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Influence of dietary phospholipids on early ontogenesis of fish

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Abstract:

The aim of this paper is to provide explanations of how dietary phospholipid (PL) globally improves fish larval development, including growth and survival, digestive functions and skeletal development, and to propose optimal PL levels and sources in fish larval diets. Dietary incorporation of 8–12% PL related to dry matter (d.m.) promotes growth and enhanced survival in various species. Marine source PL, incorporating highly unsaturated fatty acids, was most efficient than soybean lecithin. This beneficial effect was explained by an enhancement in digestive functions, assessed by digestive enzyme activities and histomorphology. Nevertheless, 1.5–2.5% highly unsaturated fatty acids related to diet d.m. supplied by PL improved growth, survival and skeletal development, while 5% induced different skeletal deformities. The high incidence of deformities was associated with the down-regulation of genes involved in development, such as RXRα, RARα, RARβ and BMP-4, observed in the early stages in larvae fed a high highly unsaturated fatty acids level.

Keywords: phospholipid • fish larvae • digestive functions • skeletal development • gene regulation

1. Introduction

The essentiality of phospholipid (PL) in fish larvae nutrition has been demonstrated since several decades: indeed, from the early 1980, Kanazawa, Teshima, Inamori, Iwhashita & Nagao A. (1981) and Kanazawa, Teshima, Kobayashi, Takae, Iwhashita & Uehara (1983) demonstrated that PL significantly improved growth and survival in ayu (*Plecoglossus altivelis*). It was found that dietary PL globally improved fish larvae development. Studies conducted on a large number of species, marine as well as fresh water, confirmed an essential dietary requirement for PL. Optimal level of dietary PL incorporation was found to depend on the species, the PL source and classes, and the criteria used to evaluate this effect: growth, survival, stress resistance, malformations in larvae. Tocher, Bendiksen, Campbell & Bell in a recent review (2008) reported the dietary requirements of different species both at larval and juvenile stages. These authors evidenced a trend suggesting that quantitative PL requirements decrease from larval stages to small juveniles. No requirements were observed in fish greater than 5 g. Coutteau et al. (1995) suggested that fish larvae may have a limiting capacity in PL biosynthesis, insufficient to sustain the high cell development occurring during larval growth. Sargent, Tocher & Bell (2002) assumed that fish larvae did not develop PL synthesis capacities as they are largely provided in their natural environment in dietary PL. Nevertheless, the biochemical limiting step(s) for PL synthesis in larvae is still unknown as metabolic pathways for de novo biosynthesis of PL in fish were found to be globally the same as those of mammals (Sargent *et al.,* 2002).

However, both optimal PL level and composition, as well as the role of dietary PL remain unclear for most of the species. Sargent, Mc Evoy, Estevez, Bell, Bell, Henderson & Tocher (1999) considered that the egg lipid composition give a valuable approximation of the optimal lipid composition of a larval diet. Therefore, the ideal diet for fish larvae should have a lipid composition close to that of the fish egg. Fish eggs, mainly those without oil globule such as cod *Gadus morhua* (Finn, Hendersen & Fyhn 1995) and halibut *Hippoglossus hippoglossus* (Rønnestad, Finn, Lein & Lie 1995) have a high PL level at hatching, mainly phosphatidylcholine (PC). During embryogenesis and yolk-sac stages, phospholipid, mainly PC, contributed up to 60% to the catabolic components. Fatty acids supplied by PC are catabolised non selectively, in proportion to their presence in the egg, almost half of these fatty acids being polyunsaturated (Finn et al 1995). Other species such as red drum *Scianops ocellatus* (Vetter, Hodson & Arnold 1983) or common dentex *Dentex dentex* (Mourente, Rodrigez, Grau & Pastor 1999), spawning eggs with a lipid globule, contain more triacylglycerol and use them primarily as well as steryl and wax esters. Nevertheless, Hilton, Poortenaar & Sewell (2008) showed recently that eggs of yellowtail kingfish (*Seriola lalandi*) that contain a lipid globule, exhibited also a high PL level. So, PL is a major component of developing eggs, by providing energy and as component of the future cell structures.

Different functions and roles of dietary PL for improving fish larval development have been suggested: they are major component of cell membranes, they enhance neutral lipid absorption and transport, but they also improve diet quality, mainly palatability, and supply essential components, such as choline and inositol (Tocher *et al.* 2008). Nevertheless, their function in larval development must also to be explained taking into account their composition, both in classes and fatty acids. Indeed, different PL sources have been used in feeding experiments in fish larvae, such as vegetable sources, soybean lecithin, supplying mainly linoleic acid, or marine PL supplying considerable amounts of highly unsaturated fatty acids (HUFA). It was difficult to attribute the observed effects to PL entity or to fatty acid composition. The aim of this paper is to propose further explanations of how dietary PL globally improves fish larval development, including growth and survival, digestive functions and skeletal development.

2. Effect of dietary phospholipid on fish larval growth and survival

Since the pioneer work of Kanazawa et al (1981), research on the effect of dietary PL on marine fish larval development was limited by practical constraints on the way of supplying dietary PL to larvae. Indeed, nutritional experiments were conducted using enriched live preys, which already contains their own PL. Nevertheless, in Atlantic halibut *Hippoglossus hippoglossus* fed live preys, the superior performance of copepod nauplii compared to artemia was attributed largely to their higher content in PL, around 50% of total lipid (Mc Evoy, Naess, Bell & lie 1998). Formulated diets supplied as microparticles only began to be efficient for sustaining marine fish larval growth around 10 years ago (Cahu, Zambonino Infante, Escaffre, Bergot & Kaushik 1998; Yufera, Fernandez Diaz & Pascual 1999; Cahu & Zambonino Infante, 2001; Koven, Kolkovski, Hadas, Gamisiz & Tandler 2001). From this date, studies for determining marine fish larval nutritional requirements led to more reliable results. Microparticulate diets were previously available for freshwater species, allowing valuable data to be collected.

2.1. Effect of total PL and PL classes

Geurden, Radunz-Neto & Bergot (1995) conducted experiments on carp *Cyprinus carpio*, using casein-based isolipidic semi-purified diets. A dietary addition of 2% PL related to diet dry matter (d.m.) induced a significant survival improvement: after 25 days of dietary treatment, around 90% of larvae were alive in the groups fed diet incorporating 2% PL, when half of the larvae died in the groups fed diet devoid in PL. Growth was also largely improved: final weight was 2 to 3 times higher in the larvae receiving PL. A further PL dietary incorporation, 4%, did not induce additional improvement. Further experiments conducted with casein-based diets enriched with different PL classes suggested that purified soybean PC has a growth promoting effect for early stages larvae, but would induce mortality. Conversely, soybean phosphatidylinositol (PI) incorporation supported high survival (Geurden, Marion, Charlon, Coutteau & Bergot 1998). Kanazawa et al. (1993) obtained a growth improvement with PC, but not with PI or phosphatidylethanolamine (PE) in Japanese flounder larvae, while PI was more efficient than PC in improving growth of ayu *Plecoglossus altivelis* larvae. The role of the different PL fractions in larval development is not clear and molecular mechanisms underlying these observations have not yet been elucidated. PC and PI supply fish larvae with choline and inositol, respectively, which are considered as vitamins for fish (Halver 2002). But these molecules are not limiting in fish diets and the experiments of Geurden *et al* (1995) suggested that the effect of dietary PI and PC cannot be explained by the supply of inositol and choline. Recently, a strong positive correlation between larval growth and dietary PL content was established from an experiment conducted in pikeperch *Sander lucioperca* fed with 1.4, 5 and 9% PL from first feeding (Hamza, Mhetli, Ben Khemis, Cahu & Kestemont 2008). The increase in dietary PL from 1.4 to 9.5% led to a 50% enhancement in larval weight at 34 day post-hatching (dph), i.e. 240 mg versus 160 mg, the intermediate PL level leading to a final weight of 190 mg. In this case, survival was not affected, suggesting that PL is not essential for sustaining survival, or is necessary only at a low level in this freshwater species. Another hypothesis is that survival was reduced by cannibalism observed in this experiment from 22 dph, preventing the possibility to evidence a higher survival in the group fed high PL level.

PL incorporation in formulated diets fed from first feeding to European sea bass *Dicentrarchus labrax* larvae led to conclusive results. An experiment was conducted by incorporating increasing levels of soybean lecithin, including 60% PL, concurrently with a decreasing level of fish oil, mainly composed of neutral lipid (NL), in the same basal

compound diet. This resulted in 4 formulated diets containing 25% lipid, but different PL concentration: 3, 6, 9 and 12% of diet dry matter (d.m). Sea bass fed from mouth opening with these diets exhibited a final weight related to the PL concentration at 40 dph, from 2 mg for the group fed the lower PL level to 32 mg for the group fed the highest PL level. Survival rate was graded in the same order and went beyond 73% in the group fed 12% PL, when it reached only 22% in the group fed the lowest PL level (Cahu, Zambonino Infante & Barbosa 2003). Levels of PC and PI were 3.5% and 1.6% respectively in the diet inducing the best results. These values are close to the 2% PC incorporation that had a growth promoting effect in carp and the 2% PI which induces high survival in carp (Geurden *et al.* 1998).

PL dietary incorporation will lead to great progress in larval production in hatcheries, as efficient microparticulate diet profitably replace Artemia in larval feeding. A 9% PL concentration in diet highly improved survival in seabream *Sparus aurata* fed compound diet from first feeding, up to the average survival generally obtained by feeding live prey in the hatchery. In this case, no effect was observed on growth but abnormal livers and calculi in urinary bladder were observed, suggesting that other nutritional requirements were not satisfied with this microdiet (Seiliez, Bruant, Zambonino Infante, Kaushik & Bergot 2006). Two recent studies reported significant expected improvements in the larval rearing of very interesting new species for aquaculture, as a result of dietary PL incorporation. Growth and survival of cobia *Rachycentron canadum* larvae were significantly improved by dietary PL incorporation. This improvement was associated with an increase in high density lipoprotein and lower level of very low density lipoproteins (Niu, Liu, Tian, Mai, Yang, Ye, Zhu 2008). A study conducted on Pacific bluefin tuna *Thunnus orientalis* led to very promising results for this species in which larval rearing constitutes a real bottleneck to aquaculture development (Seoka, Kurata, Tamagawa, Biswas, Biswas, Yong, Kim, Ji, Takii & Kumai 2008). Indeed, live prey feeding, even with enriched Artemia, led to stunted larval growth in bluefin tuna (Sekoa, Kurata, Hatanaka, Biswas, Ji & Kumai 2007). Feeding with microparticulate diets including 12% PL extracted from salmon roe and containing mainly PC led to enhanced growth and survival compared to larvae fed enriched Artemia. Role of PC in transporting dietary free fatty acids to the tissues was reported in seabream larvae, and can partially explain the positive effect of dietary PC on fish larval growth (Hadas, Koven, Sklan & Tandler 2003).

2.2. Effect of phospholipid source, i.e. HUFA composition

Soybean lecithin is often used as PL source added in experimental compound diets, as it is easy to obtain and presents a reliable composition. Soybean lecithin contains around 62% total PL (including 45%PC, 20%PE, 16% PI), 5% triacylglycerol and 15% cholesterol. Contrary to fish oil, it does not contain HUFA, namely EPA (eicosapentaenoic acid, C20: 5n-3) and DHA (docosahexaenoic acid, C22: 6n-3). In experiments conducted with isolipidic diets, a replacement of fish oil by soybean lecithin induced a change in diet fatty acid composition, and mainly EPA and DHA decreased when soybean lecithin level increased in the diet. In this case, growth and survival in sea bass and pikeperch were positively related to dietary PL concentration but negatively related to EPA + DHA (Cahu *et al.,* 2003, Hamza *et al.,* 2008). Best growth and survival were observed in the group receiving only 1.5% EPA and DHA, while those receiving 5% exhibited the poorest performances. It was not possible to conclude whether the effect may be attributed mainly to dietary HUFA or PL concentration. Moreover, a large part of the HUFA fraction was supplied by NL, when several data suggested that HUFA supplied as pre-formed PL are more efficient for sustaining growth and survival (Salhi, Izquierdo, Hernandez-Cruz, Bessonart & Fernandez-Palacios 1999; Bell, Mc Evoy, Estevez, Shields & Sargent 2003; Gisbert, Ortiz-Delgado & Sarasquete 2005). Further results were obtained by feeding sea bass from first feeding exclusively with compound diet

incorporating 1.1 and 2.5% EPA+DHA related to diet d.m., and supplied either by PL or by NL. Marine lecithin extracted from fish roe was used as PL sources with high HUFA level. All the larvae fed diet including 2.5% EPA+DHA supplied as NL died before day 37 post-hatching, growth was poor in larvae fed diet including 1.1% EPA+DHA, and significantly higher in the groups receiving HUFA in PL. The best growth was observed in the group receiving 2.5% EPA+DHA supplied as PL (Villeneuve, Gisbert, Zambonino Infante, Quazuguel & Cahu 2005a). Moreover, the same authors showed that a higher EPA+DHA dietary incorporation as PL, 5% related to diet d.m., did not further improve growth.

3. Effect of dietary PL on digestive tract development

3.1. Effect on digestive functions

In sea bass as in pikeperch (Cahu *et al.* 2003, Hamza *et al.* 2008), an earlier maturation of digestive functions was associated with high dietary PL content. Indeed, in these two species fed high PL levels, the early decrease in amylase activity suggested an efficient maturation of pancreas, whereas the increase in alkaline phosphatase (AP) and aminopeptidase N showed the maturation of intestine. Concurrently, the increase in the ratio AP/ leu-ala peptidase observed in pikeperch, associated to increasing level dietary PL, suggested an earlier shift from a larval mode to an adult mode of digestion. Indeed, proper maturation of digestive tract, assessed by the ratio of the activities of a brush border membrane enzyme such as AP and a cytosolic enzyme such as leu-ala peptidase, has been associated with good growth and survival (Zambonino Infante & Cahu, 2001).
In addition, the maturation of digestive tract, assessed by the activity of intestinal enzymes, occurred earlier in sea bass fed PL including 2.5 EPA+DHA supplied as PL compared to the same amount supplied as NL, corroborating a proper larval development (Gisbert *et al*., 2005). The same diet given to Atlantic cod, *Gadus morhua*, from 17 dph resulted in similar conclusions: EPA+DHA supplied as PL resulted in a higher weight at 45dph, associated with an earlier maturation of intestine (Wold, Hoehne-Reitan, Cahu, Zambonino Infante, Rainuzzo & Kjørsvik 2007).

When the effect of dietary PL clearly appeared on fish larval growth, the effect of NL is not very clear. Several authors suggested that high dietary NL levels depress growth (Brinkmeyer & Holt; 1995, Gawlicka, Herold, Barrows, de la Noue & Hung 2002; Morais, Conceiçao, Rønnestad, Koven, Cahu, Zambonino-Infante & Dinis, 2007). The two main digestive enzymes involved in lipid digestion, lipase and phospholipase A2 (PLA2), exhibit different patterns during larval development and seem to be differently regulated. Lipase is expressed very early during development: bile salt-dependant lipase was assayed as early as first feeding in gilthead seabream (Izquierdo, Socorro, Arantzamendi & Hernadez-Cruz 2000) and from hatching in turbot *Scophthalums maximus* (Hoehne–Reitan, Kjørsvik & Gjellesvik 2001a). It was correlated to the ingestion rate in early stages, but not to lipid content of live prey fed to turbot (Hoehne–Reitan, Kjørsvik & Reitan 2001b). In 40 dph sea bass larvae, lipase expression was affected by NL concentration in diet, but the response was not linear. Lipase activity was the same in larvae fed 13 and 17% neutral lipid (mainly triacylglycerol) and but was 4 times higher in the groups fed either 20 or 23% NL (Cahu *et al*., 2003). The uncoordinated response between mRNA coding for lipase and the enzyme activity suggested a post-transcriptional hormonal regulation, possibly by secretin as was shown in mammals (Scheele, 1994). In the same way, lipase activity in sea bass was not affected by diets containing 7.5 and 15% NL, but was affected by the source of NL, i.e. their fatty acid composition (Morais, Cahu, Zambonino Infante, Robin, Rønnestad, Dinis & Conceição 2004). Chain length and degree of unsaturation were expected to affect lipase

activity in sea bass larvae, as fish lipases have a preference for HUFA as substrate (Gjellesvik, 1991). In contrast, assays conducted in gilthead seabream (Izquierdo & Henderson 1998) and in seabass (Zambonino Infante & Cahu 2001) failed to demonstrate PLA2 activity in fish larvae at first feeding, maybe due to the sensitivity of the method. But PLA2 activity, as well as mRNA coding for this enzyme, appeared to be accurately correlated with the dietary PL content in 40 dph seabass fed increasing PL levels from first feeding (Cahu *et al.* 2003). In the same way, a 3-point increase, 3% to 6%, in dietary PL induced a large increase in PLA2 activity in 24 dph red drum *Scianops ocellatus* (Buchet, Zambonino Infante & Cahu 2000). This accurate regulation of PLA2 in fish larvae may largely explain the more efficient capacity the larvae have to utilize dietary PL than NL and the PL effect on growth and survival.

3.2. Effects of phospholipids in the histomorphological organization of the digestive system

In vertebrates, different parts of the digestive tract have been shown to employ different cellular mechanisms in response to diet quantity and quality. Consequently, the use of the intestine and digestive accessory glands as target organs for evaluating the nutritional and physiological status in fish is well known. The intestine is involved in important physiological digestive functions, being the primary site of food digestion and nutrient uptake, while the liver is the central metabolic organ of the body with a predominant role in intermediary metabolism, and important functions in lipid storage, digestive and detoxification processes. Thus, the optimum utilization of dietary nutrients ultimately depends on the effectiveness of functions in the intestine and liver and, consequently, the structural alteration of the histomorphological organization of these organs can provide useful information about the quality of the diet, the metabolism, and the nutritional status of the fish (Gisbert, Ortiz-Delgado & Sarasquete 2008). In this sense, several studies have evaluated the effects of diets containing different lipid classes (NL and PL) and sources (vegetal and marine) in larval fish performance by means of histological and stereological techniques to evaluate their effect on the digestive tract organization and estimate the accumulation of lipids in the liver and intestine (Diaz, Guyot, Vigier, & Connes 1997; Fontagné, Geurden, Escaffre & Bergot 1998; Fontagné, Burtaire L., Corraze G. & Bergot 2000; MacQueen-Leifson, Homme, Lie, Myklebust & Strøm 2003a; MacQueen Leifson, Homme, Østensen, Lie, & Myklebust 2003b; Gisbert *et al*., 2005; Morais, Koven, Rønnestad, Dinis & Conceição 2005; Morais, Caballero, Conceição, Izquierdo & Dinis 2006; Wold, Hoehne-Reitan, Cahu, Zambonino-Infante, Rainuzzo & Kjørsvik 2008, among others).

Dietary PL has been described to exert a marked effect on lipid absorption and transport (Fontagné *et al*., 2000; Izquierdo *et al*., 2000; Morais *et al*., 2006) and accumulation of lipid droplets in the enterocytes of fish larvae (Diaz *et al*., 1997; Fontagné *et al*., 1998; MacQueen-Leifson *et al*., 2003; Gisbert *et al*., 2005). In particular, the size and ultrastructural characteristics of lipid inclusions are valuable biomarkers to evaluate lipid absorption and metabolism in nutritional studies. According to their size, three types of inclusions can be distinguished in fish enterocytes: particles (20-70 nm in diameter) resembling mammalian VLDL; lipoprotein particles (70-500 nm in diameter) considered as chylomicrons; and large inclusions of triacylglycerols measuring up to 6µm and described as lipid droplets (Diaz *et al*., 1997). Changes in the size, type and location of lipid inclusions may be dietary-dependent. Thus, the size of lipoprotein particles increases with the fat content of feed and the degree of unsaturation of the lipids ingested. Feeding larvae with phospholipid-poor diets and/or triacylglycerol rich diets commonly results in the accumulation of lipid vacuoles in the basal zone of the enterocytes, which indicates good digestion and absorption of dietary fat but a reduced transport capacity (Fontagné *et al*., 1998, 2000; Salhi *et al*., 1999; Izquierdo *et*

al., 2000; Lu, Zhao, Zhao & He 2008). This accumulation has led to the suggestion that intestinal lipid inclusions are a temporary storage form of re-esterified fatty acids when the rate of lipid absorption exceeds the rate of lipoprotein synthesis, or because of an inability to metabolize lipids (Kjørsvik, van der Meeren, Kryvi, Arnfinnson & Kvenseth 1991). Lipid accumulation in the enterocyte intercellular spaces as reported in gilthead sea bream (Salhi *et al*., 1999) and European sea bass (Gisbert *et al*., 2005) is also indicative of dietary-induced problems in lipid transport from the intestinal mucosa into the body circulation.

Under normal conditions, the rapid development of the intestinal enterocytes during larval growth is combined with increasingly effective lipoprotein synthesis. This is accompanied by a considerable decrease in the number of large lipid vacuoles in the enterocytes, as well as by an important increase in the number of small lipid particles in the intercellular spaces (Deplano, Díaz, Connes, Kentouri-Dinavach & Cavalier 1991; Sarasquete, Polo & Yúfera 1995). However, unbalanced diets may further affect this process. Therefore, the excessive abundance of lipid inclusions of varying size in the enterocytes could be the result of a failure in the lipoprotein synthesis mechanism, as the endogenous synthesis of PL may be insufficient to maintain an optimal rate of lipoprotein production (Izquierdo *et al*., 2000). The formation of large lipoproteins and lipid droplets in enterocytes is closely related to an excess of fats in immature enterocytes caused by the high fatty acid contents of live preys and compound diets (Deplano *et al*., 1991). In some cases, large accumulation of lipids in the enterocytes, the so-called intestinal steatosis, may cause some pathological damage since large lipid inclusions produce epithelial abrasion, cellular necrosis, and/or inflammatory reactions along the intestinal mucosa (Deplano, Connes, Díaz & Paris 1989).

In teleost fish, it is generally accepted that the anterior intestine is the major site for lipid absorption. However, it has been recently reported in European sea bass larvae that differences in lipid absorption and accumulation in the anterior and posterior intestinal mucosa are also influenced by dietary lipid classes, their levels, and the n-6/n-3 ratio (Gisbert *et al*., 2005). Larvae fed high levels of neutral lipids (11%) showed important intracellular and intercellular accumulation of fat in the anterior intestine, while the anterior intestinal mucosa of fish fed low and moderate PL levels (13-11%) and neutral lipids (3-6%) had a normal appearance and organization. Similarly, lipid deposition in the posterior intestine depended on the dietary lipid class, since larvae fed different PL levels showed large lipid deposits whereas fish fed triacylglycerol had a lower lipid accumulation in this region. This result probably revealed a specialization of the posterior intestine in the absorption and transport of PL (Gisbert *et al*., 2005). However, in Atlantic cod, diets containing between 12.5 and 10.7% PL and differing in the amount of HUFAs incorporated in the PL or in the neutral lipid of the fraction did not have any effect on lipid absorption, transport and accumulation of lipid droplets in larval enterocytes (Wold *et al*., 2008). The former authors concluded that the observed low lipid accumulation in enterocytes of all dietary treatments was attributed to a sufficient amount of PL in the diets.

MacQueen-Leifson et al. (2003a) evaluated the effects of microdiets containing soya PL, marine PL or triacylglycerol on the ultrastructure of enterocytes in turbot larvae, and reported that enterocytes from larvae fed soya PL showed swollen and translucent mitochondria, whereas the appearance of this cellular organelle was completely normal in fish fed diets containing marine PL or triacylglycerol. The former authors concluded that such alterations in the normal morphology and appearance of mitochondria were due to an increase in membrane permeability and/or fluidity caused by the inclusion of high levels of linoleic acid provided by the soya PL diet.

The liver is a metabolically sensitive organ that may be examined to determine potential side effects of lipidic metabolism because the hepatic energy storage responds sensitively and rapidly to nutritional changes in fish larvae (Hoehne-Reitan & Kjørsvik, 2004). In well-fed larvae, glycogen and lipids tend to accumulate in the liver to varying degrees. The liver volume generally increases, and the Golgi apparatus and rough endoplasmic reticulum tend to increase as the larvae develop. In contrast, in larvae fed unbalanced diets, these cellular organelles are generally underdeveloped. Microscopically, the structural modifications of the hepatocytes might be useful biomarkers reflecting a nutritional pathology (see review in Gisbert *et al.*, 2008). Lipid accumulation in the liver has been interpreted as a disturbance in the hepatocellular lipid transfer and metabolism (Segner & Witt, 1990). It has been described in larvae of gilthead sea bream (Salhi *et al*., 1999), European sea bass (Gisbert *et al*., 2005), Atlantic cod (Wold *et al*., 2008) and yellow catfish (Lu *et al*., 2008) that the use of dietary PL results in a decrease in the degree of lipid accumulation in the hepatocytes. This is explained by a beneficial effect of polar lipids on lipid transport from the liver to extrahepatic tissues, probably by enhancing lipoprotein synthesis (Sheridan, 1988). In contrast, diets rich in NL produced a high cytoplasmatic lipid accumulation in the liver, resulting in a displacement of the nucleus and/or modifications of the nuclei shape and size, and chromatin density (Gisbert *et al*., 2008).

3.3. Effect of dietary phospholipid on fish skeletal development

Since 1981, an effect of PL on fish larval skeletal development has been suggested. Dietary incorporation of chicken egg lecithin led to reduced incidence of malformations, especially twisted jaw and scoliosis, in ayu larvae (Kanazawa *et al*., 1981). By feeding carp larvae with different PL classes, Geurden *et al* (1998) concluded that particularly PI prevents skeletal deformities. Only 2% of the carp larvae fed a diet incorporating 1.3% PI related to d.m. exhibited skeletal deformities while this value reached 32% in larvae fed 1.3% PC. In Cahu et al. (2003) experiment, skeletal deformity incidence was the lowest in sea bass larvae fed 12% PL, corresponding to 1.6% PI. Only 2% of the larvae exhibited one or more skeletal deformities, whereas one third of larvae in the group fed 3% PL, including 0.2% PI, exhibited deformities. The effect of PI on larval development is not well understood, but its role as a precursor of second messenger regulating calcium entry into the cells has been put forward. PI is involved in a signaling system controlling biological processes in the early development of vertebrates (Berridge & Irvine, 1989). Moreover, arachidonic acid is an abundant fatty acid in the sn-2 position in PI, which is hydrolysed and used for eicosanoid synthesis. Eicosanoids are implicated in large range of physiological functions in fish, including cardiovascular and neural functions and immune responses (Tocher, 2003). Some of these functions may have been improved in larvae fed diets including PI.

A recent experiment, conducted by feeding cod larvae on compound diets including EPA+DHA supplied either as PL or NL, gave new data on the effect of PL on fish skeletal development (Kjørsvik et al., unpublished). Ossification of the vertebral column in cod, first observed at 21dph in neural arches, occurred significantly earlier in groups fed diets containing PL, when measured both by larval size and larval age. At 31 dph, larvae fed PL exhibited 15 ossified neural arches and 13 ossified vertebrae when only 6 neural arches and 4 vertebrae were ossified in larvae fed NL. Development of fin rays was affected by the level of EPA+DHA supplied as PL in diet. Larvae receiving 3% EPA+DHA as PL had a higher number of fin rays than larvae fed lower EPA+DHA level supplied either by PL or NL.

HUFA supplied as dietary PL has also been evidenced as having a large effect on skeletal development in sea bass; they can either have a beneficial effect or be a potent teratogenic

agent, depending on concentration (Villeneuve *et al*., 2005a). An experiment was conducted by feeding sea bass larvae from mouth opening with diets incorporating 13% PL, with either 1.1, 2.5 or 5% EPA+DHA. The different skeletal malformations were assessed at the end of the experiment as described in Villeneuve, Gisbert, Le Delliou, Cahu & Zambonino Infante (2005b): malformation in head, neurocranium and splanchnocranium resulting in mouth prognosis; partially undeveloped operculum, and malformations in vertebral column, such as lordosis, scoliosis and kyphosis. Larvae fed low HUFA level in PL exhibited very few abnormalities: less than 7% of the larvae exhibited deformities, which is lower than the deformity incidence usually observed in hatchery, and 13% of the larvae fed 2.5% HUFA in PL exhibited deformities. Incidence of deformities was very high in the group fed the highest HUFA level: almost half of the larvae exhibited one or several skeletal deformities. In the three groups, the most affected skeletal part was the vertebral column: around 80% of the deformities consisted in lordosis, scoliosis and kyphosis.

Explanations of the effect of EPA+DHA supplied as PL on larvae skeletal development have been searched in the regulation of some genes involved in early development of vertebrates, and of course, affected by HUFA. Highly unsaturated fatty acids, mainly DHA, are natural ligands for Peroxisome Proliferator Activated Receptors (PPARs), which dimerize with Retinoid X Receptor (RXR) to modulate the expression of very numerous genes involved in cellular growth and differentiation such as Insulin Growth Factor (IGF), in morphogenesis such as Hox, in osteoblast differentiation such as Bone Morphogenetic Protein (BMP-4). A down regulation of RXRα, RARα, RARβ and BMP-4 was noted in the group fed 5% EPA+DHA supplied as PL and inducing high incidence of skeletal deformities (Villeneuve et al., 2005a). It was suggested that the high DHA+EPA level induced a high PPAR transcription, leading to an excess of PPAR/RXR heterodimers, resulting in a negative feedback in RXR . As a consequence, less RXR is available for heterodimerization with other nuclear receptors, in particular RARα in the retinoid pathway. This would lead to a repression of genes such as IGF, involved in cell differentiation and regulated by RARα (Fu, Noguchi & Kato 2001; Balmer & Blomhoff, 2002). This down regulation of IGF-1 would explain in part the high teratogenic effect of elevated EPA+DHA level in PL.

Moreover, it appeared that larvae are particularly sensitive to this teratogenic effect during a very short window of time. When the diet including high EPA+DHA in PL was fed to larvae during 5 days in later development, from 13 to 18 dph or 18 to 23 dph, no negative effect was revealed. But feeding larvae with this diet at first feeding, from 8 to 13 dph, resulted in the same abnormality incidence as in the larvae that have received this diet over all their development, until 40 dph.

Besides deformities, it appeared that the diet with high HUFA level in PL induced the development of supernumerary vertebra. The average number of vertebra is 25 in sea bass, but 13% of larvae in the group receiving high HUFA in PL exhibited 26 vertebra (Villeneuve Gisbert, Moriceau, Cahu & Zambonino Infante 2006). We can point out that this was the first time that an effect of diet on vertebra number in fish was reported; the same study evidenced also an effect of dietary vitamin A on vertebra number. Until now, plasticity in the number of vertebra has been associated with hatching temperature in halibut (Lewis, Lall & Eckard Witten 2004) or triploidy in trout (Kacem, Meunier, Aubin & Haffray 2004). Some genes, such as Hox, involved in spatial organization of morphogenesis, are known to be expressed during a specific window of time. The expression of some genes was assessed in the larvae fed excess of HUFA at first feeding, and exhibiting an extra vertebra, and compared to the gene expression in the group fed a convenient HUFA level. The authors concluded that a down regulation of RARα gene expression associated to an up-regulation of BMP gene in larvae

fed excess of HUFA during 5 days at first feeding may have induced an increase in pre-adipocyte differentiation into osteoblast, leading to a supernumerary vertebra.

4. Conclusion

An integrative effect of dietary phospholipids on fish growth and survival has been reported for a long time. This effect begins to be better explained, thanks to observations made on digestive functions, metabolism and skeletal development. Recent data shows that an extensive investigation on genes regulated by nutrients, carried out with micro-arrays including a large number of genes, brought crucial information. In particular, two different sets of genes are differentially expressed depending on the period of the larval development and, interestingly, the genes involved in organogenesis, digestion and energy metabolisms are up-expressed before day 23 post-hatching (Darias, Zambonino Infante, Hugot, Cahu & Mazurais 2008). The alterations in the gene expression, induced by nutrients, will be very useful to understand the molecular pathways influenced by PL and HUFA, and affecting fish larvae development.

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