Nutritional requirements for finfish larvae

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Abstract

From on-start feeding, fish larvae nutritional reserves are very limited and their survival dramatically depends on exogenous feed. Hence, complete and balanced nutrition is critical of rearing success during early life stage. But most fish larvae, particularly marine ones, despite on the wild fed on a wide range of live preys, under culture conditions are forced to fed on a very limited number of preys (two or three) which frequently are not part of their natural food and hence their nutritional composition is not always the most suitable for maximum growth, development and survival of the larvae. Moreover, along larval development the fish will undertake several morphological and physiological changes which in nature are simultaneous with changes in behaviour and even habitat and type of prey fed. All these changes will affect to nutrient availability and feed utilization by the larvae in order to match their nutritional requirements. In practice, most of these problems will be simplified by the proper development of inert diets which are able to cover nutritional requirements at different moments of larval development. In order to achieve those diets we need, among many other important things, to have a complete knowledge of nutrient requirements for the different fish species.

Whereas protein composition of live preys is genetically determined, lipid qualitative and quantitative composition is greatly affected by their diet and significantly varies among batches of the same type of prey, as well as among different species. Early studies on the 80´s had determined that lipids are the most important factor affecting the nutritional quality of live preys and since then a vast amount of the research conducted on larval nutrition have focussed on these nutrients. Essential long chain polyunsaturated fatty acids, as key components of bio-membranes play many important roles in their functioning and are particularly indispensable for larval development. Their presence and quantity in the diet are determining to the efficiency of digestion, absorption, and transport of some nutrients, and to the capacity of dietary energy deposition and utilization. They markedly affect eye and brain development as well as larval behaviour. Finally, as sources of eicosanoids they regulate several physiological functions including some related with larval development, immune function and stress resistance, globally affecting larval growth and survival and rearing success. Recently, molecular studies have denote the presence and activation by the fatty acid composition of the diet of a delta-6 desaturase like gene, involved in long chain polyunsaturated fatty acids synthesis. Besides, the different essential fatty acids compete among them at many different points of fish physiology, dietary unbalances among them leading to detrimental consequences for the larvae. To complicate the picture a bit more, the molecular form in which they are administered is determinant of the utilization efficiency of the dietary essential fatty acids. Other lipids such as phospholipids are considered indispensable for fish larvae, since they distinctly promote the limited ability of larvae to absorb, to re-acylate and to transport triglycerides and provide additional sources of nutrients. Fat-soluble vitamins and pigments have also prove to play important roles along larval development and their inadequate dietary levels either by shortage or excess are negative for the larvae. In a similar manner to what is found in juveniles, dietary protein utilization has been found to be affected by the dietary source, particularly during early larval stages and playing a central role in the development and maturation of the larval gut. Besides, certain free amino acids also constitute a very important source of energy, act as attractants and play a significant role in gut function and development. Finally, despite their importance, only a very limited number of studies have focused other nutritional aspects of larval development such as water-soluble vitamin and mineral requirements and energy utilization by fish larvae.
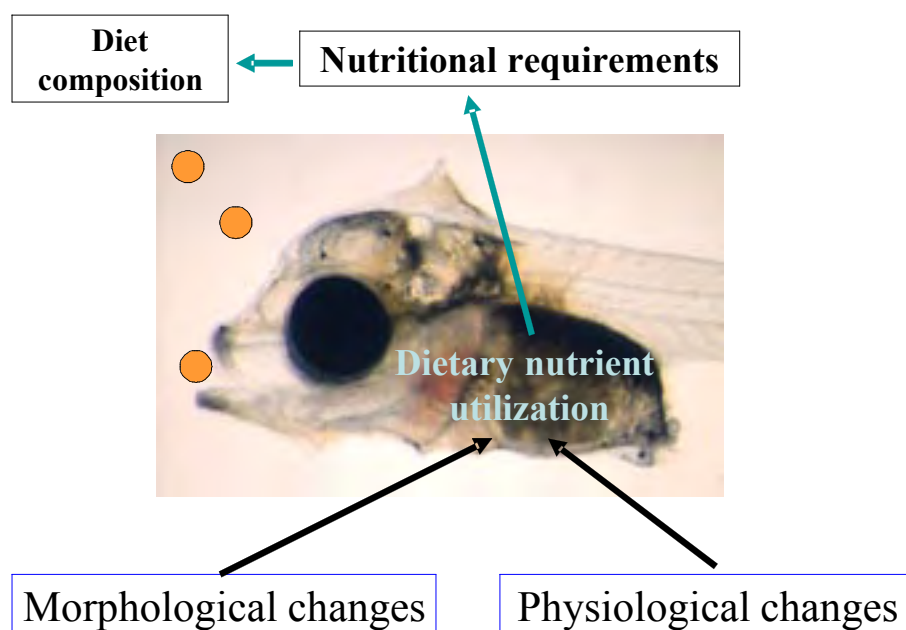
Key words

Arachidonic acid, broodstock nutrition, docosahexaenoic acid, eicosapentaenoic acid, fish nutrition, larval nutrition, essential fatty acids.

Abbreviations

EFA: essential fatty acids; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; ARA: arachidonic acid; PUFA: polyunsaturated fatty acids with 18 or more carbon atoms and 2 or more double bounds; HUFA: highly unsaturated fatty acids with 20 or more carbon atoms and 2 or more double bounds; PI: phosphatidyl inositol; AA: amino acids; EAA: essential amino acids.

Introduction

From on-start feeding, fish larvae nutritional reserves are very limited and their survival dramatically depends on exogenous feed. Hence, complete and balanced nutrition is critical of rearing success during early life stage. But most fish larvae, particularly marine ones, despite on the wild fed on a wide range of live preys, under culture conditions are forced to fed on a very limited number of preys (two or three) which frequently are not part of their natural food and hence their nutritional composition is not always the most suitable for maximum growth, development and survival of the larvae. Moreover, along larval development the fish will undertake several morphological and physiological changes which in nature are simultaneous with changes in behaviour and even habitat and type of prey fed. All these changes will affect to nutrient availability and feed utilization by the larvae in order to match their nutritional requirements. In practice, most of these problems will be simplified by the proper development of inert diets which are able to cover nutritional requirements at different moments of larval development. In order to achieve those diets we need, among many other important things, to have a complete knowledge of nutrient requirements for the different fish species.

Requirements for essential fatty acids

Three very long chain polyunsaturated fatty acids, namely docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6) have a variety of very important functions in fish species, particularly in larvae. Despite freshwater fish seem to have sufficient $\Delta 5$ and $\Delta 6$ desaturases and elongases activities to produce ARA, EPA and DHA if their precursors linoleic (18:2n-6) and linolenic (18:3n-3) acids are present in the diet, such enzymatic activity is very restricted in marine fish larvae and as a consequence, DHA, EPA and ARA have to be included in the diet and are considered essential. A $\Delta 6$ desaturase-like gene has been isolated in larval gilthead sebream (Seilez et al., 2003). More recently,

experiments in our laboratory have found that its expression is affected by the diet, denoting a higher activity of this enzyme when low EFA and high 18 carbon atoms polyunsaturated fatty acids are provided in the rotifers. Inadequate contents of those EFA in the diet give rise to several behavioural and morphological alterations such as poor feeding and swimming activities, poor growth and dropping mortality, fatty livers, hydrops, deficient swim bladder inflation, abnormal pigmentation, disgregation of gill epithelia, immune-deficiency and raised cortisol levels (Izquierdo, 1996; 2004).

Since environmental factors such as temperature, salinity and light affect lipid composition of fish tissue (Izquierdo, 2004), EFA requirements could be also affected by environmental conditions. For instance, larvae of the euryhaline species *Galaxias maculatus* have been found to be higher in EPA, DHA and ARA acids when they were obtained from marine environments in comparison with those from freshwater (Dantagnan et al., submitted), denoting the important role of some of these fatty acids in osmotic regulation. Moreover, before first feeding, synthesis of those EFA was activated in larvae from freshwater environment but not in those obtained in the estuary, suggesting the influence of environment salinity on activation of elongation and desaturation enzymes.

In the wild, types and contents of EFA differ among the different steps of the trophic chain, and EFA requirements would then rely on the trophic behaviour of each fish species. Being fish larvae visual feeders, larval trophic behaviour is closely related to the development of the visual capacity. In sparids, such as gilthead seabream and red porgy (Roo et al., 1999) the most important changes in the eye structure occur along the lecitotrophic stage as a preparation for prey capture, rod photoreceptors necessary for accurate vision at low light intensity appearing in gilthead seabream about 18th day after hatch. N-3 PUFA, and particularly DHA play a critical role in neural and retinal tissue functions. Bell and Dick (1993) found that both rod and cone photoreceptors in herring eye, accumulate and selectively retain DHA, and thus, feeding herring a DHA poor Artemia during the period of rod development resulted in impaired vision at low light intensities. Moreover, elevation of dietary DHA and eicosapentaenoic acid (EPA) increase eye diameter in gilthead seabream (Izquierdo et al., 2000; Roo et al., submitted) and this fact, together with a high density of cone photoreceptors in these larvae, implied a total higher number of cones and a potentially improved visual accuracy (Roo et al., submitted). Thus, restriction in light intensity applied in some commercial hatcheries, particularly during the first two weeks of larval development when only cone type receptors of maximum light capture effectiveness at high light intensity are sufficiently developed, may impose higher DHA requirements in broodstock and larvae than in fish cultured at higher light intensities. Besides, inadequate lighting regimes may constitute an stress factor in larval culture conditions, which in turn increase the EFA demand in this fish.

Despite the retention of EFA, particularly DHA, in seabream brain, appearance of larval swimming reaction to a visual stimulus is delayed in fish fed low EFA rotifers, suggesting the delay in functional development of brain and visual system (Benítez et al., submitted). Moreover, larvae fed low EFA rotifers showed lower cruising and escaping swimming speed than those fed high EFA.

Along larval development several authors have shown a requirement of EFA for gilthead seabream very close to 1.5 % n-3 HUFA in dry matter when larvae were fed either live preys (Rodríguez et al., 1998) or microdiets (Salhi et al., 1999), regardless dietary lipid level (Salhi et al., 1994). Much higher requirements are estimated in the literature when EPA contents are 2 or 3 times higher than those of DHA (Rodríguez et al., 1994, 1997), due to the very high incorporation of EPA into the larval polar lipids and the displacement of DHA from certain polar lipids (Izquierdo et al., 2000). However, as it happens in the other life stages, provided other nutrients such as antioxidants are also balanced, elevation of dietary n-3 HUFA up to 8 % keeping a DHA/EPA ratio of 1.7 further improves larval growth and survival (Liu et al., 2002). High n-3 HUFA requirements have been also estimated for red porgy (3.39 % at 1.35 DHA/EPA, Hernández-Cruz et al., 1999) and Dentex dentex (Mourente et al., 1999) despite in the latter the high EPA content in Artemia may have caused an overestimation of the requirements as we have seen in gilthead seabream (Rodríguez et al., 1997). On the contrary, carp larvae seemed to require as low as 0.05% n-3 fatty acids from cod liver oil (Radunz Neto et al., 1993) to cover the essential fatty acid requirements along this period of life.

The particular structure of DHA provides this fatty acid with many important functions in fish metabolism. Its incorporation into cell membranes regulates membrane integrity and function, this fatty acid being an important component of phosphoglycerides, particularly phosphatidyl ethanolamine and phosphatidyl choline,

in larvae. It is specifically retained in starved or low-EFA fed fish, possibly due to the lower cell oxidation rates than other fatty acids (Madsen et al., 1999). It is necessary for growth, survival, flat fish metamorphosis and disease prevention. It may be a substrate for some lypoxigenases and several studies have shown that it has a greater potential as an essential fatty acid for marine fish larvae than EPA (Watanabe et al., 1989; Watanabe, 1993), its requirement being more limiting for growth and survival than those for n-3 HUFA (Izquierdo, 1996). Minimum dietary levels in diets for larval gilthead seabream seem to be 0.8 (Izquierdo, 2004). In larvae, high levels (5 % in dry basis) of dietary DHA in microdiets for gilthead seabream did not caused any excess problem, but further promoted growth and larval survival (Liu et al., 2002). Regarding other sparids, requirements along larval development seem to be about 1.5 % for red porgy larvae when DHA/EPA ratios are about 1.4 (Hernández-Cruz et al., 1999) and close to 2.3 % for *Dentex dentex* fed a very low DHA/EPA ratio (0.32) (Mourente et al., 1999).

Eicosapentaenoic acid is also particularly important for larval growth (Watanabe et al., 1989) playing general and particular roles in fish metabolism. Its presence in rotifers enhances non-specific lipase activity in larval seabream (Izquierdo et al., 2000), neutral lipids esterified with EPA being a preferred substrate for this enzyme. In marine fish it is a main component of polar lipids and it regulates membrane integrity and function, indeed its incorporation into phosphoacylglycerides enhances fluidity of cell membrane (Sipka et al., 1996) in a higher degree than ARA (Hagve et al., 1998) but lower than DHA (Hashimoto et al., 1999). Moderate dietary levels of this fatty acid also enhance DHA incorporation into larval PL (Izquierdo et al., 2000, 2001), causing a sparing effect on such an important fatty acid. It is a good substrate for some cycloxygenases, being precursor of some prostanoids in marine fish and also a main substrate for some lypoxigenases, being the main precursor for leukotriene synthesis in some species. Its competition with ARA for these two types of enzymes enables it to be an important regulator of eicosanoid synthesis. Best growth, survival, resistance to stress and spawning quality have been obtained in larval gilthead seabream with EPA dietary levels of 0.7-0.8 (Rodríguez et al., 1998; Salhi et al., 1999) in dry basis. In larvae, increase of EPA up to 2.9 % in dry basis when DHA/EPA levels where high (1.72) and ARA contents were only 0.05 significantly improved growth, survival and resistance to a shock temperature stress of gilthead seabream (Liu et al., 2002), denoting its high value as EFA. However, increase of dietary EPA up to 1.8 reduced growth when ARA levels are as high as 1.8 % and DHA/EPA about 1.3, denoting how the EFA value of EPA is dependant on the dietary levels of DHA and ARA.

Arachidonic acid is a main component of a minor but very important polar lipid class, phosphatidyl inositol (PI). In vitro, ARA is a preferred substrate for most cycloxigenases, being the main precursor for prostaglandin synthesis, whereas in some marine fish in vivo, EPA is the main substrate due to its high presence in the diet. ARA also constitutes a good substrate for several lypoxigenases, its derivative hydroxi-fatty acids having important physiological functions in marine fish. Its content in the PI of cell membranes possibly regulates eicosanoid synthesis. In gilthead seabream larvae, increase of ARA up to 1 % enhances survival and growth when DHA and EPA dietary contents are 1.3 and 0.7, respectively (Izquierdo, 1996; Bessonart et al., 1999). Increase in ARA contents in the rotifers also prevent post-stress mortality (Koven et al., 2001). ARA seems to play also important roles in turbot juveniles (Castell et al., 1994) and in flatfish pigmentation (Estévez et al., 1997).

Evidences of competition among two or more of these essential fatty acids have been suggested for digestive enzymes, fatty acid binding proteins, phosphoacylglycerides synthetases, lypoxigenases and cyclooxigenases, and probably in beta-oxidation as it happens in rats (Izquierdo, 2004). Not only absolute dietary values for each of these essential fatty acids but also optimum dietary ratios among them must be define since both factors will affect at least to their incorporation into the tissue lipids and hence membrane fluidity and function, the energy values obtain from their beta-oxidation and the production of metabolically active compounds. Thus, optimum DHA/EPA ratios have been defined for turbot larvae around 2 (Reitan et al., 1994) and for seabream around 1.2 at least (Rodríguez et al. 1997). Considering both the sum of the three EFAs and the ratios among them, if we plot the dietary value of the ratio (DHA+EPA+ARA)*DHA/EPA/ARA against growth in some of our recent studies (Figure 6), we found a significant correlation. If we apply the same equation to dietary fatty acids in other gilthead seabream studies (Rodríguez et al., 1994, 1995, Salhi et al., 1998, Liu et al., 2002, Koven et al., 2001, Fernández et al., 1995 and others), we found that for ARA values higher than 0.5% the closer the value of the equation (DHA+EPA+ARA)*DHA/EPA/ARA to 50 the better the growth performance.

Phospholipids

Feeding larvae low dietary contents of PL reduces growth and lipid transport from larval enterocytes to hepatocytes (Kanazawa 1993; Izquierdo et al., 2000). For instance, feeding larval gilthead seabream diets without lecithin supplementation produces accumulation of lipidic vacuoles in the basal zone of the enterocyte and esteatosis in the hepatic tissue, both of them being markedly reduced by a 2% addition of soybean lecithin, denoting an enhancement in the lipid transport activity in gut and liver (Izquierdo et al., 2000). This reduction in lipid transport could be related with a limited capacity for "de novo" synthesis of phospholipids in the larvae. Reacilation of phospholipids in the enterocyte is known to occur through the glycerol-3-phosphate pathway in both the rough and the smooth endoplasmic reticulum (Izquierdo *et al.*, 2000). But since marine fish larvae fed microdiets show enterocytes with a poor development of endoplasmic reticulum and Golgi system, reacilation capacity may be limited in these larvae. Moreover, inappropriate dietary lipids have been found to markedly affect re-esterification pathways in seabream gut (Caballero et al., submitted), modifying the type of lipoprotein formed. For instance, addition of soybean oil promotes PC synthesis by both gycerol-3-phosphate acyltransferase and monoacylglycerol pathways, thus providing material for VLDL formation, whereas addition of rapeseed oil inhibits lipid re-esterification, particularly into TG (Caballero et al., submitted).
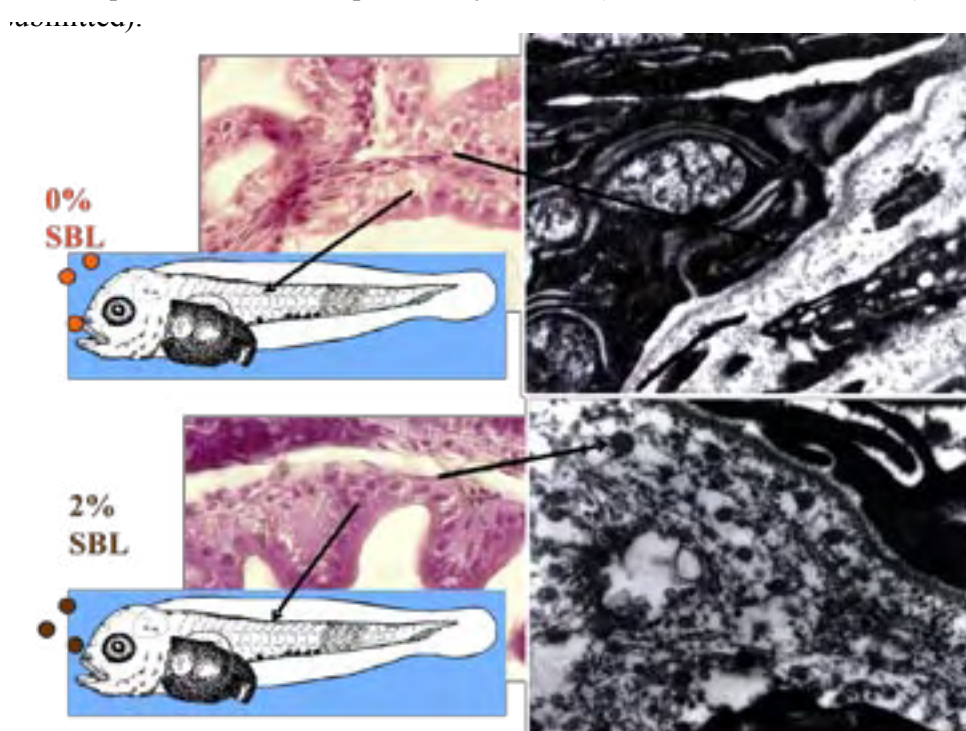

Figure 2 Inclusion of different types of phospholipids in larval microdiets markedly enhance reacilation, lipoprotein synthesis and lipid transport.

On the contrary, when gilthead seabream larvae are fed TG of marine origin, rich in n-3 HUFA it was observed an accumulation of lipid vacuoles in the basal zone of the enterocyte and hepatic steatosis, denoting the good absorption of dietary TG but also a reduced lipid transport to peripheral tissues, whereas feeding with marine PL markedly reduced lipid accumulation in both type of tissues. A higher lipid content due to accumulation of TG and cholesterol esters was found in larvae fed marine TG, whereas in larvae fed marine PL relative proportions of PC and phosphatidyl-ethanolamine (PE) were higher and richer in n-3 HUFA (Salhi et al., 1999). These results agree well with the higher incorporation into larval polar lipids of fatty acids from dietary polar lipids than from dietary triglycerides. In studies with labelled fatty acids dietary n-3 HUFA PL, significantly improved the incorporation of free eicosapentaenoic acid, but not of free oleic acid, into larval polar lipids in comparison to n-3 HUFA rich TG. This specific incorporation of eicosapentaenoic acid when dietary polar lipids are rich in n-3 HUFA could be related to the enhancement of lipid transport, mobilization and deposition in the peripheral tissues by n-3 HUFA rich dietary phospholipids. As a consequence, growth of larval gilthead seabream was improved when they were fed microdiets containing marine PL instead of marine TG despite the slightly lower dietary n-3 HUFA levels of the former (1.5% versus 1.8%, respectively) (Salhi et al., 1999).
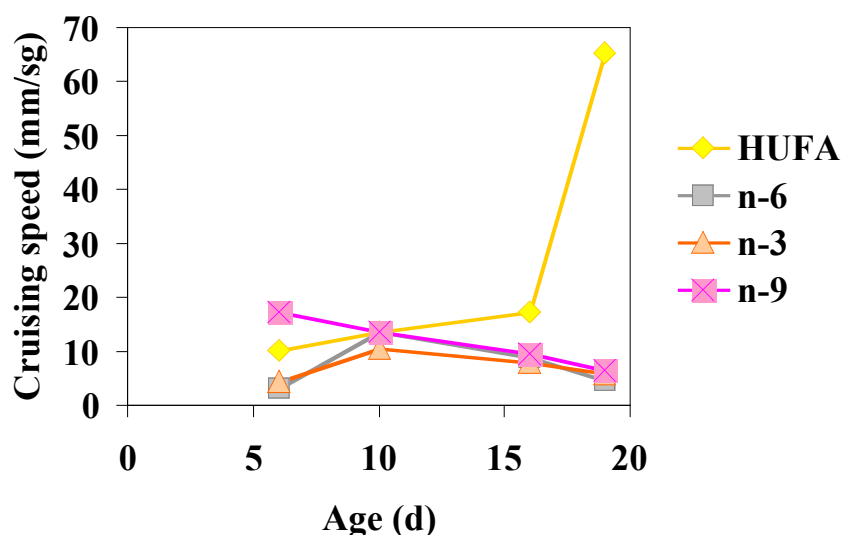
Figure 3. Effect of feeding larval seabream rotifers with different fatty acid composition.

But incorporation of dietary free fatty acids seems to be even lower than that of triglycerides. Thus, labelled oleic acid was better incorporated into both polar or neutral lipids of seabream larvae when it was provided in the diet esterified in a triglyceride than as a free fatty acid, suggesting again a limited capacity of reacilation or transport for dietary long chain free fatty acids or its preferential utilization as energy source in the enterocyte.

Enzymatic, histological and biochemical evidences suggest that marine fish larvae are able to digest and absorb n-3 HUFA rich TG more efficiently than free fatty acids, but feeding with PL, particularly if they are rich in n-3 HUFA, will enhance PL digestion and specially lipid transport allowing a better n-3 HUFA incorporation into larval membrane lipids and promoting fish growth. This confirms former studies which suggest that in addition to the dietary level of essential fatty acids, the molecular form in which they are present in the diet is also important for good growth and survival of marine fish larvae (Izquierdo, 1988; 1996; Izquierdo *et al.*, 1989).

Accumulation of lipidic vacuoles in the basal zone of the enterocyte caused by feeding diets without lecithin supplementation in gilthead seabream disappeared when 0.1% PC was added regardless of its (squid or soybean) origin (Izquierdo et al., 2000). However, squid PC was more efficient in reducing hepatic steatosis than soybean PC, suggesting a combined effect of dietary PC and n-3 HUFA to further enhance hepatic lipid utilization. Indeed both types of molecules have been found to promote lipoprotein synthesis.

Vitamins

The improvement in production of microdiets for larval feeding has greatly facilitated the determination of the vitamin requirements in fish larvae, allowing to experimentally isolate vitamin deficiencies and describing several types of abnormalities. Most described water-soluble vitamin requirements are much higher for larvae than for juveniles of the same species, not only due to the higher metabolic demand in the former, but also for the high ratio surface/volume in larval diets making the diets more prone to oxidation and leaching. Thus, whereas in juveniles vitamin premix accounts for about 2-3% of the diet, in larval microdiets they may reach up to 6-8% of the diet.

Most water soluble vitamin contents of hatchery microalgae and live prey seem to be able to match the requirements of fish larvae, except for the low levels of pyridoxine described in certain studies (González, 1997). However, fat soluble vitamin contents of microalgae and live prey greatly varied among sample batches and with culture conditions, frequently originating hypo and hypervitaminosis.

Vitamin E and vitamin A decreased in seabream from fertilization to the onset of exogenous feeding and a continuous uptake of both nutrients from live preys is observed from day 10th after hatching. However a decrease in the larvae vitamin A content is found when rotifers are substituted by *Artemia* nauplii. Enrichment

of *Artemia* nauplii with fat-soluble vitamins improves amber-jack growth (*Seriola dumerilii*) and seabream microdiet supplementation with 1756 IU of a retinol and beta-carotene mixture significantly improves larval growth. However, bioavailability of beta-carotene seems to be very poor in gilthead seabream in comparison with retinol and astaxanthin which seems to have a provitamin A function in larvae of this species. Regarding vitamin E requirements, progressive elevation of dietary alpha-tocopherol acetate levels from up to 1500 mg/kg in larval seabream diets containing free ascorbic acid significantly reduced larval survival, whereas the same increase in alpha-tocopherol when vitamin C was supplemented as ascorbic acid polyphosphate caused a significant improvement in larval growth without affecting survival, suggesting a pro-oxidative effect of alpha-tocopherol over vitamin C in the former.
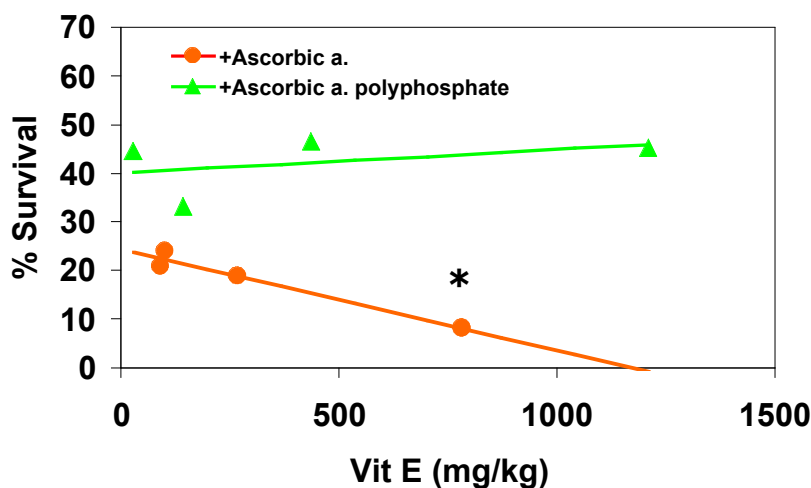

Figure 4. Effect of dietary Vit E in seabream performance in diets containing ascorbic acid in a free or polyphospate form.

Protein and amino acid requirements

Fast growing fish larvae have a high demand for protein requiring more elevated dietary contents than juveniles and adults, microdiets designed for larval rearing containing between 50 and 70 % protein. From the 20 most common amino acids 10 have been found to be essential or indispensable for all studied fish and are required for optimum growth despite fish are not able to synthesize them: Leu, Ile, Val, Thr, Phe, Met, Trp, Arg, His, and Lys. Another two amino acids, Tyr and Cys are only non essential if Phe and Met are present in the diet. At least all those amino acids should be also required by fish larvae. Moreover, the importance of other minor amino acids such as taurine, recently pointed out as essential for best growth and survival of several species of sparids should not be neglected. Methods to determine quantitative requirements of each of those aa in fish larvae include feeding microdiets with graded levels of one amino acid at a time in a test diet containing either all crystalline amino acids, a mixture of casein, gelatin and crystalline amino acids, or a semipurified diet using an imbalanced protein (zein, corn gluten) formulated so that the amino acid profile is identical to the test protein except for the amino acid being tested. As studied by Kanazawa and co-workers for fish larvae of several species, diets are designed to contain protein levels at or slightly below the optimum protein requirement for that species to assure a maximum utilization of the limiting amino acid. Hence, quantitative requirements of several aa have been determined for red sea bream and Japanese flounder larvae (López-Alvarado, 1995). Relations among aa, such as competition or common synthesis pathways, need also be considered. Moreover, aa leaching in the relatively long water staying microdiets, cause difficulties to accurately determine physiological requirements. Hence other methods previously utilized in juveniles have been applied to fish larvae. For instance, from the early 80´s it has been shown that there is not difference between the relative proportions of individual essential aa required in diet and the relative proportions of the same 10 aa present in fish carcass. Since the essential aa profile of fish muscle protein does not differ greatly between individual fish species the pattern of requirement for individual species will also be similar. Thus, analysis of the larval aa composition has been frequently used to predict its essential aa requirements (Watanabe and Kiron, 1994).

Comparison of live prey and fish larvae aa profile would also allow us to predict if such feed would cover the larval aa requirements. For instance, when turbot larvae and live food eaa profiles are compared, the profile of

the latter seems to be deficient in some eaa such as leucine, arginine, threonine or methionine (Conceiçao et al., 1997), depending on the larval age and type of prey, whereas rotifers seem to be deficient in threonine and leucine for larval seabream.

Other methods utilized in juveniles consider that when an essential amino acid is deficient in a diet the major proportion will be used for protein synthesis and only a little fraction will be oxidized to carbon dioxide to obtain energy, whereas if that amino acid is supplied in the diet in excess plasma levels will increase and it will be more available for oxidation. A force feeding method including labelled eaa has been recently developed for fish larvae (Conceiçao et al., 2003), denoting a high retention of labelled doses of eaa in the body (>60%), and low catabolism as measured by liberated $14CO_2$ (< 25%). In contrast, non essential aa were faster catabolized (>40%).

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