FEEDING MARINE FISH LARVAE WITH LIPID SOURCES ALTERNATIVE TO FISH OIL

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Abstract

Despite global production of fish oil has been stagnant for the last decade, its demand as a source of n-3 PUFAs for aquafeeds has been continuously increasing. In marine fish larvae, fish oil replacement levels by vegetable oils will depend on the use of live preys or microdiets. The high content of lipids in enrichments for live preys may allow a high percentage of fish oil substitution, depending on the requirements of each species but also on the utilization of enrichment lipids by rotifers or Artemia. In microdiets, despite larvae have high EFA requirements, the high protein at this stage of development impose high dietary protein contents which in turn provide additional sources of EFA. Complete substitution of fish oil by vegetable oils in enrichments for seabream larvae reduces growth and affects larval normal behaviour. But fish oil replacement by vegetable oils in microdiets for seabream, did not affected growth and survival. Moreover, feeding larvae with vegetable oils increased up to 6 times the relative expression of delta 6 desaturase like gene in larvae fed rapeseed and soybean oils. Since the lack of essential fatty acids for marine fish constrains the use of vegetable oils in larval feeds, alternative EFA sources are being developed, such as marine micro algae which constitute a well recognized "single cell oil source" high in PUFA. Substitution of either fish oil or booster oils in microdiets for seabream by homogenized *C. cohnii* promotes good growth and survival, particularly in very young larvae, whereas substitution by *Phaeodactilum tricornutum* at this larval stage damages intestine epithelia and reduces survival. Finally, substitution by *Schyzotrichium sp*. slightly reduces larval seabream growth.

Importance of fish oil as the main lipid source in aquafeeds and larval nutrition

Due to its high digestibility and elevated content on essential fatty acids, fish oil has been traditionally used as a main lipid source in fish diets. But fish oil production is dependant on fisheries which constitute a declining natural resource seriously endangered and its continue exploitation is linked to detrimental environmental consequences (Tonon *et al.,* 2002). Hence, global production of fish oil has been stagnant around 1.2 millions tons/year for the last decade. Besides, the demand for fish oil as a source of n-3 PUFAs is increasing together with the fast development of aquaculture and its use in food and pharmacy. At present more than 70% of the fish oil production is consumed by aquafeeds (Tuominen and Esmark, 2003) and it is expected world fish oil supply will not be enough to cover completely the lipid sources demand for aquaculture within the next ten years. Thus, further development of aquaculture seems to be dependant on the availability of other lipid sources alternative to fish oil (Bell *et al.,* 2003). Moreover, competition for fish oil for its inclusion in human nutritional supplements and agricultural feeds other than for aquaculture will soon make fish oil a highly prized commodity (Harel *et al.,* 2002). In addition,

fish oil use has also other types of problems such as a poor oxidative stability (Swaaf *et al.,* 1999), pollutants accumulation (Tonon *et al.,* 2002), contamination with heavy metals and seasonal and species variation in of a very complex fatty acid profile (up to 50 different fatty acids may be present) (Medina *et al.,* 1998). For all these reasons, there is a high interest in searching for other lipid sources which are able to substitute fish oil in diets for aquaculture.

As the main source of essential fatty acids, together with fish meal, minimum fish oil inclusion in fish diets will depend on quantitative requirements. The reported essential fatty acid requirements may vary between species (NRC, 1993) and are higher in larval stages (1-4% of feed dry matter; Izquierdo, 1996) than in adult fish (0.5-2.0% of feed; NRC 1993). Hence, whereas a fish juvenile is able to survive for months on a diet almost completely deprived from EFA (Izquierdo, 2005), a larvae would die in 10-15 days (Izquierdo *et al.,* 1988). Moreover, DHA is known to have a higher efficiency as an essential fatty acid than EPA (Watanabe *et al.,* 1989; Watanabe 1993), the former being particularly accumulated in the olfactory nerve, retina and central nervous system (Sargent *et al.,* 1993). The content of DHA in fish meal and fish oil is will vary dependent on species and season, but the average content in fish meal is 1.7% (Nutreco ARC, unpublished) and in fish oil 5% (NRC, 1993). Taking into account that fish feed contain about 1.5-2.5% DHA, the market demand for year 2010 would be close to 400.000 metric tons, whereas world production of fish meal and oil only renders less than 200 000 tons of DHA (Rosenlund, unpublished). Despite larval diets for marine fish will require higher concentrations of DHA, the global amount of this fatty acid required for this type of diets will be very small compared with the total feed production.

Plant oils as alternative lipid sources

Some vegetable oils such as soybean and linseed oils are considered as good alternative lipid sources in diets for juvenile salmonids and freshwater fish (Bell *et al.,* 2001; Rosenlund *et al.,* 2001; Caballero *et al.,* 2002). However, in marine fish the use of vegetable oils as a sole lipid source is limited by the low ability of these species to convert linoleic and linolenic acids, abundant in many vegetable oils, into arachidonic (ARA), eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) which are essential for marine fish and high in fish oil. For that reason, partial replacement of fish oil by vegetable oils would be only possible when these essential fatty acids are present in the diets in sufficient quantities to meet the essential fatty acid requirements.

In juveniles of marine fish, recommended fish oil replacement levels found in literature will vary according to the species EFA requirements, the amount of fish meal included as it constitutes a complementary source of EFA, the type of fish oil used the total dietary lipid content and the feeding period (Izquierdo *et al.,* 2005). For instance, up to 60% fish oil may be replaced by palm oil in diets for yellowtail without altering fish growth (Watanabe, 2002), as well as in seabream or sea bass if high lipid content diets are used (Izquierdo *et al.,* 2003), since both fish meal and the portion of fish oil included is able to meet the essential fatty acid (EFA) requirements of these species. Nevertheless, after three months of feeding high lipid diets with a 60% fish oil replacement by certain vegetable oils, particularly soybean oil, liver lipid content and lipid deposition in the hepatocytes markedly increased (Caballero, 2002) and some immune parameters were affected (Montero *et al.,* 2003) suggesting undesirable effects on fish health when longer feeding periods are tested. Besides, increase of substitution levels up to 80 %, significantly reduced growth and conversion indexes (Izquierdo *et al.,* 2006). Regarding the fish oil type, it is advantageous to use fish oils very rich in n-3 HUFA such as Peruvian anchovy oil (Rosenlund *et al.,* 2001), which would allow a higher replacement by vegetable oils, producing fish fillets with a similar n-3 HUFA content than fish fed 100% menhaden fish oil.

In marine fish larvae, fish oil replacement levels will depend firstly on the type of feed used, basically live preys or microdiets. The high content of lipids in enrichments for live preys may allow a high percentage of fish oil substitution, depending on the requirements of each species but also on the utilization of enrichment lipids by rotifers or Artemia. In microdiets, despite larvae have high EFA requirements, the high protein at this stage of development impose high dietary protein contents which in turn provide additional sources of EFA. Moreover, the high energy requirements also impose high dietary lipid levels which allow a higher percentage of replacement by vegetable oils, which indeed are very digestible and constitute a good energy source.

Inclusion of vegetable oils in rotifers enrichment

In order to study the effect of fish oil substitution by different vegetable oils in enrichment emulsions for rotifers an experiment was conducted with seabream larvae (Benítez-Santana et al., in press). From day 4th after hatching, larvae were fed with rotifers (*Brachionus plicatilis*) twice a day (at 9h00 and 15h00) for the following 20 days with four types of rotifers: "FO rotifers" enriched with fish oil, "SO rotifers" enriched with soybean oil, "LO rotifers" enriched with linseed oil and "RO rotifers" enriched with rapeseed oil. Each type of rotifers was tested in triplicate larval rearing tanks.

Complete fish oil replacement in rotifers enrichment by any of the vegetable oils assayed markedly reduced seabream larval growth, in relation with the lower n-3 HUFA and DHA levels found in these rotifers in comparison with fish oil enriched ones (Benítez-Santana *et al.,* in press). Essential fatty acid requirements in gilthead seabream along larval development are around 1.5% n-3 HUFA in dry matter, regardless the use of different prey types (Rodríguez *et al.,* 1998) or microdiets (Salhi *et al.,* 1999). The n-3 HUFA contents in rotifers enriched with vegetable oils were lower than 0.19 being well bellow the minimum level necessary to cover the fatty acid requirements of this species (Benítez-Santana *et al.,* in press).

Whole body composition of the larvae was similar in larvae fed fish oil enriched rotifers with respect to the initial larvae, being higher in n-3 HUFA and DHA than larvae fed vegetable oils enriched rotifers (Benítez-Santana *et al.,* in press). Besides, fatty acids composition in both central nervous system and eyes, revealed a retention of n-3 HUFA, particularly DHA, even in larvae fed rotifers enriched with vegetable oils, confirming their importance for the development of such tissues. Nevertheless, EFA contents in larvae fed rotifers enriched with vegetable oils was lower, in agreement with the different behaviour found in this larvae. For instance, a reduction in burst swimming speed after a visual stimulus was obtained in larvae fed vegetable oils enriched rotifers in comparison with fish oil, in agreement with the visual incapacity found in yellowtail (*Seriola quinqueradiata*) fed DHA-deficient diets (Masuda *et al.,* 1999).

Swimming activity before stimulus was also reduced by feeding rotifers enriched with vegetable oils (Benítez-Santana *et al.,* in press). Despite reaction against sonorous stimulus was not affected by feeding vegetable oils, appearance of reaction after visual stimulus was delayed to day 19th in larvae fed LO rotifers and it was also delayed and reduced by feeding the other vegetable oils. Higher burst swimming speed in larvae fed LO is in agreement with the higher response to acute stress found in juveniles of the same species fed with linseed oil (Montero *et al.,* 2003). Higher burst and cruise swimming speed in the larvae fed rotifers enriched with fish oil, would be related to the higher levels of DHA in larval eyes and brain, since this fatty acid is involve in several neural tissue related functions such as neurocytes myelination and synapse construction, both functions being sensitive to nutritional deficiencies (Krigman and Hoga, 1976).

In summary, complete substitution of fish oil by vegetable oils in enrichments for seabream larvae reduces growth and affects larval normal behaviour, reducing cruise speed, and particularly delaying the appearance of the visual stimulus, suggesting a delay in the functional development of brain and vision, in agreement with the minor EFA and DHA found in eyes and brains of these larvae.

Substitution of fish oil by vegetable oils in microdiets for seabream

Starting diets for marine fish larvae contain high levels of proteins from fish, squid, krill or other meals which generally contain a good amount of essential fatty acids in the polar lipid fraction, suggesting that it is possible to obtain a higher replacement than in live prey enrichments. In order to determine the ability of larval gilthead seabream to utilize different vegetable oils as alternative lipid sources to replace fish oil, seventeen day old gilthead seabream larvae were fed during 17 days four different microdiets formulated with either sardine fish oil (FO), soybean (SO), rapeseed (RO) or linseed (LO) oils and a fifth diet containing defatted squid meal and linseed oil (ELO) (Izquierdo *et al.,* in press).

Good growth, both in terms of total length and body weight, and survival of gilthead seabream larvae fed either FO or RO, SO and LO suggested the good utilization of these vegetable oils when dietary n-3 HUFA levels are high enough to cover the larval requirements for essential fatty acids. Thus, n-3 HUFA levels in the microdiets containing FO, RO, SO or LO were higher than 3% dry weight diet are able to fulfill the essential fatty acid requirements of this species (Izquierdo, 2005). Thus, complete substitution of fish oil by either rapeseed, soybean or linseed oils in microdiets for gilthead seabream seems to be possible when the EPA and DHA requirements are covered by the dietary contents of fish or squid meal. In juveniles of the same species, it is possible to reduce a 60% of the fish oil in diets without compromising growth survival, fillet organoleptic properties or fish feed utilisation, when fish are fed either for a medium (3 months, Izquierdo *et al.,* 2000; Izquierdo *et al.,* 2003) or for a long feeding period (Menoyo *et al.,* 2004; Izquierdo *et al.,* 2005). On the contrary, Increase fish oil substitution levels up to 80% significantly reduced growth and conversion indexes (Izquierdo *et al.,* 2006). Thus, higher substitution levels are obtained in larval diets than in juvenile ones. Despite essential fatty acid requirements are higher for larvae (3%) than for juveniles (0.8%) of gilthead seabream (Izquierdo, 2005), the lower protein requirements of the later markedly reduces the inclusion of protein sources such as fish or squid meals which in turn constitute a considerable source of essential fatty acids. Hence, complete substitution of fish oil by vegetable oils in diets for larvae rendered about 3.5% n-3 HUFA, whereas it only rendered 0.3% in diets for juveniles. However, reduction of n-3 HUFA obtained by defattening of squid meal and complete replacement of dietary lipids by linseed oil, did not covered the essential fatty acid requirements of gilthead seabream larvae and significantly reduced larval growth and survival.

Feeding larvae with vegetable oils, increased up to 6 times the relative expression of delta 6 desaturase like gene in larvae fed rapeseed and soybean oils (Izquierdo *et al.,* submitted) in comparison with those fed fish oil and denotes the nutritional regulation of desaturase activity through its gene expression in this fish species. However, feeding LO did not increased to such a high extend the expression of delta 6 desaturase gene, perhaps due to the fact that this oil contains LA but also high level of LNA which is competitor in desaturation and elongation of C_{18} PUFA. Moreover, very high contents of lineseed oil in diet ELO completely inhibited desaturase gene expression, denoting that regulation of desaturase gene expression depends on the type of dietary vegetable oil fed.

The results of this study showed for the first time a significant effect of dietary lipids on the regulation of delta 6 desaturase expression. Feeding gilthead seabream larvae with too high linseed oil contents significantly reduced the expression of desaturase gene, whereas feeding soybean and rapeseed oils increased such expression up to 4 times.

Use of single cell oils in larval feeds

Since the lack of essential fatty acids for marine fish constrains the use of vegetable oils in larval feeds, alternative EFA sources have been the objective of study along the last years. Marine micro algae constitute a well recognized "single cell oil source" high in PUFA, but its production in commercial hatcheries is very limited. Microalgae species can provide numerous high-value products (Pulz *et al.,* 2001), and vary significantly in their nutritional value (Enright *et al.,* 1986; Brown *et al.,* 1997). PUFAs are widespread in the marine food chain and the primary producers are marine algae (Swaaf *et al.,* 2003b). The PUFA content of microalgae depend not only on the species, but also on factors related to culture condition including composition of the medium, pH, aeration, light intensity, temperature, age of culture (Tonon *et al.,* 2002) and duration of the photoperiod (Medina *et al.,* 1998).

The marine dinoflagellate *Crypthecodinium* cohnii constitutes an excellent heterotrophic producer of DHA. *Crypthecodinium* cohnii is a chloroplastless heterotrophic marine microalgae, which shows two different phases: swarming flagellated cells and cysts. Utilizing several carbon sources *C. cohnii* accumulates lipid over 40% of its biomass dry weight, with up to 30 of total lipid being DHA (Swaaf *et al.,* 2003a and b).

Another interesting single cell source of lipids is the diatom *Phaeodactylum tricornutum* which utilizes lipids as main storage products (Reis *et al.,* 1996, Mirón *et al.,* 2002), producing large quantities of polyunsaturated fatty acids, including considerable amounts of EPA (Arao *et al.,*1987; Dunstan *et al.,* 1994). In P. tricornutum the fatty acids constitute between 8 and 10% of the algal cell biomass, where EPA constitute between 27 and 30% of the total fatty acids present, or 2.6–3.1% of the dry biomass.

As part of the EU-funded project PUFA-feed, a series of studies were conducted in our laboratory in order to determine the effect of inclusion of several types of single cell oils on starter diets for seabream. For instance, inclusion of non-homogenised C. cohnii in starter diets for seabream reduced growth in terms of total length and body weight, but it did not affected survival, suggesting an inefficient utilization of dietary nutrients. In a similar way non-homogenized *Schyzotrichium sp.* included in microdiets for seabream produced significantly smaller larvae than a control diet containing sardine oil.

Diet acceptance was determined calculating the percentage of gut occupation by the microdiet by image analysis in pictures of 30 larvae/tank. 37 day-old-larvae (20 larvae per tank) were taken for image study. Larval abdominal cavity was observed in a stereoscope (Leica Wild M3Z®) and the area of the gut occupied by digested matter was measured on the optic micrographs taken at a magnification of 25X, using Image Pro Plus® (Media Cybernetics inc., Silver Springs, MD, USA) semiautomatic image analysis system. There were no significant differences in the area of gut occupied by digested matter among the experimental diets. There was a good acceptability of the microdiet containing non- homogenized C. cohnii and no difference was found between microdiet ingestion.

However, when C. cohnii was homogenized and included in substitution of a commercial booster rich in EFA, growth and survival was not affected and both parameters were enhanced in comparison with a diet containing fish oil (capelin oil) as a single lipid source. Incorporation of DHA and other fatty acids was proportional to their contents in diet, regardless the inclusion of *C. cohnii* suggesting the good utilization of homogenized *C. cohnii*, in comparison with the non-homogenized ones. Moreover, a higher resistance to epitheliocystis incidence was found in larvae fed with *C. cohnii*.

Homogenized *Schyzotrichium sp*. was also better utilized by gilthead seabream than the non homogenized *Schyzotrichium sp.*, confirming as well as it happened with *C. cohnii* that homogenization, hence partitioning of the cell wall improves the nutritional value of both microorganisms. However, growth expressed in body weight tend to be lower when *Schyzotrichium sp.* was included in seabream microdiets and 22:5n-6 accumulated in larvae.

Feed intake by the seabream of the studied ages was not affected by the dietary inclusion 2 and 4% of homogenized *C. cohnii* and 5% of *Phaeodactylum tricornutum* (Atalah *et al*., in prep.). Besides, partial substitution of fish oil by the homogenized *C. cohnii* along a long period of time improved growth and survival in gilthead seabream, particularly during their early life stages.

However, feeding with *Phaeodactylum tricornutum* reduced seabream survival in very young larvae causing degeneration of the anterior intestine epithelial (Atalah *et al.*, in prep). A further experiment was conducted to determine the effect of these microorganisms in larval health. For that purpose, seabream postlarvae fed for one month with either homogenized *C. cohnii* or *P. tricornutum* were exposed to *Photobacterium damselae subsp. piscicida*. Mortality was found only at day 21 after inoculation and only in the group fed 4% *C. cohnii,* which reached up to 18% of the population. However no *Photobacterium damselae subsp. piscicida* was found in any of the spleen, kidney or liver of fish fed *P. tricornutum.*

In summary, substitution of either fish oil or booster oils in microdiets for seabream by homogenized *C. cohnii* promotes good growth and survival, particularly in very young larvae, whereas substitution by *Phaeodactilum tricornutum* at this larval stage damages intestine epithelia and reduces survival. Finally, substitution by *Schyzotrichium sp*. slightly reduces larval seabream growth. These studies showed that it is possible to substitute fish oil by homogenized microorganisms, however their production costs are still very high and they should be reduced in order to be economically feasible.

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Table 1

Averages of Δ6 desaturase-like gene expression per diet in sea bream larvae.

Diet	Average	Std. error
ExtraLinseed Oil	0.401	2.278
Fish oil	1.171	2.278
Linseed oil	2.849	2.278
Soybean oil	7.013	2.278
Rapeseed oil	7.941	2.278

Figure 2. *Abdominal cavity and gut from larvae fed a C. cohnii containing diet.*

Figure 3*. Abdominal cavity and gut from larvae fed a diet without C. cohnii.*

Figure 4*. Area of the gut larvae occupied by digesta.*

Figure 5. *Effect of dietary substitution of booster or fish oil by C. cohni in growth of larval seabream.*