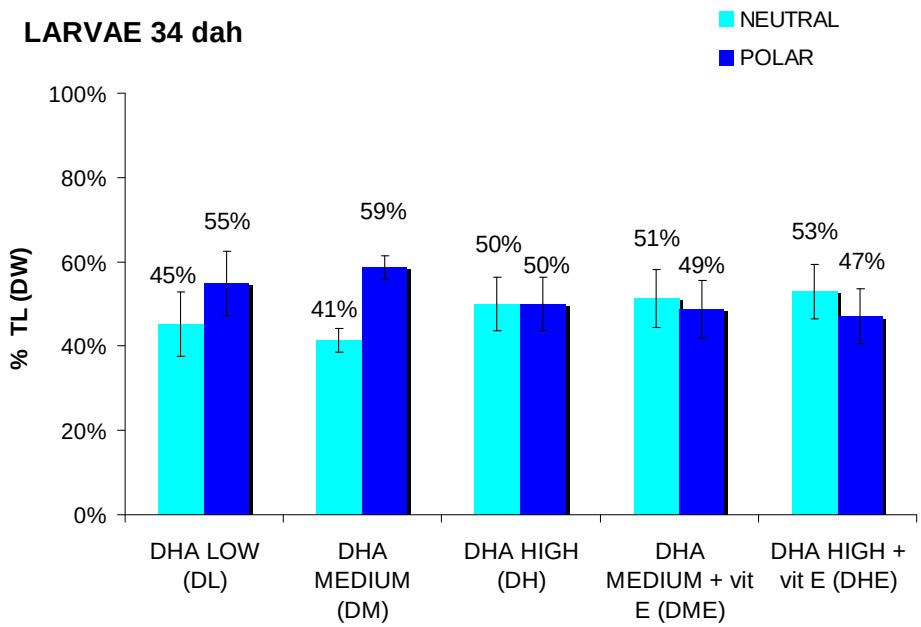


Figure 46 Neutral and polar lipids contents (%) of 34 dah larvae.

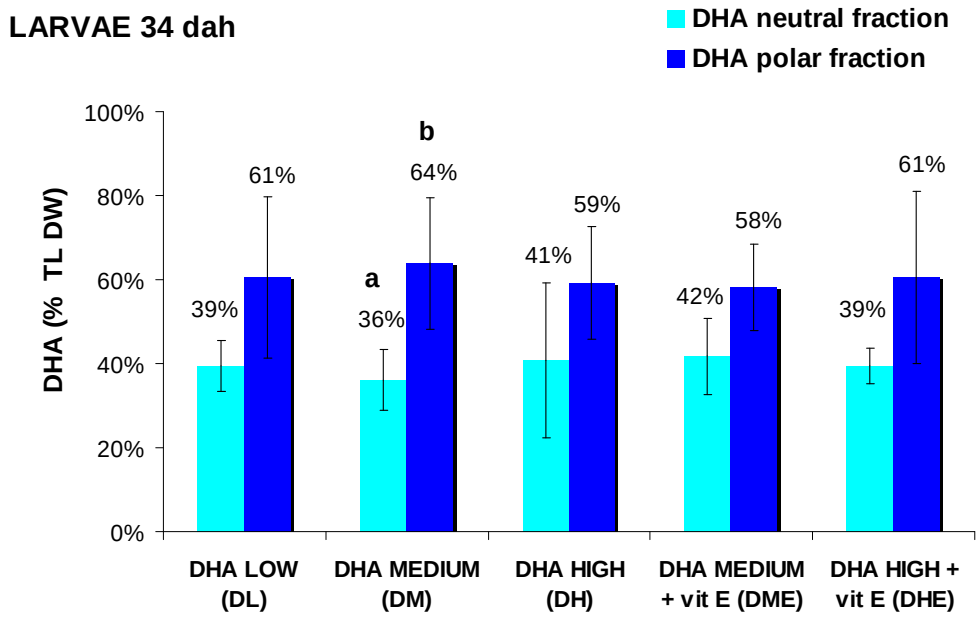


Figure 47 DHA percentages (mean ± s.d.) in 34 dah larvae fed rotifers with different DHA and vit E contents. Different letters indicate statistical differences ($P < 0.05$).

Table XXV Fatty acids profiles (mean ± s.d.) of the lipids neutral fraction of 34 dah larvae (n=3 per treatment)

LARVAE 34 dah ***Neutral Lipids***	**DHA LOW (DL)**	**DHA MEDIUM (DM)**	**DHA HIGH (DH)**	**DHA MEDIUM + vit E (DME)**	**DHA HIGH + vit E (DHE)**
14:00	0.07 ± 0.00	0.07 ± 0.0	0.08 ± 0.02	0.05 ± 0.02	0.07 ± 0.02
14:1n-5	0.04	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.00
15:00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01
15:1n-5	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
16:0ISO	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
16:00	0.77 ± 0.06	0.76 ± 0.09	0.84 ± 0.07	0.84 ± 0.13	0.82 ± 0.17
16:1n-7	0.24 ± 0.05	0.21	0.27 ± 0.04	0.25 ± 0.02	0.29 ± 0.05
16:1n-5	0.05 ± 0.01	0.04 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
16:2n-6	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
16:2n-4	0.05 ± 0.01	0.04 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.01
17:00	0.04 ± 0.01	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.01
16:3n-4	0.03 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.03 ± 0.00	0.03 ± 0.01
16:3n-3	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.00
16:3n-1	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
18:00	0.57 ± 0.09	0.48 ± 0.04	0.58 ± 0.08	0.57 ± 0.08	0.56 ± 0.07
18:1n-9	1.31 ± 0.29	1.08 ± 0.06	1.35 ± 0.09	1.34 ± 0.1	1.73 ± 0 .67
18:1n-7	0.34 ± 0.09	0.26 ± 0.02	0.37 ± 0.11	0.37 ± 0.09	0.39 ± 0.04
18:1n-5	0.03 ± 0.01	0.02 ± 0.00	0.03 ± 0.01	0.022 ± 0.001	0.03 ± 0.01
18:2n-9	0.04 ± 0.01	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
18:2n-6	0.78 ± 0.06	0.87 ± 0.07	1.01 ± 0.44	0.75 ± 0.11	0.79 ± 0.12
18:2n-4	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01
18:3n-6	0.03 ± 0.01	0.025 ± 0.003	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
18:3n-3	0.91 ± 0.31	0.67 ± 0.11	0.84 ± 0.17	0.97 ± 0.17	0.98 ± 0.20
18:4n-3	0.19 ± 0.05	0.15 ± 0.02	0.17 ± 0.04	0.19 v 0.03	0.23 ± 0.02
20:00	0.02 ± 0.01	0.02 ± 0.00	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.00
20:1n-9+n-7	0.10 ± 0.00	0.12	0.14 ± 0.04	0.11 ± 0.02	0.11 ± 0.01
20:1n-5	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
20:2n-9	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
20:2n-6	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.02	0.03 ± 0.00	0.04 ± 0.00
20:3n-6	0.02 ± 0.00	< 0.01	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
20:4n-6	0.16 ± 0.03	0.13 ± 0.02	0.12 ± 0.02	0.15 ± 0.03	0.14 ± 0.00
20:3n-3	0.06 ± 0.02	0.05 ± 0.00	0.06 ± 0.00	0.07 ± 0.01	0.07 ± 0.01
20:4n-3	0.06 ± 0.02	0.05 ± 0.00	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
20:5n-3	0.32 ± 0.05	0.30 ± 0.02	0.33 ± 0.13	0.30 ± 0.07	0.34 ± 0.05
22:1n-11	0.06 ± 0.02	0.09 ± 0.02	0.08 ± 0.04	0.06 ± 0.01	0.05 ± 0.01
22:1n-9	0.05 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
22:5n-6	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
22:5n-3	0.10 ± 0.02	0.08 ± 0.01	0.09 ± 0.03	0.09 ± 0.02	0.08 ± 0.01
22:6n-3	0.54 ± 0.10	0.65 ± 0.10	0.76 ± 0.32	0.65 ± 0.16	0.69 ± 0.07
14:1n-7	0.02 ± 0.00	--	0.001	--	0.001
16:4n-3	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
16:4n-1	< 0.01	--	< 0.01	--	--
18:3n-4	< 0.01	< 0.01	< 0.01	< 0.01	0.01
18:4n-1	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
20:3n-9	< 0.01	0.01 ± 0.00	< 0.01	< 0.01	--
22:4n-6	0.01 ± 0.00	< 0.01	< 0.01	< 0.01	0.01 ± 0.00
Total saturated	1.50 ± 0.17	1.41 ± 0.13	1.68 ± 0.14	1.57 ± 0.19	1.56 ± 0.30
Total monounsaturared	2.23 ± 0.44	1.90 ± 0.04	2.40 ± 0.30	2.30 ± 0.22	2.76 ± 0.75
Total n-3	2.20 ± 0.36	1.964 ± 0.005	2.32 ± 0.29	2.36 ± 0.40	2.47 ± 0.18
Total n-6	1.06 ± 0.08	1.12 ± 0.09	0.54 ± 0.45	1.03 ± 0.14	1.07 ± 0.13
Total n-9	1.76 ± 0.66	1.27 ± 0.07	1.53 ± 0.18	1.50 ± 0.11	1.92 ± 0.67
Total n-3HUFA	1.078 ± 0.16	1.12 ± 0.12	1.29 ± 0.48	1.18 ± 0.27	1.25 ± 0.04
DHA/22:5 n-6	40.10 ± 0.46	38.77 ± 2.18	53.52 ± 13.48	37.74 ± 2.24	49.50 ± 5.55
EPA/ARA	2.92 ± 1.44	2.24 ± 0.15	2.56 ± 0.70	2.07 ± 0.07	2.41 ± 0.31
DHA/EPA	1.71 ± 0.24	2.18 ± 0.23	2.32 ± 0.06	2.16 ± 0.26	2.08 ± 0.45
DHA/ARA	4.86 ± 1.98	4.86 ± 0.18	5.97 ± 1.73	4.44 ± 0.44	4.93 ± 0.62
oleic acid/DHA	2.54 ± 0.94	1.70 ± 0.37	1.99 ± 0.80	2.14 ± 0.51	2.59 ± 1.22
oleic acid/n-3HUFA	1.24 ± 0.38	0.98 ± 0.16	1.14 ± 0.41	1.17 ± 0.22	1.39 ± 0.56
n-3/n-6	2.08 ± 0.36	1.76 ± 0.15	6.41 ± 3.88	2.29 ± 0.28	2.35 ± 0.45
ARA/EPA	0.39 ± 0.15	0.45 ± 0.03	0.41 ± 0.10	0.48 ± 0.02	0.42 ± 0.05
ARA/DHA	0.23 ± 0.08	0.21 ± 0.01	0.18 ± 0.04	0.23 ± 0.02	0.20 ± 0.02

– indicates component no detected.

Table XXVI Fatty acids profiles (mean ± s.d.) of the lipids polar fraction of 34 dah larvae (n=3 per treatment)

LARVAE 34 dah ***Polar Lipids***	**DHA LOW (DL)**	**DHA MEDIUM (DM)**	**DHA HIGH (DH)**	**DHA MEDIUM + vit E (DME)**	**DHA HIGH + vit E (DHE)**
14:0	0.03 ± 0.02	0.11 ± 0.09	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.01
14:1n-5	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
15:0	0.02± 0.01	0.04 ± 0.02	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0 .00
16:0ISO	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
16:0	1.69 ± 0.40	2.32 ± 0.72	1.46 ± 0.11	1.45 ± 0.19	1.38 ± 0.24
16:1n-7	0.18 ± 0.08	0.25 ± 0.08	0.21 ± 0.05	0.23 ± 0.09	0.19 ± 0.03
16:1n-5	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
16:2n-6	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.00	0.02 ± 0.01
16:2n-4	0.06 ± 0.01	0.07 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
17:0	0.06 ± 0.01	0.05 ± 0.00	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
16:3n-4	0.01 ± 0.00	0.01	0.01	< 0.01	< 0.01
16:3n-3	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
16:3n-1	0.07 ± 0.03	0.06 ± 0.02	0.05 ± 0.02	0.07 ± 0.02	0.06 ± 0.01
16:4n-3	0.04 ± 0.01	0.04 ± 0.02	0.03 ± 0.01	0.05 ± 0.02	0.05 ± 0.01
18:0	0.78 ± 0.16	0.79 ± 0.09	0.61 ± 0.07	0.63 ± 0.11	0.58 ± 0.11
18:1n-9	1.50 ± 0.15	1.38 ± 0.18	1.18 ± 0.06	1.29 ± 0.23	1.18 ± 0.18
18:1n-7	0.31 ± 0.06	0.29 ± 0.01	0.27 ± 0.02	0.23 ± 0.05	0.30 ± 0.09
18:1n-5	0.02 ± 0.00	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
18:2n-9	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.00
18:2n-6	1.07 ± 0.26	0.98 ± 0.13	0.84 ± 0.02	0.71 ± 0.12	0.67 ± 0.14
18:2n-4	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
18:3n-6	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.00
18:3n-3	0.53 ± 0.10	0.32 ± 0.02	0.38 ± 0.14	0.46 ± 0.17	0.43 ± 0.01
18:4n-3	0.06 ± 0.01	0.04 ± 0.00	0.04 ± 0.01	0.05 ± 0.02	0.04 ± 0.00
20:0	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
20:1n-9+n-7	0.07 ± 0.01	0.11 ± 0.05	0.055 ± 0.003	0.05 ± 0.01	0.05 ± 0.02
20:1n-5	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
20:2n-9	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	< 0.01
20:2n-6	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
20:3n-6	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.01
20:4n-6	0.18 ± 0.03	0.14 ± 0.02	0.12 ± 0.03	0.15 ± 0.04	0.13 ± 0.03
20:3n-3	0.05 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.01	0.05 ± 0.01
20:4n-3	0.07 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.02	0.06 ± 0.01
20:5n-3	0.54 ± 0.11	0.45 ± 0.05	0.39 ± 0.04	0.39 ± 0.12	0.37 ± 0.09
22:1n-11	0.01 ± 0.00	0.04 ± 0.03	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
22:1n-9	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	< 0.01	< 0.01
22:4n-6	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
22:5n-6	0.03 ± 0.00	0.04 ± 0.00	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.04
22:5n-3	0.15 ± 0.04	0.12 ± 0.02	0.10 ± 0.02	0.11 ± 0.03	0.11 ± 0.03
22:6n-3	0.83 ± 0.33	1.14 ± 0.22	1.10 ± 0.23	0.91 ± 0.18	1.05 ± 0.36
14:1n-7	< 0.01	0.01	< 0.01	< 0.01	< 0.01
15:1n-5	< 0.01	< 0.01	0.01 ± 0.00	< 0.01	0.01 ± 0.00
16:4n-1	--	--	0.01	0.03	--
18:3n-4	< 0.01	< 0.01	< 0.01	--	< 0.01
18:4n-1	< 0.01	< 0.01	0.01 ± 0.00	< 0.01	< 0.01
20:3n-9	0.01	--	< 0.01	< 0.01	--
Total saturated	2.62 ± 0.57	3.35 ± 0.97	2.20 ± 0.16	2.21 ± 0.31	2.07 ± 0.36
Total monounsaturared	2.20 ± 0.24	2.19 ± 0.35	1.83 ± 0.01	1.92 ± 0.33	1.84 ± 0.32
Total n-3	2.28 ± 0.41	2.21± 0.33	2.14 ± 0.36	2.08 ± 0.54	2.16 ± 0 .52
Total n-6	1.40 ± 0.31	1.26 ± 0.17	1.08 ± 0.04	1.00 ± 0.16	0.93 ± 0.19
Total n-9	1.63 ± 0.17	1.55 ± 0.25	1.27 ± 0.07	1.23 ± 0.16	1.27 ± 0.19
Total n-3HUFA	1.64 ± 0.48	1.80 ± 0.30	1.68 ± 0.30	1.52 ± 0.36	1.64 ± 0.49
DHA/22:5 n-6	31.11 ± 1.12	32.97 ± 3.87	49.02 ± 6.80	30.51 ± 0.20	44. 40 ± 7.87
EPA/ARA	2.96 ± 0.10	3.25 ± 0.10	3.56 ± 1.11	2.68 ± 0.06	2.93 ± 0.18
DHA/EPA	1.51 ± 0.27	2.53 ± 0.20	2.83 ± 0.28	2.34 ± 0.27	2.80 ± 0.28
DHA/ARA	4.49 ± 0.97	8.22 ± 0.42	9.99 ± 2.84	6.26 ± 0.60	8.23 ± 1.06
oleic acid/DHA	1.96 ± 0.58	1.25 ± 0.40	1.10 ± 0.21	1.44 ± 0.13	1.16 ± 0.20
oleic acid/n-3HUFA	0.96 ± 0.21	0.79 ± 0.23	0.71 ± 0.11	0.86 ± 0.12	0.74 ± 0.11
n-3/n-6	1.64 ± 0.19	1.75 ± 0.03	1.98 ± 0.25	2.08 ± 0.30	2.34 ± 0.34
ARA/EPA	0.34 ± 0.01	0.31 ± 0.01	0.30 ± 0.08	0.37 ± 0.01	0.34 ± 0.02
ARA/DHA	0.23 ± 0.04	0.12 ± 0.01	0.11 ± 0.03	0.16 ± 0.02	0.12 ± 0.01

– indicates component no detected.

3.2.7 TBARS OF 15 DAH OLD LARVAE

The lowest TBARS (thiobarbituric acid reactive substances) levels were the ones of DL and DME groups (9.76 and 11.64 nmol(M) g^{-1} respectively), followed by DM group (446.41 nmol(M) g^{-1}) and DH and DHE groups in 15 dah larvae (551.95 and 563.83 nmolM g^{-1} respectively) (Figure 48) ($P > 0.05$). No differences were found among different treatment groups at 34 dah (data not showed).

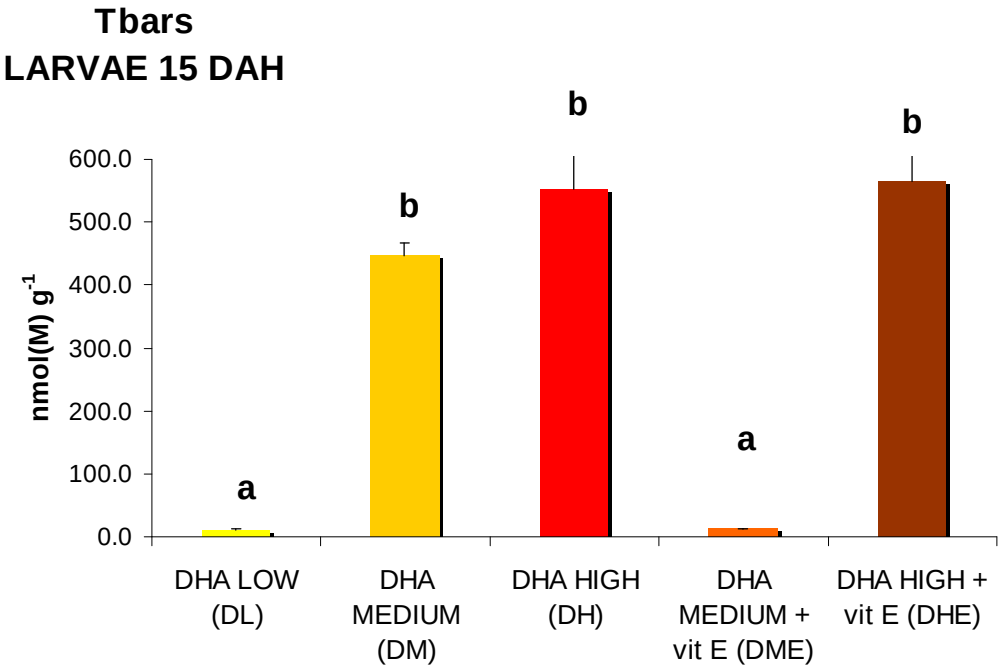


Figure 48 TBARS of 15 dah larvae expressed as nanomoles of malonaldheyde (M) per gram of sample.

4. DISCUSSION

4. DISCUSSION

4.1 TRIAL I: DIFFERENT WHOLE MOUNT STAINING PROTOCOLS AND SKELETAL DEVELOPMENT IN OLD STORED FORMALDHEYDE LARVAE

Whole mount staining protocols for bone and cartilage visualization are used since long for developmental or teratogenical studies both in tetrapods and fish. Differences in the application of these techniques may vary in the fixative (buffered formaldehyde, Bouin, formol-acetic-alcol), and concentrations and times of application of the different dyes etc. The simultaneous use of alcian blue and alizarin red, even if is still used in fish for developmental studies (Liu, 2001; Gavaia *et al.*, 2002; Socorro, 2006; Roo, 2009), has been questioned and avoided in other situations for several reasons. Alcian blue is specific for mucopolysaccharides, while alizarin red stains areas of calcium salt deposition. In fish, the origin of ossified tissue is complex, bone can develop directly into bone (intramembranous bone) or from cartilagineous precursors (cartilage replacement bone), Alcian blue, seems to stain both uncalcified bone and cartilage in early-stage larvae (Kohno and Taki, 1983). On the other side, alizarin red is not specific for hydroxyapatite, the main mineral phase of bone, but it indicates deposition of calcium salts also in non-ossifying embryonic connective tissue (Faustino and Power, 1998). Another problem discussed, principally in small specimens, regards the use of alcian blue staining,

prior to alizarin staining, because of the acidic solution (glacial acetic acid) in which the dye is dissolved. Indeed, some bony structures in early stages of development may be demineralized during cartilage staining (Vandewalle *et al.* 1998). In recent studies, separated single staining have been generally performed (Witten and Villwock, 1997; Kjørsvik *et al.*, 2009; Moren *et al.*, 2009) or new acid-free alcian blue solutions have been proposed (Walker and Kimmel, 2007; Bensimon-Brito *et al.*, 2010).

In this study, the visualisation of cranial structures was not strongly affected by the double staining as either single or double stainings visualized mineralised skull bones. Upper and lower jaw, together with bones from the opercular series and neurocranium, were among the first elements to mineralize in *S. aurata*, contemporary to caudal fin elements (hypural I and II and some of the PCR). Therefore, being these structures earlier and strongly mineralized, they could resist better to the slight decalcification by the acidic solution of alcian blue. On the contrary, in specimens that started to show first vertebral column elements, like neural spines and centra (28 dah larvae), double stain seemed to affect negatively their visualization, that, on the contrary were perfectly visible with single alizarin red staining.

In any case, it must be pointed out that, these results were developed starting from old stored buffered formaldehyde larvae that could have lost the buffering proprieties due to the long time of storage affecting

staining performance. Other studies in which the biological material (stored in the same fixative) have been processed for double staining in less time, gave right information on skeletal development of some Sparids species (Socorro, 2006; Roo, 2009). In other cases, to avoid misinterpretation of whole mount staining results, double staining was coupled with studies on the expression levels of osteocalcin in order to ratify a correct visualization (Darias *et al.*, 2010).

Skeletal development of mesocosm larvae, long stored in buffered formaldehyde and single stained with alizarin red, did not showed any particular deviation from the typical osteological development already described for this species and other Sparids (Koumoundourous *et al.*, 1997a; Sfakianakis *et al.*, 2004; Sfakianakis *et al.*, 2005). The effect of long time storage seemed to do not compromise the general developmental scheme of sea bream larvae. Differences were found in the onset of notochord flexion, the ossification of v. I, and some caudal fin complex elements appearance.

Flexion of the notochord occurred in fish of 5.8-6 mm SL (larvae from 17 up to 21 dah) in agreement to Faustino and Power (1998) studies. On the contrary, Koumoundourous *et al.* (1997a) found flexion in a higher size range (7.5 – 8.1 mm TL), that could be related to differences in water temperature or rearing system. Thus, in the present study average temperature was of 20ºC higher than in Koumoundourous *et al.* (1997a)

research, wheras larval density was similar between both studies. In agreement, the onset of ossification of the caudal fin (5.5 – 6 mm TL in this study vs 6.5 mm TL), as well as first mineralizaiton of the urostyle and the occasional presence of mineralized hypural V occurred before than described by Koumoundourous *et al.* (1997a).

The first ossified elements at 4 mm SL (10 dha) were the lower and the upper jaws, the chleithrum, brachiostegals and the preoperculum in agreement with other studies for this species and other Sparids (Cubbage and Mabee, 1996; Faustino and Power, 1998; Faustino and Power, 2001; Liu, 2001; Sfakianakis *et al.*, 2004; Sfakianakis *et al.*, 2005; Socorro, 2006).

Vertebral neural spines were the first elements to ossify starting from neural spine II and following in a caudal direction (Faustino and Power, 1998; Gavaia *et al.*, 2002; Sfakianakis *et al.*, 2004; Sfakianakis *et al.*, 2005; Socorro, 2006). The vertebral centrum of the first vertebra, as well as the pattern of mineralization in a ventral direction, of this vertebra and the haemal and pre-haemal ones, remarked the same pattern of other Sparids and can be recognized at 28 dah at a SL of 6.6 – 6.8 mm well after the pre haemal and haemal centra with a pattern of mineralization around the notochord proceeds ventrally for cranial, pre haemal and haemal centra. At 10.9 mm SL the vertebral column presented complete mineralization as reported by other studies (Faustino and Power, 1998).

At this length corresponded also the mineralization, in the caudal complex, of the uroneural I in accordance with the previous study, but well before reported by Koumoundourous *et al.* (1997a) (14 mm TL vs 18.3 mm TL). In the same way, the last elements to mineralize (epurals) were present at a lower length than the ones reported by Koumoundourous *et al.* (1997a), but in the size range suggested by Faustino and Power (1998).

No studies actually exist on sea bream vertebral dimension development with age. The present study tried to elucidate vertebral development in length of sea bream larvae reared in mesocosm system in order to give information on a normal development of axial skeleton of this species.

Studies on salmons have indeed highlighted that different production strategies may affect vertebral dimension and their biomechanical carachteristics (Fjelldal *et al.*, 2006; Fjelldal *et al.*, 2007).

Results from the present study, confirm the general developmental pattern described for sea bream (Faustino and Power, 1998). Indeed, in mesocosm sea bream larvae analyzed in this study since 35 dah, haemal and pre haemal regions account for the majority of total vertebral length both considering length at neural (dorsal) and haemal (ventral) sides (30 and 27% of total vertebral length respectively) in comparison to cranial and caudal regions (18 and 21% respectively)and height

Length of pre haemal vertebrae, both considering dorsal and ventral measures, almost remain constant from 35 to 50 dah, since the onset of

vertebral centra (VB) mineralization starts from the ventral (haemal) side of each vertebra in these regions (Faustino and Power, 1998) and at 35 dah they are almost complete. Vertebrae of the haemal region, on the contrary, slightly increased the proportion occupied in length with age (from 28% to 31%), in accordance to the cranial to caudal pattern of mineralization (Faustino and Power, 1998).

Vertebrae from the cranial region and caudal region, on the contrary, showed a higher variation occupying an increasing proportion to the total length with age. The cranial centra that at 35 dah account for a 26% of vertebral total length, occupied only a 18% at 50 dah, with a drop of 68% in the space occupied along the entire development, probably due to the higher developmental rates of the following centra. An opposite condition, could be noticed in the caudal centra that, along with age, accounted for a higher proportion of the entire length.

This is in accordance to the development of sea bream axial skeleton in which, the caudal verterbrae and the first one are the last to complete the development (Faustino and Power, 1998; Koumoundourous *et al.*, 2001).

Regarding vertebral height, trunk (pre-haemal and haemal) centra, and in particular pre –haemal, showed always a higher height at the posterior side of the centra (p.h.) showing that the posterior height of these vertebrae is always higher than the anterior one at all ages considered and probably suggesting a higher grow rate of the developing vertebral

centra in caudal direction. On the contrary, both cranial and caudal centra showed a more homogenous growth around the notochord regardless the age as corroborated by a.h./p.h. ratio close to the unity.

4.2 TRIAL II: EFFECTS OF DIFFERENT DHA ENRICHMENT LEVELS ON THE BONE FORMATION AND EARLY MINERALIZATION OF THE AXIAL SKELETON OF *Sparus aurata*

Rotifers composition reflected the enrichments products content, with increased DHA and vit E levels in the rotifers supplemented by these nutrients, denoting high assimilation rates and a successful enrichment.

Vitamin E content of the enrichments, was in the range of raccomended values for enriched and natural zooplankton [114÷1040 mg kg^{-1} DW] (Mourente *et al.*, 1999).

The contents in ARA and EPA from all the enriched rotifers were enough to cover the requirements described for best growth in sea bream larvae (Rodríguez *et al.*, 1998; Izquierdo, 2005).

DL enriched rotifers had a 31% lower content in DHA than the recommended requirement for this species (0.8% DW) (Rodríguez *et al.*, 1998), whereas in the other rotifers it was in the range required (up to 5% DW) (Izquierdo, 2005; Liu *et al.*, 2002).

Rotifers enriched with DH and DHE and DM and DME emulsions showed comparable values with the ones reported in Roo *et al.* (2009) who used the same type of initial products.

Total lipids' content in rotifers increased in DL group in respect to the relative enrichment, while decreased in the other groups. Enriched rotifers reflected the composition of total fatty acids of the enrichments. Anyway, ARA and EPA were incorporated in the same quantity they were present in the original enrichment in DL, DM, DME groups, while rotifers from DH and DHE groups showed a 50% less of the latter fatty acid than the quantity present in the enrichments.

DHA rotifers' content was positively correlated with the content in the enrichments, even though also in the case of this fatty acid, a drop in concentration could be detected in live preys in respect to the enrichments.

Rotifers differently metabolized the enrichments in particular with a general decrease of the DHA that account for ~ 20% and up to the 60% of the original value in the enrichments DM/DME and DH/DHE respectively. On the contrary, rotifers fed with DL treatment metabolized more efficiently the DHA normally showing an increase of 32% in respect to the original enrichment content. These results probably indicate that especially EPA and DHA are less efficiently incorporated into rotifers' bodies in respect of ARA as the concentration of these fatty acids in the enrichments is not reflected in live preys. Anyway, in DH, DHE groups,

the EPA drop in concentration was lower than the drop in DHA, indicating a better accumulation of EPA in respect of DHA as suggested by Rodríguez *et al.* (1996). The author indicates that rotifers, being principally found in freshwater habitat and by feeding on freshwater microalgae (richer in EPA than in DHA), could have enzymatic systems more adapted to incorporate EPA more readily than DHA.

Due to these differences in DHA and EPA content, also the DHA/EPA ratio was altered in rotifers. In particular these values ranged between 0.27 (DL), 2.6 (DM, DME), 5.2 (DH, DHE), with values of DL rotifers far above from the ones reported by Rodríguez *et al.* (1998) and Sargent *et al.* (1999) for best performance in sea bream larvae. Minimum DHA/EPA ratio suggested for marine fish larvae (2:1) (Sargent *et al.*, 1999) was achieved only in rotifers of DM, DH, DME, DHE.

However, rotifers from DL group were the only that showed an increased DHA/EPA ratio in respect to the original enrichment while the other groups showed in all cases a decrease of this ratio in respect of the respective enrichments. This same trend could be observed for rotifers' n-3 HUFA content.

EPA/ARA ratios were close to 4 (for DL, DM and DME groups) and close to 6 (in DH, DHE group), and in line with the values reported in Atalah (2008) for dry feeds for best performance of sea bream larvae.

Explications for drops in concentration of n-3 HUFA have been related to rotifers' rearing temperature and to the enrichment period. Low temperature stabilized total lipids content (Olsen *et al.*, 1993) while its increase induces proportional loss principally of n-3 (Rainuzzo *et al.*, 1989). In this study, temperatures in the enrichment tanks were about 20ºC, but the enrichment process last less than the 12 hours cited in those studies, and no differences were found between total fatty acids composition in a previous trial in which rotifers were enriched for 6 or 8 hours (data not shown) in accordance also with Rodríguez *et al.* (1996).

Moreover, it must to be pointed out that a 20% of the rotifers DH and DHE generally died during the enrichment (personal observation). A similar result was obtained by Rodríguez and collaborators (1996), who showed a sharp decline in rotifers' population with increasing enrichments oil emulsions. The authors suggested that either high quantities of the enrichment could have pollute the water or that the excess of lipids itself could be harmful to the rotifers.

Anyway, only the use of rotifers DH and DHE, permit to have larvae at 15 dah with a DHA content very close to the one of the eggs from they derived (eggs: 3.85% *vs* DH/DHE larvae 3.37% DW). Indeed, both DM/DME and DL larvae showed lower content in DHA in relation to that of the eggs (DL: 0.97%, DM/DME: 2.5% DW), suggesting a closer approach of DH and DHE rotifers to satisfy the essential fatty acids

requirements of the larvae during first feeding. Indeed, eggs composition has been suggested as an indicator of nutritional requirements of newly hatched larvae (Robaina and Izquierdo, 2000).

Eggs composition showed comparable levels of the principal fatty acids reported for this and other Sparids species (Fernández-Palacios *et al.*, 1997; Lane and Kohler, 2006; Samaee, 2010), being palmitic acid the most representative. Anyway, principally linoleic acid showed higher percentages in respect to the previous cited works while palmitoleic acid (16:1n-7) showed a lower percentage in respect to the studies of Fernández-Palacios *et al.* (1997) and Samaee (2010). In most cases, the increase of linoleic acid in eggs has been attributed to the broodstock nutrition on commercial feeds richer in vegetable sources.

Both in larvae of 15 and 34 dah no differences were found in proteins, total lipids or ash content among the treatment groups. Lowering in ash content has been suggested to be inversely correlated with skeleton abnormalities (Barahona-Fernández, 1982).

Proximal composition of larvae generally reflected the one of live feed up to 15 dah. In particular, larvae fed DL rotifers showed an increase of n-3 HUFA principally due to an increase of ~ 26% in DHA, of ~ 61% of EPA (20:5n-3), and showed an increase of ~ 36% of ARA (20:4n-6) in respect to live preys.

DM, DME groups reflected rotifers' composition, both for EPA and DHA content, while showed a strong drop in ARA concentration of ~ 92% in respect to live preys. DH, DHE groups showed a decrease in n-3 HUFA principally due to a decrease of ~ 31 and ~ 41 % of DHA respectively.

Elevated levels of specific fatty acids compared to dietary amount have been referred to the incorporation efficiency (Castell *et al.*, 1994) and in particular, being DHA mobilized after the other fatty acids of the n-3 series, it is more conserved, because of its fundamental role in the regulation of membrane integrity and function (Kolkovski *et al.*, 2009).

In larvae of 34 dah, the effects of enriched rotifers was less marked even though DL larvae still presented the lowest level of DHA among the groups. Probably a "wash-out" process due to alimentation with *Artemia* sp. (offered from 15 dah) and weaning diet (from 26 dah) may partially explain this effect. Similar results were found for red porgy larvae (*Pagrus pagrus*) by Roo and collaborators (2009) who did not find any effect of rotifers' enrichment on larvae starting from 35 dah after 7 days co-feeding with Artemia.

Specific composition of larval neutral and polar lipids showed that after 13 days of exogenous feeding (15 dah), larvae presented higher percentages of neutral lipids instead of the polar fraction, except for larvae from DL group in which both neutral and polar lipids account with the same percentage to total lipids composition. In the other treatments,

neutral lipids account for ~ 66% of TL while polar lipids for ~ 36% of TL for DH, DME, DHE treatments and for 45% for DM larvae.

Rodríguez *et al.* (1998) showed a marked depletion of lipids from the polar lipid fraction in the very first days of feeding probably due to selective catabolism of phospholipids that provide phosphate and choline for intermediary metabolism and to facilitate the transport of neutral lipids. Normally, dietary phospholipids have a marked effect on lipid transport (Sahli *et al.*, 1999; Izquierdo *et al.*, 2000) and may accelerate the incorporation of TAG (triacylglicerols) when the digestive capacity is not completely developed (Teshima *et al.*, 1987). In other species it has been shown that in developing larvae, essentially fatty acids are not oxidized, but are temporary stored in the neutral lipid fraction, and only after mobilized for structural purposes (Tocher *et al.*, 1985; Fraser *et al.*, 1988; Wiegand, 1996).

Fatty acids profiles of 15 dah larvae did not agree completely to the data reported in Bessonart (1997) both regarding neutral and polar fractions. Apart from DM larvae, where ARA, EPA and DHA were equally stored in both fractions, larvae from the other treatments, showed a higher retention of these fatty acids in the neutral fraction.

Apart from DHA, both neutral and polar fractions were constituted principally by palmitic, oleic and stearic acids, while in Bessonart (1997) palmitoleic acid was reported as the second most representative in the neutral fraction and the fourth in the polar one

In 34 dah larvae, a higher percentage of body lipids was generally represented by polar lipids in all treatment groups even though without marked differences between the two fractions, except for larvae of DM group, in which polar lipid fraction represented almost 2/3 of total lipids. In all cases, DHA and EPA were preferentially stored in the polar lipid fraction contrary to ARA, presented in higher percentages in the polar fraction, indicating their importance as cell membrane constituents (Sargent *et al.*, 1999).

Many studies focused on the importance of vitamin E as one of the principal components (together with vitamin C) of the animal antioxidant system, with particular reference to marine species (Mourente *et al.*, 1999; Tocher *et al.*, 2002; Lewis-McCrea and Lall, 2007; Mourente *et al.*, 2007; Betancor *et al.*, 2010). Malonaldheyde (MDA) is one of the main oxidative breakdown products of PUFAs and it is measured by thiobarbituric acid reactive substance (TBARS). The efficacy of this indicator has been proved both for the evaluation of tissue lipid peroxidation and for the efficacy of the antioxidant system (Mourente *et al.*, 1999; Tocher *et al.*, 2002).

Larvae fed rotifers with low level of n-3 HUFA (1.38% DW) and in particular with low levels of DHA (0.25% DW) showed the minimum values of TBARS as well as DME fed larvae. On the contrary, DM, DH and DHE larvae showed the highest values of TBARS. TBARS of liver

and muscles have been found to be negatively correlated with vit E levels in the diets both in sea bream and in halibut (Tocher *et al.*, 2002; Lewis-McCrea and Lall, 2007). Anyway, Betancor *et al.* (2010) found that bass larvae fed diets with high levels of DHA even though supplemented with vit E, presented muscular damage and higher incidence of ceroids pigments in the hepatocytes, as symptom of vitamin E deficiency and/or imbalances between anti and pro-oxidants. Therefore, it can be suggested that, in this case, in DL fed group there was not an alteration of the oxidative status of the larvae because of the overall low content of HUFA (and in particular DHA), while in the other groups the oxidative status has been altered but, in the case of DME group, supplementation of vit E could have controlled this condition. Finally, in both groups fed highly DHA enriched rotifers (DH, DHE), neither the increment of vit E could have been contained lipids peroxidation. Actually it is not possible to exclude the participation of other nutrients (such as vitamin C) or phospholipids that have been suggested to have a sparing effect on tocopherols enhancing their antioxidant activity (Lambelet *et al.*, 1984).

No differences in growth both in terms of weight or length have been found in any sampling point of this study. Anyway, larvae fed DL treatment starting from 20 dah showed a lower weight and length in respect to the other treatments.

A various range of effects on growth in sea bream larvae and other teleosts species due to the supplementation of different PUFAs in live feeds have been reported as first requirements are not well established for all marine species (Rodríguez *et al.*, 1998; Ali *et al.*, 2007; Roo *et al.*, 2005; Roo *et al.*, 2009).

Gapasin and Duray (2001) did not found any increase in growth in milkfish (*Chanos chanos*) fed rotifers with 1.28 (%DW) of DHA, while Roo and collaborators (2005) showed that larvae fed enriched rotifers with 19.7 (%TFA) induce an increment in length at the end of the larval phase for sea bream. In cold water species, Copeman *et al.* (2002) reported increased growth for yellowtail flounder (*Limanda ferruginaea*) fed enriched rotifers with 28.2 (%TFA) respectively, comparable to DHA values of rotifers of DH and DHE treatment in this study, induce an increment in length at the end of the larval phase. In *Dentex dentex* best growth is achieved feeding larvae with rotifers with 7.2 (%DW) of DHA (Ali *et al.*, 2007). Anyway, Rodríguez *et al.* (1998) suggest that best growth for sea bream larvae can be achieved with a minimum DHA/EPA ratio of 1.4/1 and a total n-HUFA of 1.5 (%DW), condition that was achieved only in DM, DME, DH, DHE groups.

Interaction between dietary vit E and different levels of PUFAs gave different results in terms of growth in teleost fishes. In bass, fed microdiets, increasing DHA up to 5% supplemented only with 1500 mg kg^{-1} of vit E showed the minimum growth of larvae, even though an

increase of DHA up to 3% is beneficial in terms of growth (Atalah *et al.*, 2008; Betancor *et al.*, 2010). In bream fed microdiets, 2.5% (DW) DHA and, EPA 1.5% (DW) supplemented with 3000 mg kg^{-1} of vit E gave the best results in growth after 14 feeding days (Atalah, 2008). In this study, increasing DHA up to 5% in rotifers and supplementing enrichements up to 15000 mg kg^{-1} of the products did not enhanced growth, as similar length and values were found in DM, DH, DME, DHE groups. In particular, DM and DH groups even showed slightly higher values in contrast to the same treatments supplemented with higher levels of vit E (DME, DHE).

Increasing vitamin E has been showed to be beneficial in some cases. Tocher and collaborators (2002) showed that increasing vitamin E up to 1247 mg kg^{-1} in dry diet enhances survival and growth in sea bream juveniles. The same conclusions were achieved by González Pérez (1995) for sea bream larvae. No effects in growth or survival have been found also in halibut juveniles (Tocher *et al.*, 2002; Lewis-McCrea and Lall, 2007) or turbot, even if fed with graded levels of oxidized lipids. Brown *et al.* (2005), did not evidenced enhancement of larval survival in another demersal marine fish (*Latris lineata*), but generally indicated an overall better biological performance in larvae fed vit E enriched rotifers.

The same trend seen in growth was also seen in overall survival that was not affected both due to DHA levels or vit E. In all groups high survival rates have been recorded both at 15 and at 34 dah in respect to

other studies with the same or other Sparids species treated with different nutritional regimes in terms of fatty acids (Bessonart *et al.*, 1999; Rodríguez *et al.*, 1989; Ali *et al.*, 2007; Roo *et al.*, 2009). Generally these authors found high mortalities rates at the beginning of the exogenous phase, event that was not recorded in the present study. Even though no significant differences were found among treatment groups, a lower survival trend was found in DH and DHE groups.

The increase of vitamin E, in some other cases has been found to be detrimental to growth and survival (Hamre *et al.*, 2010) and to have pro-oxidant effects in juvenile halibut (Hamre *et al.*, 1997) and sea bream (Atalah, 2008) in the absence of sufficient amounts of vitamin C.

Survival after stress was not affected by rotifers' enrichments as also described in González Pérez (1995) with sea bream larvae fed microdiets enriched with vit E. On the contrary, in sea bass larvae, both Betancor *et al.* (2010), and Atalah (2008) found positive correlation between total or after stress survival and dietary vit E for a fixed level of DHA. In particular improvement in survival was evident in larvae fed high levels of DHA. They suggest an optimal value of 3000 mg kg^{-1} supplementation for dry feeds for sea bass larvae.

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.*, 2005a;

Imsland *et al.*, 2006). In particular, the nutritional quality of start feed for larvae has been indicated as extremely important for all the biological performance of different finfish species (Imsland *et al.*, 2006; Park *et al.*, 2006; Roo *et al.*, 2009). Rotifers, contrary to wild zooplankton present low levels of several nutrients (Van der Meeren *et al.*, 2008), and often enrichments/enrichments techniques do not fulfil completely larvae requirements (Hamre *et al.*, 2008) affecting therefore both health and production quality of fish fries.

Generally low levels of n-3 PUFA has been indicated as one of causative factors for higher incidence of skeletal anomalies (Park *et al.*, 2006; Roo *et al.*, 2005; Roo *et al.*, 2009), but also the contrary has been proved (Cahu *et al.*, 2009), In particular if an excess of DHA is not accompanied by appropriate levels of antioxidant substances (Park *et al.*, 2006; Cahu *et al.*, 2009; Kolvkoski *et al.*, 2009; Betancor *et al.*, 2010).

In this study total skeletal anomalies were not statistically different among groups and generally did not show acute manifestations. Slight skeletal malformations, anyway, may be recovered as, differently from higher vertebrates, in which congenital malformations are irreversible, lower vertebrates show high capacity for tissue or organs regeneration (Bongiorno *et al.*, 2007).

The highest, although not significantly, values of total anomalies were found in larvae fed high levels of DHA without supplementation of vit E (DH group, 10%). Sea bass fed dry diets with 5% DHA supplemented

with 15000 mg kg^{-1} vit E, showed higher percentages of muscular lesions (Betancor *et al.*, 2010) denoting that this level may be insufficient to couple with the increased DHA in food for larvae that may not have sufficient antioxidant capability at this stage (Mourente *et al.*, 1999).

In general, the two groups that were fed live feed rich in DHA (DH, DHE groups) showed higher percentages of jaws malformations, while DM and DME groups showed defects of the vertebral spines. Boglione *et al.* (2001) reported an overall incidence of jaw deformities in reared sea bream of 6%, while always indicate the presence of vertebral spinal deformities that may affect up to the 3% of the reared population. Cranial skeleton elements are generally the first to show mineralization (Faustino, 2002), being functional to predation. Larvae fed high DHA preys (DH, DHE groups), showed a more rapid mineralization of vertebral elements, therefore it is probable that high DHA may have affected the development of the very first ossifying elements. Similar observations, even though with higher incidence percentages, have been reported by Fernández *et al.* (2008) and related to high levels of vit A.

Lordosis that was the principal anomaly reported for reared sea bream larvae by Andrades *et al.* (1996) and Boglione *et al.* (2001) with frequencies of 27% and 14.45% respectively, in this study presented the highest percentages in DL group (~5%) being the most important anomaly followed by slight kyphosis (2%).

Surprisingly, only one specimen, belonging to the DH group showed anomalies of the brachiostegals, contrary to what reported for the same species in intensive system in which opercular anomalies account for 2% (Boglione *et al.*, 2001) and even up to 6% (Roo *et al.*, 2005).

The presence of calculi in the present study was pretty high if compared from the one reported in Boglione *et al.* (2001) (0.4% for reared specimens). In mice, Kruger and Schollum (2005), showed that animals fed high DHA levels had a lower excretion of calculi and a higher retention of calcium in the kidneys, probably due to the cardinal structural and transport role of DHA at membrane level. In this study, DL and DME groups were the groups that showed a higher percentage of calculi in respect to DH and DHE groups, but in any case other causes may have masked the possible role of this fatty acid in the reduction of the anomaly.

Regarding the presence of an additional vertebra, no differences were found among the treatments groups even though the lowest percentages were found only in DM group in respect to the other groups that, on the contrary, showed a ~ 20% of individuals with an additional vertebra. Boglione *et al.* (2001) showed a natural variation in the meristic characters also in wild specimens, but with a higher variability in hatchery-reared specimens. It has been suggested that establishment of vertebral meristic characters occurs as early as embryogenesis (Taning,

1952; Fowler, 1970), and that rearing condition during early developmental stages exert a strong influence on the presence of additional elements (Boglione *et al.*, 2001; Pulcini *et al.*, 2010). In other studies, a positive and significant relationship between additional vertebral centra and dietary vit A are reported (Fernández *et al.*, 2008). The authors together with Mazurais *et al.*, 2008 suggest that, high levels of dietary vitamins positively influences osteogenesis and therefore the normal differentiation pattern influencing the formation of an additional caudal vertebra (that are the last to be formed).

However, in this study, no definitive conclusions could have been drawn on the effects of dietary fatty acids or vit E, even though vit E supplementation seemed to affect positively the presence of an additional vertebra. In any case, it is probable that this eventual additional vertebra may be lately remodelled as one, as seen in some specimens, based in the assumption that evolution tends to reduce the number of elements (Bensimon-Brito *et al.*, 2010).

In the present study, statistical differences were found regarding the number of mineralized vertebrae at each size with an increase explained by a logarithmic function. Darias *et al.* (2010), showed that ossification process in sea bass larvae follows an exponential kinetic that is represented before 8.3 mm TL principally to the mineralization of the skull, and thereafter principally by the increase of mineralization of the vertebral centra. In this case, ossification of the skull was not

considered, but only the number of mineralized centra, fact that may explain the correspondence with a different type of mathematical function.

Larvae fed live food with lower DHA always showed a lower percentage of specimens with complete mineralized column and a lower number of complete vertebral centra for a fixed SL, while DHE group showed an inverse situation. Anyway, this last group presented at lower SL a high number of mineralized centra but up to 8.4 mm SL this number does not increase more. On the contrary, the other treatment groups, even if at lower length showed an inferior number of mineralized vertebral bodies, gradually increased this number up to reach at 9.285-9.485 mm the same number of DHE group.

Kjørsvik and colleagues (2009) indicated that cod fed with n-3 HUFAs incorporated in the polar lipid fraction instead of neutral lipids, showed a more narrow length range in which vertebral column elements complete their mineralization. In this study, only the contemporary presence of high levels of DHA and of vit E seems to accelerate the mineralization of vertebral centra and specifically at low SL, slowing thereafter. Larvae from DH, and from DME groups on the contrary, showed relatively a lower number of mineralized vertebrae at identical SL in comparison with DHE group. Moreover, percentage of specimens with complete vertebral column was lower than larvae fed the same live feed but without

supplementation of vit E but a more rapid increase in the completion of mineralization of the vertebral column.

In tetrapods, long chain n-3 PUFAs exert a stimulatory effect on bone mineral deposition (Watkins *et al.*, 2001), but when oxidized reduce bone appositional rate (Turek *et al.*, 2003).

In this study, data from the TBARS, suggest a high overall degree of oxidation for both DHE and DH groups, but the supplementation of vit E (DHE group) may have exert an enanchment of the mineralization rate of the vertebral centra. Moreover, a role on the timing of notochord formation (Fernández *et al.*, 2008) and vertebral centra formation (Mazaurais *et al.*, 2008) in fishes has been already suggested for other fat soluble vitamins (e.g. vit A). The effects of vit E on genes expression and regulation at cartilage membrane level have been reviewed in tetrapods (Brigelius-Flohé and Galli, 2010). Therefore, apart from the antioxidant function of vit E, its regulatory role and interaction with highly unsatured fatty acids in the very first steps of fish bone formation (osteogenesis and cell differentiation) should be further investigated.

5.
CONCLUSIONS

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

– Acidic double staining with alcian blue and alizarin red may affect negatively the visualization of some bone structures on long term stored formaldehyde sea bream larvae, in particular vertebral centra. Therefore, alizarin red single staining is preferable to acidic double staining in this situation.

– Sea bream larvae bred in mesocosm, long-stored in formaldehyde and single stained with alizarin red, showed a developmental pattern similar to the one reported in the literature with slight differences probably due to the initial rearing conditions.

– High levels of DHA in the enrichment products may affect negatively the viability of the enriched rotifers without improving their nutritional quality, but on the contrary, reducing the overall population.

– DHA level in live preys showed a significant positive correlation to DHA in larvae up to 15 dah, while yet after 34 days (9 day feeding with different food from rotifers) no significant effects can be evidenced.

– Supplementation of DHA up to 5% (DW) in live food does not improve overall survival or survival after stress in the first larval phase of sea bream larvae, even though seems to affect larval growth in terms of weight in respect of low (< 0.5% DW) DHA enriched live food.

– Supplementation of vit E seems to reduce the oxidative status of the larvae up the age they are fed with rotifers. In particular levels of 15,000 mg kg^{-1} of vit E in the enrichments seems to be suitable to overcome oxidation if DHA in live preys is about of 2% DW, while it is not sufficient when the concentration of DHA is increased up to 5% DW.

– Supplementation of vit E seems not to particularly affect incidence of skeletal anomalies, even though a 5% (DW) of DHA in live preys without increment of vit E induce larvae with a higher incidence of overall anomalies.

– Low levels of DHA in live preys negatively affect the number of mineralized vertebral bodies in respect larvae fed live preys with 5% DHA with or without supplementation of vit E and 2% (DW) DHA enriched live food supplemented with vit E.

– High levels of DHA and vit E in rotifers increase the number of specimens with complete vertebral column at a fixed standard length, in particular at smaller lengths, while low levels of DHA without the supplementation of vit E delay the mineralization of vertebral centra.

6. REFERENCES

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ANNEX I

Principal fatty acids profile from rotifers' enrichment trial previous to the preparation of the experimental enrichments.

Rotifers enriched with DHA Protein Selco™			rotifers enriched with DHA Protein Selco™ + capsules MorDHA omega-3 I.Q™		
	LIPIDS (DW)	20.81		LIPIDS (DW)	20.16
	%TFA	%DW		%TFA	%DW
Total saturated	24.22	5.04	**Total saturated**	21.41	4.32
Total monounsaturated	37.31	7.77	**Total monounsaturated**	26.75	5.39
Total n-3	24.13	5.02	**Total n-3**	39.34	7.93
Total n-6	8.85	1.84	**Total n-6**	9.63	1.94
Total n-9	23.54	4.90	**Total n-9**	15.69	3.16
Total n-3HUFA	21.82	4.54	**Total n-3HUFA**	37.58	7.58
14:0 (myristic acid)	1.49	0.31	**14:0 (myristic acid)**	1.14	0.23
16:0 (palmitic acid)	17.41	3.62	**16:0 (palmitic acid)**	16.25	3.28
16:1 n-7 (palmitoleic acid)	10.40	2.17	**16:1 n-7 (palmitoleic acid)**	7.71	1.55
18:0 (stearic acid)	3.91	0.81	**18:0 (stearic acid)**	2.90	0.58
18:1 n-9 (oleic acid)	17.76	3.70	**18:1 n-9 (oleic acid)**	12.16	2.45
18:2 n-6 (linoleic a.)	6.39	1.33	**18:2 n-6 (linoleic a.)**	6.39	1.29
18:3 n-3 (alfa linolenic acid)	1.12	0.23	**18:3 n-3 (alfa linolenic acid)**	1.11	0.22
20:1 n-9 (eicosanoic acid)	2.24	0.47	**20:1 n-9 (eicosanoic acid)**	1.67	0.34
ARA	1.42	0.30	**ARA**	1.12	0.23
EPA	6.18	1.29	**EPA**	3.05	0.61
DHA	13.20	2.75	**DHA**	25.82	5.20
DPA (22:5n-6)	0.67	0.14	**DPA (22:5n-6)**	0.81	0.16
DHA/22:5 n-6	19.76		**DHA/22:5 n-6**	32.02	
EPA/ARA	4.35		**EPA/ARA**	2.72	
DHA/EPA	2.14		**DHA/EPA**	8.47	
DHA/ARA	9.29		**DHA/ARA**	23.09	
oleic acid/DHA	1.35		**oleic acid/DHA**	0.47	
oleic acid/n-3HUFA	0.81		**oleic acid/n-3HUFA**	0.32	
n-3/n-6	2.73		**n-3/n-6**	4.08	
ARA/EPA	0.23		**ARA/EPA**	0.37	
ARA/DHA	0.11		**ARA/DHA**	0.04	