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## Nutritional requirements of marine fish larvae and broodstock

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**SUMMARY-** As the Mediterranean production of marine cultured fish increases, the demand for good quality seed has been continuously arising. To satisfy that increasing demand spawning quality and seed production success must be improved by controlling the nutritional quality of broodstock diets and first-feeding regimes. Gonadal development and fecundity in fish are greatly affected by broodstock nutrition in several fish species. During the last decade, increasing attention has been paid to the role of different components of broodstock diets such as protein, essential fatty acids, vitamin E, vitamin C, carotenoids and phosphoglycerides. Some authors have also studied the nutritional quality of raw components used in broodstock diets for these species. However, few of them deal with Mediterranean fish species. A considerable amount of research has been devoted lately to study the nutritional requirements of marine fish larvae. Comparison of the biochemical composition of eggs and larvae at different stages, patterns of loss and conservation of nutrients during feeding and starvation, and feeding experiments controlling live-prey or microdiets nutrient composition are some of the most frequently used methods to study nutritional requirements of marine fish larvae. Most of this research effort was initially related with the essential fatty acid (EFA) requirements of fish larvae, as early studies showed that one of the principal factors affecting the nutritional value of live preys for marine fish larvae was their EFA content. Only very few studies have been dedicated to the study of the dietary phosphoglyceride or fat-soluble vitamin requirement of marine fish larvae, and the nutritional role of other fat soluble compounds such as pigments are still mostly unknown.

**Key words:** Fish, nutrition, broodstock, feeding, larvae, marine.

**RESUME -** "Besoins nutritionnels des larves et des géniteurs de poissons marins". Face à l'augmentation de la production méditerranéenne de poissons marins d'élevage, la demande de larves de bonne qualité s'est toujours posée. Afin de satisfaire cette exigence grandissante, il est nécessaire d'améliorer la qualité de la ponte et la production de larves, en contrôlant la qualité nutritionnelle des régimes pour géniteurs et des régimes de premier âge. Le développement des gonades et la fécondité chez les poissons sont très dépendants de la nutrition des géniteurs chez plusieurs espèces de poissons. Pendant la dernière décennie, une attention grandissante a été portée au rôle des différents composants des régimes pour géniteurs, comme la protéine, les acides gras essentiels, les vitamines E et C, les caroténoïdes et les phosphoglycérides. Des auteurs ont également étudié la qualité nutritionnelle des matières premières utilisées pour les régimes des géniteurs en ce qui concerne ces espèces. Cependant, il n'y a eu que peu d'études faites sur les espèces piscicoles méditerranéennes. Beaucoup de recherches ont porté dernièrement sur l'étude des besoins nutritionnels des larves de poissons marins. Quelques-unes des méthodes les plus utilisées pour étudier les besoins nutritionnels des larves de poissons marins sont la comparaison de la composition biochimique des oeufs et des larves à différents stades, de la tendance à perdre ou garder les nutriments en alimentation et sous-alimentation, ainsi que les expérimentations nutritionnelles contrôlant la composition en nutriments des micro-régimes ou des proies vivantes. La plus grande partie de cet effort de recherche a porté initialement sur les besoins en acides gras essentiels

(AGE) des larves de poissons, les premières études ayant montré que les principaux facteurs qui déterminent la valeur nutritionnelle des proies vivantes est leur teneur en AGE. Il n'y a eu que peu d'études consacrées aux phosphoglycérides alimentaires ou aux besoins en vitamines liposolubles des larves de poissons marins, et au rôle nutritionnel d'autres composés liposolubles tels que les pigments, qui sont encore pratiquement inconnus.

**Mots-clés :** Poisson, nutrition, géniteurs, alimentation, larves, marin.

## INTRODUCTION

As the Mediterranean production of marine fish increases, then so the demand for good quality seed is increasing. Within these marine species all the processes of gonadal development up to oviposition are closely related to variations in temperature and photoperiod (Devauchelle, 1984). The control of these two parameters permits egg production to be adjusted to hatchery needs at any season. However, spawning quality and seed production success still needs to be improved by controlling the nutritional quality of broodstock diets and first-feeding regimes.

The spawning quality of Mediterranean marine fish species may be increasing by improving the nutritional quality of broodstock diets, as has been shown in other fish species (Barton 1981; Craik and Harvey 1984; Watanabe 1985; Devauchelle et al., 1988; Eskelinen 1989; Watanabe et al., 1991; Watanabe and Kiron, 1995). Gonadal development and fecundity in fish are greatly affected by broodstock nutrition in several fish species (Bromage, 1995). During the last decade, increasing attention has been paid to the role of different components of broodstock diets such as protein (Watanabe et al., 1985), essential fatty acids (Watanabe et al., 1984; Zohar et al., 1995; Hardy et al., 1990), vitamin E (Watanabe et al., 1991a), vitamin C (Sandnes et al., 1984; Blom and Dabrowski, 1995), astaxanthin (Watanabe and Kiron, 1995) and phosphoglycerides (Watanabe et al., 1991b; Table 1).

Table 1. Essential nutrients in diets for marine broodstock

Nutrient	Species	Reference
Fatty acids	<i>Pagrus major</i>	Watanabe et al., 1984a,b
	<i>Sparus aurata</i>	Fernández-Palacios et al., 1995
Vitamin E	<i>P. major</i>	Watanabe et al., 1991a
	<i>S. aurata</i>	Fernández-Palacios et al., in press
Carotenoids	<i>P. major</i>	Watanabe et al., 1991b
Phospholipids	<i>P. major</i>	Watanabe et al., 1991b
Vitamin C	<i>Salmo salar</i>	Eskelinen, 1989
	<i>Gadus morhua</i>	Mangor-Jensen et al., 1993
Protein	<i>P. major</i>	Watanabe et al., 1984d
	<i>D. labrax</i>	Cerdá et al., 1994

Regarding larval nutrition, feeding of marine fish larvae is often limited to the administration of a few live prey organisms such as rotifers and *Artemia*. This reduced range of food organisms available for the larvae may lead to nutritional imbalances or nutritional deficiencies (Watanabe, 1993). For this reason a great deal of research has recently been devoted to study the nutritional requirements of marine fish larvae (Izquierdo, 1996). The bulk of this research effort has been related to the essential fatty acid (EFA) requirements of fish larvae, as early studies had shown that one of the principal factors affecting the nutritional value of live prey organisms was their EFA content (Watanabe et al., 1983). Only very few studies have been dedicated to dietary phosphoglycerides or fat-soluble vitamin requirements, and still less concerning the nutritional value and effects of other fat-soluble compounds such as the carotenoid pigments in larval nutrition (Table 2).

Table 2. Essential nutrients in diets for marine fish larvae

Nutrient	Species	Reference
Fatty acids	<i>Pagrus major</i>	Izquierdo et al., 1989a
	<i>Sparus aurata</i>	Rodríguez et al., 1994
	<i>Psetta maxima</i>	Gatesoupe & Le Milinaire, 1985
	<i>Paralichthys olivaceous</i>	Izquierdo et al., 1992
Vitamin E	<i>S. aurata</i>	González et al., 1995
Phospholipids	<i>P. major</i>	Kanazawa et al., 1983b
	<i>P. olivaceus</i>	Teshima et al., 1987
	<i>S. aurata</i>	Koven et al., 1993

The present study deals with the nutritional requirements of broodstock and larvae of marine fish cultured in the Mediterranean Sea, summarizing some of the results obtained by the authors over the last decade.

## BROODSTOCK FEEDING

### Spawning quality evaluation

Unpredictable and variable egg quality is a major limiting factor for the successful mass production of juveniles of marine fish species (Kjorsvik et al., 1990). Methods for determining egg quality vary greatly. For example, the percentage of buoyant eggs floating on the water surface has been broadly used to evaluate egg quality in some sparids (Watanabe et al., 1984a; Harel et al., 1992); good quality pelagic eggs usually being more buoyant than poor quality eggs (McEvoy 1984) and in some species such



as red sea bream buoyant eggs generally have high hatching rates and normal development (Watanabe et al., 1984a). However, the percentage of buoyant eggs of gilthead sea bream varies with the separation method used (Fernández-Palacios, unpublished data), depending upon the incubator size and separation time. Furthermore, during the first hours after spawning unfertilized eggs are still buoyant. It follows thereby that criteria other than the percentage of buoyant eggs may be a more accurate indicator of egg quality in gilthead sea bream.

Concerning the numerous different parameters studied by Fernández-Palacios et al. (1995) to determine the spawning quality in gilthead sea bream, the total number of eggs produced per kg female and percentage of morphologically normal eggs expressed as eggs viability were found to be more sensitive than the other parameters studied. Moreover, in gilthead sea bream, morphological criteria such as symmetrical early cleavage, as in red sea bream (Sakai et al., 1985), and egg transparency, as in turbot (McEvoy 1984), seemed to be good early indicators of eggs viability (Fernández-Palacios et al., 1995).

### **Dietary essential fatty acids levels**

One of the nutritional factors that have been found to greatly affect spawning quality in fish has been the dietary essential fatty acids content (Watanabe et al., 1984a,b). In gilthead sea bream, when broodstock were fed a minced cuttlefish diet or a commercial diet plus minced cuttlefish, a close relationship between lipids and fatty acid composition of broodstock diets and of eggs was found (Mourete and Odriozola 1990a). However, the influence of dietary levels of these fatty acids on reproduction and spawning quality of gilthead sea bream remains unknown. Recently, Harel et al. (1992) fed gilthead sea bream broodstock with diets containing up to 1% n-3 HUFA (highly unsaturated fatty acids) and observed that the composition of the female organs which are associated with reproduction were modified by the essential fatty acid levels of the diet and could effect egg quality over a short time period.

Spawning quality in gilthead sea bream has been shown to be improved by the elevation of the dietary n-3 HUFA up to 1.6% (Fernández-Palacios et al., 1995); similar results have been found in other sparids (Watanabe et al., 1984 a,b,c and 1985 a,b). These dietary levels are higher than those obtained for salmonids (around 1.0% n-3 HUFA) and similar to those obtained for red sea bream by Watanabe and coworkers in Japan. However, high levels of dietary n-3 HUFA reduced the total amount of eggs produced and caused yolk sac hypertrophy in some larvae, with a consequent decrease in larval survival rates.

Since the egg composition and spawning quality of gilthead sea bream broodstock was effected by dietary essential fatty acid levels only three weeks after feeding (Fernández-Palacios et al., 1995), this suggests that dietary essential fatty acids are easily incorporated into the eggs and that spawning quality in this species may be improved by modifying the nutritional quality of broodstock diets even during the course of spawning season (Fernández-Palacios et al., in press). In continuous spawners with short vitellogenetic periods such as sparids (Zohar et al., 1984) spawning quality seems to be affected by dietary lipids during the verge of the spawn (Watanabe et al., 1985b) or even during spawning. By contrast, in batch spawners with up to 6 months

of vitellogenesis (Frémont et al., 1984) such as in salmonids to improve their spawning quality broodstock must be fed a good quality diet for several months before the spawning season (Watanabe et al., 1984d; Corraze et al., 1993). Thus, although fatty acid profiles of fish muscle and developing eggs of coho salmon (Hardy et al., 1989) reflect dietary fatty acid profiles only after two months of feeding, Harel et al. (1992) have shown that the tissue lipid composition of gilthead sea bream broodstock reaches an equilibrium with the dietary lipids after only 15 days of feeding.

Dietary levels of EPA and Arachidonic acid have also been found to correlate with fertilization rate in gilthead sea bream broodstock (Fernández-Palacios et al., 1995; Fernández-Palacios et al., in press). EPA is known to be a precursor of prostaglandins (PG) from series III, one of the major PG components synthesized in marine fish (Stacey and Goetz 1982). Arachidonic acid is also a precursor of PG from series II. Some PGs produced by the female goldfish, such as PGFs, have been shown to play an important role as pheromones, stimulating male sexual behaviour and synchronizing male and female spawnings, thus affecting directly to the success in fertilization (Sorensen et al., 1988). Distribution of EPA in sperm may also be affected by dietary fatty acids, as has been shown in rainbow trout (Watanabe et al., 1984d, Labbe *et al.* 1993), and may also be responsible for spermatozoan activity and fertilization rates as suggested by same authors (Watanabe et al., 1984d).

The percentage of morphologically normal eggs (a parameter frequently used to evaluate eggs viability) has been found to increase with the elevation of n-3 HUFA levels in broodstock diets and the incorporation of these fatty acids into the eggs (Fernández-Palacios et al., 1995), indicating the importance of EFA for the normal development of gilthead sea bream eggs and embryo. These fatty acids have an important structural role as components of phospholipids in fish biomembranes, being related with the membrane fluidity and correct physiological functions for bound membrane enzymes and cell functions in marine fish (Bell et al., 1986). In some species, such as halibut (*Hippoglossus hippoglossus*), the n-3 PUFA (polyunsaturated fatty acids) are also regarded as major energy sources during early embryonic development (Falk-Petersen et al., 1989).

Gilthead seabream fed an EFA deficient diet also results in an increase in the number of egg lipid droplets (Fernández-Palacios et al., in press) as also found in red seabream (Watanabe et al., 1984a). By contrast, an increase of dietary n-3 HUFA levels supported higher lipid contents in the eggs (Fernández-Palacios et al., 1995). Very low hatching rates or egg viability have been associated with a higher total lipid content in the eggs of turbot, sole and seabass (Devauchelle et al., 1982; Serrano et al., 1989). However, the elevation of the lipid contents of gilthead sea bream eggs up to 20 % was not found to affect the hatching percentage or the proportion of normal eggs (Fernández-Palacios et al., 1995).

As mentioned previously, the fatty acid composition of eggs is also effected by the n-3 HUFA contents of the broodstock diets; the n-3 fatty acids and n-3 HUFA content of gilthead seabream eggs increasing with the elevation of n-3 HUFA dietary levels, mainly due to the increase of 18:3n-3, 18:4n-3 and EPA content the eggs (Fernández-Palacios et al., 1995). Thus, a positive correlation was found between dietary and egg levels of n-3 HUFA ( $r=0.92$ ,  $P<0.1$ ) with EPA contents being more readily affected by dietary n-3 HUFA than DHA egg content. In rainbow trout a n-3 deficient

diet during the last three months of vitellogenesis was found to have a moderate effect on DHA incorporation into egg lipids whereas EPA decreased by 50% (Frémont et al., 1984). However, the levels of other fatty acids in the eggs were not reportedly affected by the fatty acid composition of the diet.

It has been suggested by several authors (Sandnes et al., 1984; Craik, 1985; Harel et al., 1992) that the chemical composition of the fish eggs is related to spawning quality since egg composition must satisfy embryonic nutritional demands for development and growth. However, the results of Fernández-Palacios et al. (1995) showed that the percentage of n-3 HUFA of the eggs should not be used alone as a criterion to assess the egg quality of gilthead sea bream broodstock, as both low and high levels of n-3 HUFA within eggs have been associated with low spawning quality on the basis of the total number of eggs produced and larval survival rate.

### **Dietary $\alpha$ -tocopherol levels**

The elevation of dietary  $\alpha$ -tocopherol levels from 22 to 207 mg/kg significantly reduced the percentages of abnormal gilthead seabream eggs (Fernández-Palacios et al., in press). Moreover, the elevation of  $\alpha$ -tocopherol levels up to 127 % resulted in an improvement in fecundity, as expressed by the total number of eggs produced/female and egg viability, and as expressed as a percentage of normal eggs; the lowest fertility and larval survival rate reported in eggs from broodstock fed the lowest dietary levels of  $\alpha$ -tocopherol. However, the  $\alpha$ -tocopherol contents of eggs were not affected by an increase of dietary vitamin E levels up to 127 mg/kg, but further increases above this significantly increased the  $\alpha$ -tocopherol level within the eggs. Egg quality in red sea bream was also found to be improved by the addition of vitamin E to broodstock diets (Watanabe et al., 1991a).

### **Feed ingredients with high nutritional quality**

Some authors have suggested that cuttlefish (Mourete et al., 1989) and squid meal (Zohar et al., 1995) contain nutritional components which are essential for successful spawning in gilthead seabream. Mourente et al. (1989) related this beneficial effect to the high content of essential fatty acids in cuttlefish. However, Watanabe and co-workers (1984a) suggested that the high dietary value of cuttlefish meal was mainly due to the fat-insoluble fraction of the meal. We discussed before the importance of the essential fatty acids in broodstock diets for this fish species, but the effects of other nutrient deficiencies on spawning quality have not been investigated in detail.

Fernández-Palacios and co-workers conducted an experiment to clarify the effective components within squid meal which are able to improve egg quality in comparison with fish meal, one of the major ingredients used within commercial diets for gilthead seabream broodstock (Fernández-Palacios et al., in press). For this purpose, broodstock were fed diets based either on fish meal, squid meal, and defatted fish meal with squid oil or defatted squid meal with fish oil. These authors showed an improvement in egg quality when broodstock were fed the fat-insoluble fraction of squid meal in terms of total the number of eggs produced daily per kg of female and percentages of viable and fertilized eggs. Squid meal protein, as the major component



of the squid meal fat-insoluble fraction, seems to have been responsible for the positive effect of this fraction on egg quality (Fernández-Palacios et al., in press). As the amino acid profiles were very similar among the diets used during the study, the superior value of the squid protein based diets have been related to its higher protein digestibility for gilthead seabream (Fernández-Palacios et al., in press). In fact, slightly higher protein levels were evident within the eggs from broodstock fed squid protein based diets, the fish also producing about 40% more eggs/kg/female than broodstock fed fish meal-based diets. The high calcium content of fishmeal could not have been responsible for the poorer spawning quality of fish fed fishmeal compared with squid meal as the addition of calcium to a cuttlefish meal based diet was not found to affect spawning quality in the sparid *Pagrus major* (Watanabe et al., 1991a). Increased egg production and viability was also observed by Watanabe et al., (1984a and 1984b) when red seabream were fed with a cuttlefish meal based diet. Moreover, the replacement of 50% of the fish meal with cuttlefish meal (Watanabe et al., 1984b) resulted in improved egg viability, although the number of eggs produced per female were not affected.

The dietary replacement of protein or lipid extracted from squid meal by protein or lipid extracted from soybean meal in diets for gilthead seabream broodstock caused a reduction in hatching and 3 day-old larval survival rates (Zohar et al., 1995). This could have been due to the beneficial effect of squid meal or to the detrimental effect of soybean meal. Although it has been shown (Robaina et al., 1995) that soybean protein is an interesting protein source for use as a partial substitute for fishmeal in diets for gilthead seabream, there several antinutritional factors present which may constrain its use at high levels within diets for this species. Moreover unbalanced fatty acid composition, in terms of high n-6 polyunsaturated fatty acids and low n-3 fatty acids together with a lower phosphorous availability (Robaina, 1995) in broodstock diets based on soybean meal, could also directly reduce spawning quality as both nutrients are essential for reproduction in sparids (Watanabe et al., 1984a; Watanabe and Kiron, 1995).

Another very interesting ingredient, often included within practical diets for sparids, is the raw krill which seems to have distinct quality enhancing effect compared with fishmeal. For example, viable offspring production in red sea bream (*Pagrus major*), in terms of the percentage of buoyant eggs, total hatch and normal larvae, was more than double when krill was included within broodstock feeds (Watanabe and Kiron, 1995). The lipid fraction was shown to be responsible for the positive effect of raw krill on egg quality. In spite of the importance of this ingredient as a spawn quality enhancing factor for Pacific sparids, there is little published information concerning the nutritional value of raw krill, or their components, for broodstock of Mediterranean fish species.

## Other nutrients

Apart from fatty acids and  $\alpha$ -tocopherol, numerous other nutrients have also been shown to effect reproductive performance of marine fish (Table 1). For example, the experiments of Watanabe and co-workers (Watanabe et al., 1991a,b) concerning the spawning quality enhancement effect of raw krill showed that both polar and nonpolar lipid fractions contained important nutritional components for red sea bream broodstock. They attributed this positive effect to the presence of phosphatidyl choline and



astaxanthin from the polar and nonpolar fractions, respectively.

For over 50 years there has been a great deal of controversy concerning the relationship between egg carotenoid content and egg quality in salmonids. For a review of carotenoid nutrition and functions (including their effect on egg quality) see Tacon (1981), Craik (1985), Choubert (1986), Torrison (1990) and Torrison and Christiansen (1995). However, the results concerning the effect of carotenoid egg content on egg quality in salmonids have been contradictory. Some authors suggest that there is a relationship between egg pigmentation and the fertilization or survival rates of rainbow trout eggs (Harris, 1984; Craik, 1985) whereas others found no relationship at all (Torrison, 1984; Craik and Harvey, 1986; Torrison and Christiansen, 1995). Differences in the methodology employed by the different authors include broodstock age, lack of control of carotenoid contents, differences in carotenoid type included in diet or determined in the egg, sample size and even differences in criteria used to determine egg quality. Very few studies have been conducted controlling the level of dietary carotenoid supplied in broodstock diets (Harris, 1984; Choubert and Blanc, 1993; Watanabe and Kiron, 1995). The addition of purified astaxanthin to broodstock diets for red seabream was found to clearly improve the percentage of buoyant and hatched eggs, as well as the percentage of normal larvae (Watanabe and Kiron, 1995). By contrast, the inclusion of beta-carotene was found to have no effect on these parameters. Miki et al., 1984 have demonstrated the incorporation of either dietary cantaxanthin or dietary astaxanthin into red sea bream eggs and the absence of such transfer for dietary beta-carotene. These results could have been due to the lower intestinal absorption, of beta-carotene compared with that of cantaxanthin or astaxanthin. A preferential absorption and deposition of hydroxy and keto carotenoids was reported in fish by Torrison and Christiansen (1995). Carotenoids constitute one of the most important pigment classes in fish, with a wide variety of functions and actions including photoprotection, provitamin A source, communication, antioxidant and singlet oxygen quenching, among others.

Other investigations with red sea bream have also shown that dietary phospholipids also improve quality of eggs (Watanabe et al., 1991a,b); the beneficial effects of phospholipids attributed to their quencher activity and ability in stabilizing free radicals (Watanabe and Kiron, 1995).

Other dietary nutrients which have been found to affect the reproductive performance of marine fish have included dietary protein and vitamin C. For example, a low protein-high calorie diet was found to cause a reduction in red sea bream reproductive performance (Watanabe et al., 1984d), while reduction of dietary protein levels from 51 to 34 together with the increase of dietary carbohydrate levels from 10 to 32 reportedly reduced egg viability in sea bass (*Dicentrarchus labrax*) (Cerdeira et al., 1994). Ascorbic acid has been shown to play important roles in salmonids reproduction (Eskelinen, 1989; Blom and Dabrowski, 1995). Rainbow trout (*Oncorhynchus mykiss*) broodstock requirements for this vitamin seemed to be about eight times higher than that of juveniles (Blom and Dabrowski, 1995). However, much lower demands for ascorbic acid have been reported in broodstock diets for cod (Mangor-Jensen et al., 1993).

## LARVAL FEEDING

### Essential fatty acid intake

Some of the most frequently used methods employed for investigating the fatty acid requirements of marine fish larvae have been 1) comparison of the biochemical composition of eggs and larvae at different stages 2) pattern of loss and conservation of nutrients during feeding and starvation, and 3) feeding experiments controlling the fatty acid composition of live-prey or microdiets (Izquierdo, 1996).

As fish eggs should contain all the essential nutrients required for the development of the embryo and the growth of larvae up to the stage of yolk sac absorption, the analysis of their chemical composition could shed some light on the nutritional requirements of fish larvae. The major fatty acids in the lipids of marine fish eggs such as halibut (*Hippoglossus hippoglossus*) (Falk-Petersen et al., 1989), turbot (*Scophthalmus maximus*) (Planas et al., 1993), plaice (*Pleuronectes platessa*) (Rainuzzo et al., 1993), dolphin (*Coryphæna hyppurus*) (Ako et al., 1991), red sea bream (*Pagrus major*) (Izquierdo et al., 1989a) and gilthead seabream (*Sparus aurata*) (Mourete & Odriozola, 1990) are docosahexaenoic (DHA), palmitic, eicosapentaenoic (EPA) and oleic acids (Table 1). However, the relative importance of each fatty acid differ markedly among the studied species or even between different egg batches of the same species. Despite this, the fatty acid composition of polar lipids seems to be less affected by the broodstock diet (Mourete and Odriozola, 1990a). The major fatty acids of egg polar lipids are DHA and palmitic acid, followed by EPA and oleic acid in halibut (Falk-Petersen et al., 1986), red sea bream (Izquierdo, 1988), gilthead seabream (Mourete & Odriozola, 1990), cod (*Gadus morhua*) (Rainuzzo et al., 1991), which suggests the importance of these fatty acids for the further development of the embryos and larvae.

Another method used by some authors to obtain more information on the EFA requirements of marine fish larvae has been to compare the patterns of conservation and loss of fatty acids between starved and feeding larvae. For example, highly unsaturated fatty acids such as DHA, arachidonic acid (AA) and in some species EPA have been found to be conserved at the expense of other fatty acids during starvation of marine fish larvae such as turbot (Rainuzzo et al., 1994), cod (van der Meeren et al., 1991), dolphin (Ako et al., 1991), red sea bream (Tandler et al., 1989) and gilthead sea bream (Koven et al., 1989, Rodríguez, 1994)(Table 2). Larvae of gilthead seabream show preferential retention not only of DHA but also of AA during starvation. Docosapentaenoic acid is also strongly retained during starvation (Rodríguez, 1994). This biochemical strategy allows the preservation of valuable essential components of the biological membranes during critical periods of starvation. In all these studies, DHA is again preferentially conserved suggesting the importance of this fatty acid for larvae of marine fish.

Feeding marine fish larvae with EFA deficient rotifers, *Artemia* or artificial diets resulted in reduced growth for turbot (Gatesoupe & Le Milinaire, 1985), Japanese flounder (*Paralichthys olivaceous*) (Izquierdo et al., 1992); summer flounder (*P. dentatus*) (Bisbal and Bengtson, 1991), striped jack (*Longirostris delicatissimus*) (Izquierdo, 1988), red sea bream (Izquierdo et al., 1989a, 1989b) and gilthead seabream (Koven et al., 1990; Rodríguez et al., 1993, 1994; Salhi et al., 1994). In



these studies an increase in dietary n-3 HUFA levels significantly improved larval growth. However, Rainuzzo et al. (1994) and Dickey-Collas and Geffen (1992) could not find a relationship between n-3 HUFA levels in live prey and growth of turbot and plaice. Possibly, the range of n-3 HUFA tried by Rainuzzo et al. (1994) was too high for the culture conditions used to show an effect on growth, as it exceeded the requirement of 1.3 g/Kg on dry weight basis determined as optimum for the growth rate of turbot larvae by Le Milinaire et al. (1983). The n-3 HUFA levels in the *Artemia* fed by Dickey-Collas and Geffen (1992) to larval plaice were very low. The authors related good growth rates with the ability of plaice to synthesize DHA from EPA as suggested by Minkoff (1987). However, EPA levels in *Artemia* were also extremely low (19 g/kg total fatty acids) and the conversion was not confirmed by analysis of the larvae.

More controversy exists concerning the effect of dietary n-3 HUFA levels on larval survival. Several authors have reported a reduction in survival rates when larvae of halibut (Holmefjord and Olsen, 1991), turbot (Gatesoupe and Le Milinaire, 1985), striped jack (Izquierdo, 1988), red sea bream (Izquierdo et al., 1989a, 1989b) and gilthead seabream (Rodríguez et al., 1993, 1994; Salhi et al., 1994) were fed low n-3 HUFA levels in rotifers, *Artemia* or microdiets. However, other authors did not observe significant differences in larval survival of some flatfish species such as turbot (Rainuzzo et al., 1994), plaice (Dickey-Collas & Geffen, 1992), Japanese flounder (Izquierdo et al., 1992) or summer flounder (Bisbal & Bengtson, 1991). This fact could be related to a higher ability of flatfish to elongate and desaturate fatty acids in comparison with some other marine fish (Tzoumas, 1988; Izquierdo et al., 1992; Linares & Henderson, 1991) which in turn may be related to the different dietary characteristics of each species.

A parameter which is often used to determine the EFA condition of fish larvae is to count the survival after a so-called "activity test". In this type of test fish are submitted to an acute stress such as handling them out of the water for few seconds and subsequently returning them to the rearing tank. Survival rate is then determined 24 hours later, with most fish species fed n-3 HUFA deficient live prey being very sensitive to this type of test. Stress sensitivity has also been found to be related to the ratio of oleic acid to n-3 HUFA in the glycerophospholipids of fish (Izquierdo et al., 1989b), which was used by several authors as an indicator of EFA deficiency in fish (Watanabe et al., 1989; Koven, 1991).

The biochemical composition of fish has also been found to be modified by dietary n-3 HUFA level; n-3 HUFA levels being low within the TL and PL of larvae fed EFA deficient rotifers, *Artemia* or artificial diets (Izquierdo et al., 1989a, 1989b; Rodríguez et al., 1993; Salhi et al., 1994). Furthermore, the ratio of oleic acid to n-3 HUFA within PL is generally high within EFA deficient fish and decreases as the n-3 HUFA dietary levels increases (Izquierdo et al., 1992). Larval total lipid and moisture content has also been found to vary inversely as a function of dietary n-3 HUFA in gilthead seabream (Koven, 1991).

Besides, n-3 HUFA deficiency signs in larvae also may give rise to alterations in the behaviour or in the anatomy of the fish larvae these being easily observed by binocular microscopy. Several authors (Izquierdo et al., 1989; Rodríguez et al., 1993; 1994) have described a reduction in the swimming and feeding activities in EFA-deficient larvae; these larvae usually remain floating on the water surface.

Inadequate swim bladder inflation has also been described by Koven (1991) in gilthead seabream fed n-3 HUFA deficient rotifers. Swim bladder inflation usually takes place around day 10-12 in gilthead seabream larvae and is generally considered to be a critical factor for survival. Although a general trend between improved swim bladder inflation and dietary n-3 HUFA was observed, the correlation between these two parameters was not found to be statistically significant (Koven et al., 1990). Despite the fact that Kanazawa et al., (1982) identified the presence of EPA in the swim bladder of juvenile red sea bream, there was no clear evidence concerning the effect of EPA on swim bladder inflation. The weak swimming ability of fish larvae fed rotifers of low nutrient value is believed to be one of the major causes proposed to explain the inhibition of air-intake by the larvae for the initial inflation of the swim bladder (Kitajima et al., 1994; Watanabe and Kiron, 1995). Disgregation of gill epithelia has also been reported by Arnaiz et al., (1993) in larval turbot fed n-3 HUFA-deficient live prey.

Hydrops, described by Yamashita (1981) as subcutaneous and coelomic deposits of water in red sea bream larvae, has also been related with EFA deficiency according to some authors (Izquierdo et al., 1989; Watanabe et al., 1989; Rodríguez et al., 1993, 1994).

Hypomelanosis on the ocular side in marble sole larvae (*Limanda yokohamae*) was found in larvae fed DHA deficient microdiets by Kanazawa (1993). Abnormal pigmentation was further enhanced by a deficiency of dietary glycerophospholipids or retinol. Poor pigmentation was also related to low n-3 HUFA and especially very low DHA contents in polar lipids of turbot larvae (Rainuzzo et al., 1994). The critical stage to induce albinism in flatfish has been estimated to be at a total length of 8 mm, when the retina in the eye is formed (Kanazawa, 1993); the rodopsin within the rod cell, which is responsible for dark vision, being composed of the protein opsin, retinol and glycerophospholipids, including DHA. If these components are deficient in the diet the formation of the retina is interrupted; the visual information of the retina is not delivered to the nerve-center, the melanocyte stimulating hormone not secreted by the internal gland, and the formation of melanin being interrupted (Kanazawa, 1993). Recently, Bell et al., (1995) have shown a reduced predation activity, specially during low light intensity, in herring reared on *Artemia* containing no DHA; the eyes of deficient fish containing greatly reduced levels of di22:6n-3 molecular species of total phospholipid.

The optimum n-3 HUFA levels within rotifers, *Artemia* or microdiets are normally determined by measuring parameters affected by n-3 HUFA deficiency such as growth, survival and activity, together with measuring the improvements in the biochemical composition of the larvae and the absence of the gross signs of EFA deficiency. The n-3 HUFA requirements reported for larvae of various species of marine fish range from 0.3 to 39 g/kg on a dry basis (Izquierdo, 1996); the lowest requirement described by Dickey-Collas and Geffen (1992) for larval plaice fed *Artemia* high in linolenic acid, and the highest requirement reported for yellowtail (*Seriola quinqueradiata*) (Watanabe, 1993).

The n-3 HUFA requirements also may be different for larvae and juveniles of the same species (Izquierdo et al. 1989a); the requirement being about twice as high in the larvae as in the juveniles. This may be due to the greater need of larvae for n-3 HUFA in their metabolism and for membrane construction in accordance with their higher growth rate, or, to the high larval demand for DHA for the proper development



of neural tissue.

The optimum n-3 HUFA levels determined by different authors for the same species often differ markedly. For example, the lower n-3 HUFA requirement determined by Koven et al., (1990) for gilthead seabream larvae fed with rotifers contrast, not only with the requirements for other sparids such as red sea bream (Izquierdo et al., 1989a) and knifejaw (*Oplegniatius fasciatus*), but also differ from those reported for the same species by Mourente et al., (1993) or Rodríguez et al., (1993).

When larvae of red sea bream were fed with enriched *Artemia* containing various levels of n-3 HUFA, a better correlation was found between the DHA contents in *Artemia* and growth, survival and survival after activity test than between the n-3 HUFA contents in *Artemia* and the latter parameters (Izquierdo et al., 1989b). The comparison between EPA and DHA in rotifers in terms of EFA for red seabream larvae (Watanabe et al., 1989) showed that the increase of either of these fatty acids effectively improved growth and survival rates compared to those of larvae fed a n-3 HUFA deficient diet. However, fish fed DHA as the major n-3 HUFA source showed better growth, higher survival after activity test and lower incidence of hydrops than larvae fed EPA as the major n-3 HUFA source. Furthermore, an accumulation of EPA and a decrease in DPA and DHA in the larvae fed EPA rich rotifers, suggested a low or absent elongation and desaturation of the latter. In a similar way the levels of EPA and DPA decreased in fish fed rotifers rich in DHA, suggesting that no retroconversion from the latter occurs in red sea bream (Watanabe et al., 1989). The ratio of oleic acid to n-3 HUFA was also more effectively reduced by dietary DHA than EPA. These results suggested that DHA has a higher EFA efficiency for larval red sea bream than EPA, and this has been also confirmed more recently for other fish species (Takeuchi et al., 1990; Toyota et al., 1991; Watanabe, 1993).

Current evidence suggests that not only DHA but also other polyunsaturated fatty acids are essential for marine fish larvae. It is probable that, not only the total content of DHA or n3 HUFA is important, but also that a balanced dietary ratio of EPA/DHA may be necessary to obtain optimum growth rates. Rodríguez et al. (1994), feeding gilthead seabream larvae with rotifers containing a ratio of 2.5 EPA/DHA, obtained best growth when rotifer contained 55 g/kg n-3 HUFA on dry weight basis. But in a further experiment the same author (Rodríguez, 1994) found an improvement in larval growth when the EPA/DHA ratio in rotifers was reduced from 36 to 8, for a constant n-3 HUFA level of 30 g/kg. EPA and DHA contents in TL of larvae were significantly correlated with the levels of each fatty acid in rotifers (Fig. 3), but incorporation of DHA into larval TL was always higher than that of EPA, regardless of the content of this fatty acid in the rotifers. Although preferential hydrolysis and a specific absorption of n-3 PUFA has been demonstrated in juvenile turbot (Koven et al., 1994), no information is available regarding the digestion and absorption of individual PUFA in the larvae of marine fish. Furthermore, the results of Reitan et al., (1994) showed a correlation between the EPA/DHA ratio in the total lipids of the larvae and larval pigmentation. Additional research is currently being conducted concerning the possible competition between these individual fatty acids.

When the EPA/DHA ratio was reduced from 2.5 to 1.5 the optimum n-3 HUFA levels in rotifers required to obtain the best growth was reduced from 55 to 15 g/kg on a dry weight basis (Rodríguez, 1994). In fact, the lower n-3 HUFA requirements found for

gilthead seabream larvae by other authors (Koven et al., 1990; Mourente et al., 1993) were determined with rotifers containing lower EPA/DHA ratios than those used by Rodríguez et al. (1993, 1994).

### **Fat-soluble vitamins effects**

Analysis of the  $\alpha$ -tocopherol content of gilthead seabream eggs and larvae during the first days of larval development have shown an increase of vitamin E content up to day 10th after hatching, reflecting the high  $\alpha$ -tocopherol content of rotifers enriched with Superselco (INVE Aquaculture, Belgium). However, from this developmental stage until day 20 there was a marked decrease in the vitamin E content of the larvae. This reduction could have been related to the antioxidant function of vitamin E, as polyunsaturated fatty acid contents of larvae at this stage also suffer a reduction (Izquierdo, 1988; Rodríguez, 1994) and the tissue vitamin E levels generally closely relate with tissue polyunsaturated fatty acid levels.

*Artemia* nauplii enriched with fat-soluble vitamins were found to significantly improve the growth of yellowtail larvae, *Seriola dumerilii*, (Izquierdo, unpublished data). In other experiments, feeding of larval gilthead seabream with microdiets containing four different levels of  $\alpha$ -tocopherol (from 22.27 mg/kg to 780 mg/kg) resulted in a good larval survival when the levels were increased to 136mg/kg; further elevation of the  $\alpha$ -tocopherol levels significantly reduced larval survival (González et al., 1995). Furthermore, the dietary supplementation of microdiets with 1756 I.U. of retinol and beta-carotene was found to significantly improve the growth of larval gilthead seabream (González and Izquierdo, unpublished data).

### **Other nutrients**

Supplementation of microdiets with glycerophospholipids such as lecithin have been found to improve larval growth in ayu (*Plecoglossus altivelis*) (Kanazawa et al., 1983a), red seabream (Kanazawa et al., 1983b), knifejaw (Kanazawa et al., 1983b), Japanese flounder (Teshima et al., 1987) and gilthead seabream (Koven et al., 1993; Salhi et al., 1995). The beneficial effect of lecithin could be related with an enhancement of triglyceride absorption by dietary lecithin in the undeveloped larval digestive tract and the utilization of the lecithin in the production of lipoproteins and cellular components.

### **Importance of rearing conditions in determining nutritional requirements**

Differences in the nutritional requirements of larvae determined by several authors may also depend upon the larval rearing conditions or the enrichment method used. For example, Kjorsvik et al. (1991) reported no differences in growth rates between rotifers high or low in n-3 HUFA when algae were added to the rearing tanks. However, growth rates were correlated with rotifers high in a lipid and n-3 HUFA content of this rotifers if no algae were added to the larval tanks. Reduction in the dietary requirement with algal enrichment was also found for halibut by Holmefjord and Olsen, (1991) if algae were added to the rearing tanks. However, since many enrichment methods also include the addition of other nutrients apart from fatty acids, namely phosphoglycerides,

fat-soluble vitamins, pigments or proteins, other possible interactions with the nutrient studied will also have to be considered.

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