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Dietary Phospholipids are more Efficient than Neutral Lipids for Long Chain Polyunsaturated Fatty Acid Supply in European Sea Bass *Dicentrarchus labrax* Larval Development

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Abstract: We evaluated the effects of dietary lipid class (phospholipid vs. neutral lipid) and level of n-3 long-chain PUFA (LC-PUFA) on the growth, digestive enzymatic activity, and histological organization of the intestine and liver in European sea bass larvae. Fish were fed from the onset of exogenous feeding at 7 to 37 d post-hatch with five isoproteic and isolipidic compound diets with different levels of EPA and DHA. Diet names indicated the percentage of EPA and DHA contained in the phospholipids (PL) and neutral lipids (NL), as follows: PL5, PL3, PL1, NL1, and NL3. Histological observations showed different patterns of lipid absorption and accumulation in the intestinal mucosa depending on the level and nature of the dietary lipid fraction. Fish fed high levels of neutral lipids (11%, NL3 diet: 2.6% of EPA + DHA in the NL fraction) showed large intracellular and intercellular lipid deposits in the anterior intestine, but no such lipid accumulation was detected when larvae were fed with low and moderate levels of EPA and DHA in the phospholipid and neutral lipid fractions of the diet (PL and NL1 diets). PL were preferentially absorbed in the postvalvular intestine, and the accumulation of marine PL was inversely correlated to their dietary level. The postvalvular intestinal mucosa and liver showed signs of steatosis; large lipid vacuoles were observed in this region of the intestine and in the liver and were inversely correlated with the level of dietary neutral lipids. The best results in terms of growth, survival, and development (maturation of the digestive system and histological organization of the liver and intestinal mucosa) were obtained in the group fed with 2.3% of EPA and DHA in the PL fraction of the diet (PL3 diet), revealing that European sea bass larvae use the LC-PUFA contained in the PL fraction more efficiently than those from the NL fraction of the diet.

INTRODUCTION

Dietary lipids are the main energy source in developing larvae and a source of fatty acids needed for the synthesis of new cellular structures, which are required for normal larval growth and development (1). Lipid requirements of marine fish larvae have been extensively studied during the last two decades (1-3) and particular attention has been paid to long chain polyunsaturated fatty acids (LC-PUFA) and phospholipids (PL) (4-6, 7). Like most marine fish, European sea bass is considered to have an absolute requirement for LC-PUFA, such as eicosapentaenoic acid (EPA, 20:5*n*-3), docosahexaenoic acid (DHA, 22:6*n*-3) and arachidonic acid (ARA, 20:4*n*-6), being unable to produce these fatty acids from their precursors 18:3*n*-3 and 18:2*n*-6 (1). In this sense, the deficiency in LC-PUFA delays fish growth, induces mortality, reduces resistance to stress and results in anatomical alterations associated with nutritional disorders (6). The essential fatty acid requirements of marine fish vary both qualitatively and quantitatively and the optimal level of dietary components for marine fish is known to be around 3% of dry matter for EPA+DHA (1). However, most studies dealing with the determination of LC-PUFA requirements for first feeding marine larvae were conducted using live preys to deliver different levels of LC-PUFA (3), but these studies did not evaluate the effect of the class of lipids (neutral vs. phospholipids) incorporated into the diet, since in most cases lipids and LC-PUFA are generally provided in the diet by fish oils, which are mainly constituted by neutral lipids (6, 7).

Although it is generally accepted that there are beneficial effects in adding phospholipids to microdiets or live-prey enrichment emulsions on the larval growth, survival, stress tolerance and larval quality (reduction of skeletal deformities) (2-4, 6-8), their exact role in fish digestive physiology and development has not been completely characterized. Fish larvae are extremely sensitive to dietary phospholipid (PL) deficiency and require higher levels of dietary PL than juveniles (see review in 8). Feeding gilthead sea bream larvae with microdiets containing marine PL instead of marine triglycerides (TAG) resulted in better growth even if larvae were fed PL at lower levels than TAG (10). Recently, it has been shown that a diet containing 19% lipids including almost 9% phospholipids induced a good growth in European sea bass first feeding larvae, demonstrating that the development of skeletal deformities in European sea bass during early development was more linked to the proportion of dietary PL: neutral lipids (NL) than to the total dietary lipid content (7). Dietary PL seem to have a marked effect on lipid absorption and transport (6, 8, 11, 12). An accumulation of lipid droplets in enterocytes has been associated with PL deficiency (13-15) that might result in intestinal steatosis (16, 17), suggesting that PL have a specific role in the synthesis and secretion of chylomicrons and very low density lipoproteins from the intestinal mucosa into the circulatory system.

Different segments of the gastrointestinal tract in vertebrates have been shown to employ different cellular mechanisms in response to diet quality (18). Thus, the use of the intestine and liver as indicator organs of the nutritional and physiological status in fish is well-known (19- 22). The intestine is involved in important physiological digestive functions, being the primary site of food digestion and nutrient uptake (23), while the liver is the central metabolic organ of the body with a predominant role in intermediary metabolism, and important functions in lipid storage, and digestive and detoxification processes (23). The optimum utilization of dietary nutrients ultimately depends on the effectiveness of functions in the intestine and liver (22) and consequently, the structural alteration of the histomorphological organization of the liver and intestine can provide useful information about the quality of the diet, the metabolism and the nutritional status of the fish (18, 20, 21, 24, 25).

The present study evaluates the effect of the lipid class (PL vs. NL) and the level of *n*-3 LC-PUFA in the diet on the growth, the digestive enzymatic activities and the histological organization of the intestine and liver in European sea bass larvae.

MATERIALS AND METHODS

Animals and diets. Three-day old European sea bass (*Dicentrarchus labrax* L.) larvae were obtained from the *Ecloserie Marine de Gravelines* (Gravelines, France) and shipped to the *Fish Nutrition Laboratory* at the *Ifremer* (Centre de Brest). Fish were acclimated and divided into fifteen 35 L-cylindroconical fibber glass tanks (2100 larvae/tank) at a initial density of 60 larvae/L. Tanks were supplied with running sea water (35‰), which had been previously filtered

through a sand filter, then passed successively through a tungsten heater and a degassing column packed with plastic rings. Throughout the experiment, the water temperature and salinity were 20°C and 35‰, respectively, and the oxygen level was maintained above 6 mg/L by setting the water exchange of the tank up to 30% per hour (flow rate: 0.18 L/min). Photoperiod was 24L:0D and light intensity was 9 W/m^2 (900 lux) maximum at the water surface. All animal procedures and handling were conducted in compliance with the Guide for the Care and Use of Laboratory Animal (26).

At 4 days post hatch (dph), European sea bass larvae were divided into 5 experimental groups (4 replicates per group) that were fed from the onset of exogenous feeding at 7 dph with experimental compound microdiets until 37 dph. Five isonitrogenous and isolipidic diets (Table 1) were formulated to incorporate different levels of LC-PUFA, which differed in their lipid class composition, PL vs. NL. Fish meal, containing naturally 17% lipids, was previously defatted to control the experimental dietary lipid composition by only the addition of oils. Lipid was extracted from fish meal using dichloromethane (5 ml / g fish meal) for 20 min at 40°C, rinsed and dried until complete solvent evaporation. Diet names indicated the percentage of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) contained in the PL and NL fractions of the diet: PL5, PL3, PL1, NL1 and NL3 (Table 1-2). Marine phospholipid fraction in PL diets varied in inverse relation to soybean lecithin, whereas NL diets contained soybean lecithin in an inverse proportion to marine TAG (cod liver oil). The composition of diet PL1 was close to the diet previously used (7) that supported good growth and survival in European sea bass larvae, and for comparative purposes it was considered in this study as the control. Microdiets were processed as follows: fish meal, fish meal hydrolysate, betaine and mineral mixture were first mixed. The oil, phospholipids and the vitamin mixture were added as an emulsion to the dry mixture. Then water was added and the mixture was blended well mechanically, pelleted and dried at 50ºC for 20 min (6). This procedure prevents from undesirable reactions between ingredients (particularly between mineral and vitamins). The pellets were sieved to obtain two sizes of particles; 125-200µm used during the first five days and 200-400µm used during the rest of the experiment. At this stage, a sample of each experimental diet was taken for analysis. Diets were then vacuum-packed and stored at 4°C until use. During all the experimental period, larvae were continuously fed in excess 24 hours per day using an automatic belt feeder. Food ingestion was monitored by observing the larval digestive tract under a binocular microscope; dietary microparticles being visible by transparency.

Sampling. To evaluate growth, ten specimens were randomly sampled from each experimental tank (30 larvae per experimental diet) at 12, 16, 23, 30 and 37 dph, euthanized with an overdose of anesthetic (tricaine methanesulfonate, MS 222) and their wet body weight measured to the nearest 0.1 mg. To evaluate the level of maturation of the digestive system, larvae (20-50 larvae, depending on wet body weight) were sampled from each tank and kept at –20°C until analysis. Samples for measuring lipase (EC 3.1.1.3), and brush border intestinal enzymes (alkaline phosphatase, EC 3.1.3.1 and aminopeptidase N, EC 3.4.11.2) were taken at 30 and 37 dph, while trypsin (EC 3.4.21.4) and amylase (EC 3.2.1.1) secretions were only measured at 37 dph. For light microscopy purposes, ten specimens were randomly sampled from rearing tanks at 30 and 37 dph, preserved in 10% buffered formalin (pH 7.2) and stored in the dark at 6–8°C. Repetitive sampling did not allow to determine real survival (percentage of surviving individuals in relation to the initial number of larvae), however, relative survival, expressed as the percentage of surviving fish fed on a diet (i.e. NL1) in relation to the surviving specimens fed on the control diet (PL1), could be calculated.

Analytical methods. A minimum of thirty larvae per replicate and treatment were dissected on a glass cutting board kept on ice (0ºC) under a binocular microscope. Individuals were cut into 4 parts: head, pancreatic segment (PS), intestinal segment (IS) and tail, in order to limit the assay of enzymes to specific segments. This dissection inevitably produced a crude mixture of organs in each segment. The pancreatic segment, besides the pancreas, contained the liver, heart, muscle and spine. The intestinal segment contained the intestine, muscle and spine (27). Once dissected, pancreatic segments were homogenized in 5 volumes (v/w) of ice-cold distilled water. Amylase and trypsin activities were measured using Na-benzoyl-DL-arginine-p-

nitroaniline and starch as substrates (28, 29), respectively, in both pancreatic and intestinal segments. Lipase was measured in the pancreatic segment using p-nitrophenyl myristate as substrate (30). Purified brush border membranes from the intestinal segment were obtained according to a method developed for intestinal scrapping (31). The degree of purification of the brush border membrane, considering alkaline phosphatase and aminopeptidase N as markers of cell membrane fraction, was close to that reported in the literature (31) i.e. 11.0 and 10.8 fold, respectively. Alkaline phosphatase and aminopeptidase N, two enzymes of the intestinal brush border membrane, were quantified using p-nitrophenylphosphate (32) and L-leucine-p-nitroaniline (33) as substrates, respectively. Specific enzyme activities were expressed as µmol of substrate hydrolyzed per min and mg of protein (U/mg protein) and protein was determined using the Bradford method (34). Secretions of trypsin and amylase were calculated as the ratio of the activity in the intestinal segment, related to total activity (PS+IS) considering that enzyme activity in PS can be used as an index of the synthesis function of pancreas and in IS as an index of pancreatic secretory function (35).

Dietary total lipid content was determined using a slightly modified Folch's procedure (36), with chloroform being replaced by dichloromethane. The separation of NL and PL was carried out as Juaneda and Rocquelin (37). Fatty acids were saponified by a 2 M KOH-methanol solution and then esterified in a 0.7 M HCl-methanol solution. Fatty acid methyl esters (FAME) were separated by gas chromatography, in an Auto-system Perkin-Elmer with a flame ionization detector, BPX 70 capillary column (J&W Scientific, 25 m x 0.22 mm i.d. x 0.25 µm film thickness), split-splitless injector, with helium as carrier gas (flow rate 1.4 ml/min). The injector and detector temperatures were, respectively, 220 and 260°C. Initial temperature of the oven was 50°C, increasing to 180°C by increments of 15°C/min, maintained for 5 min, and finally increased to 220°C by increments of 3°C/min. Data acquisition and handling were carried out by connecting the gas chromatograph to a Nelson computer. Each FAME was identified by comparison with the retention time of authentic standard mixtures and the results of individual FA composition are expressed as percent of total identified FAME in the PL and NL fractions.

Light microscopy. Larvae were dehydrated in a graded series of aqueous ethanol, embedded in paraffin wax and cut in serial sagittal sections (5–7 µm thick). Slides were stained by haematoxylin and eosin for topographic observations of the liver, intestine (prevalvular and postvalvular regions) and swimming bladder. The term steatosis was applied, without pathological connotation, when clear vacuoles with diameter greater than 5 µm were observed in the enterocytes (13).

Statistical analyses. Results are given as mean $\pm$ SD. All data were checked for variance homogeneity using the Barlett's test (38). Growth, enzymatic specific activity and arcsin ($x^{1/2}$)-transformed trypsin and amylase secretions were compared by means of a one-way analysis of variance (ANOVA) followed by Newman Keuls multiple range test when significant differences were detected ($P < 0.05$).

RESULTS

Growth and survival. At the end of the rearing period (37 dph), statistically significant differences in larval growth were observed between experimental groups (ANOVA, $P < 0.05$, Fig. 1). Larvae fed PL3 exhibited the best growth performance. Larvae fed PL1 and PL5 were similar in weight, being 21% and 26% smaller than PL3 larvae respectively. Larvae from NL3 dietary group were smaller than all the other groups (50% smaller than PL3 larvae). As NL3 group died before 37 dph, the larval body weight could not be compared to those of the other experimental groups, although it was significantly lower at early stages of development (23 and 30 dph).

The best survival, i.e. the largest number of fish at the end of the experiment, was observed in groups fed PL3 and NL 1 diets (n = 710 and 404, respectively). Relative survival rate of groups PL3, NL1 and PL5 was 226.1, 128.7 and 31.5% higher respectively, than the control (PL1, n = 314).

Enzyme activities. At the end of the study, total EPA and DHA content in diets significantly affected the trypsin and amylase secretion (ANOVA, $P < 0.05$; Fig. 2). Fish fed diets containing low levels of EPA and DHA (PL1 and NL1) exhibited the highest trypsin secretion rates, while those reared with PL3 and PL5 showed lower secretion values. Amylase secretion was negatively affected by high dietary content of EPA and DHA (group PL5, Fig. 2), being 8.2% lower than in those groups fed low or medium levels of these two fatty acids (PL3, PL1 and NL1 diets). Similarly, specific activity of lipase was affected by the experimental diets (ANOVA, $P < 0.05$; Fig. 3). At 30 dph, lipase specific activity was two times higher in fish fed NL1 than in PL5 group. In 37 days-old larvae, the lipase activity in PL5 group was 55% and 45% higher than in fish fed PL3 and NL1, respectively.

The same effect was observed in the specific activities of brush border enzymes (ANOVA, $P < 0.05$; Fig. 4). At 30, the highest alkaline phosphatase specific activity was observed in fish fed PL3, while no differences were observed between the other experimental groups (PL1, PL5 and NL1), which were significantly lower than in fish fed PL3. At day 37, the lowest alkaline phosphatase activity was detected in larvae fed PL1, no differences being evidenced between the other dietary groups. No statistically significant differences were observed in aminopeptidase N specific activity of larvae fed different diets at 30 dph. At the end of the larval rearing period, aminopeptidase N specific activity was 30% lower in larvae fed PL1 (control group) compared to the rest of dietary treatments.

Light microscopy. The liver of European sea bass larvae is organized in polygonal-shape hepatocytes arranged along hepatic sinusoids. Nuclei position and lipid deposition depended on dietary levels of marine and vegetable phospholipids (Fig. 5). Large central nuclei were observed in livers containing few lipid inclusions (PL1, PL3, PL5 and NL1), while peripheral nuclei were detected in livers of larvae showing high levels of lipid deposition (NL3).

The mucosa of the intestine (prevalvular and postvalvular regions) is lined by a simple columnar epithelium with prominent acidophilic brush borders and centrally located nuclei (Figs. 6a-b, 7). The only morphological differences observed between both regions are the number and size of mucosal folds which are longer and more numerous in the anterior region. Changes in the histological organization of the prevalvular intestine were only observed in fish fed NL3 diet, which showed a large accumulation of small lipid droplets, resulting in the displacement of nuclei to a basal position (Fig. 6c). Large intercellular lipid vacuoles were also observed (Fig. 6d). The mucosa of the postvalvular intestine exhibited remarkable signs of steatosis in fish fed diets containing EPA and DHA mainly in their phospholipid fraction (PL diets) (Fig. 7a-d). Fish fed NL diets exhibited a lower lipid accumulation in this region than in the other experimental groups (Fig. 7e, f).

Fish fed on PL and NL1 diets had normally inflated swim bladder, which was organized in a stratified cuboidal epithelium and *rete mirabile* (Figure 6e). Most of the larvae (90%) fed NL3 diet did not inflate their swim bladder and showed hyperplasia of the gas gland cells, with hypertrophied cells occupying the swim bladder's lumen (Fig. 6f).

DISCUSSION

In a previous study, good growth and survival was obtained using a compound microdiet containing 11% phospholipids (7). We have used this PL dietary level in the current study to formulate experimental diets with an appropriate level of phospholipids (PL5, PL3, PL1 and NL1 diets). However, even if NL3 diet only contained 8% of phospholipids, this content was acceptable in terms of growth and larval survival rates (7). In the present study, diets were efficiently ingested by European sea bass larvae. Thus, the differences observed in growth and survival rates in this study were not related to dietary phospholipid level and/or larval food intake of compound diets.

In the present study, total DHA/EPA ratios in PL diets and in NL diets were close to 2, largely higher than 1, considered as a suboptimal ratio, either by not providing sufficient DHA or by providing an undesirable excess of EPA (1). Therefore, the observed differences in growth and survival of European sea bass in the present study could not be attributed to inappropriate DHA/EPA ratios. The tested diets, mainly differing by their LC-PUFA source included in the PL or NL fraction, induced large differences in growth and survival of European sea bass larvae. The diet containing a moderate level of EPA and DHA in the phospholipid

fraction (PL3) induced the best growth, survival and intestinal maturation in comparison to the rest of the experimental diets, including the control (PL1), while the diet with a similar level of EPA and DHA in the neutral lipid fraction (NL3) was lethal for European sea bass larvae. A high content of EPA and DHA in the PL fraction (PL5) negatively affected growth, survival and maturation of the digestive tract in larvae. The differences observed in growth between larvae feeding on a low dietary level of EPA and DHA depended on the form that both essential fatty acids were supplied; if they were included in the PL fraction (PL1) good growth was observed, whereas it was significantly lower when they were included in the NL fraction. The more efficient use of EPA and DHA supplied in the phospholipid class of the diet might be related to the ability of young larvae to better modulate phospholipase A2 expression than that of lipase, suggesting a more efficient capacity to utilize phospholipids than triglycerides (7).

PUFA, and especially DHA and EPA, which are vital constituents for cell membrane structure and function, are very susceptible to the oxidation by oxygen and other radicals. The resultant PUFA oxidation of membrane phospholipids can have dramatic consequences for cell membrane structure and fluidity, with a potential pathological effect on cells and tissues, that could result in a reduction on fish growth, loss of appetite, decreased feeding efficiency, and increased mortality (2). In the present study, differences in growth, survival and histological organization of the intestine and liver in the European sea bass larvae were not attributed to in vivo lipid peroxidation, since any of the histological lesions commonly associated to this phenomenon, such as myopathy of skeletal muscle, lipoid liver degeneration and accumulation of ceroid pigments (2), was not observed in the larvae fed high dietary levels of LC-PUFA. Moreover, diets were formulated using stable forms of vitamins, α-tocopherol acetate for vitamin E and ascorbate polyphosphate for vitamin C, at concentrations recommended by NRC (26), in order to prevent the oxidation of unsaturated fatty acids in the microparticles.

The level of secretion of the pancreatic enzymes (amylase, trypsin and lipase) has been currently used as an indicator of intestinal maturation during European sea bass development (7, 27, 43). It is generally accepted that at a similar stage of development, pancreatic enzyme secretion is higher in fish exhibiting a good growth and harmonic development than in those showing a delay in these processes. In the present study, all the experimental groups exhibited a good level of trypsin secretion, while the fish fed the highest levels of EPA and DHA in the PL and NL fractions (PL5 and NL3 diets) showed lower values of amylase secretion than the rest of the experimental groups. It should be noted that although larvae fed PL1 and NL1 diets exhibited the highest values of trypsin and amylase secretion amongst all dietary treatments, these values should not be attributed to a major maturation of the digestive system and harmonious development of larvae, since the specific activity of intestinal brush border enzymes (particularly alkaline phosphatase) was lower in these groups in comparison to fish fed PL3 diet. High secretion of pancreatic enzymes observed in the larvae fed PL1 and NL1 might be correlated to the high dietary content of linoleic acid, since C_{18} fatty acids, such as linoleic and oleic acids, play an important role in the regulation of the pancreatic secretion (44). In vitro experiments have shown that these fatty acids increased the intracellular calcium levels in enterocytes, stimulating the release of two gastrointestinal hormones, the cholecystokinin (CCK) and secretin, which regulate the pancreatic secretion of amylase, trypsin and lipase (12, 44).

Despite major advances in our understanding of lipid digestion in juvenile or adult fish, knowledge of lipolytic enzymes in young larvae is scarce, particularly in terms of luminal digestion of dietary lipids (6). The capacity to digest fats is related to lipase secretion by the pancreas and the production of bile salts in the liver, processes regulated by the levels of dietary TAG and the rate of intestinal absorption of lipids (12). In this sense, although it was not possible to determine the specific activity of pancreatic lipase in fish fed high levels of neutral lipids (NL3), since most of larvae died during the study, we observed that the specific activity of lipase in fish fed intermediate levels of neutral lipids (6.2%, NL1 diet) tended to be higher than that of fish fed PL diets at 30 dph. At the end of the experiment, this tendency in lipase activity was not observed. Such changes in specific activity might be related to the high accumulation of lipids observed in the enterocytes rather than to a lower maturation of the digestive system, since lipids accumulated in the intestinal mucosa might have disrupted the

metabolic capacity of the intestine to digest, absorb and export lipids into the circulatory system. These observations strongly suggested that large quantities of accumulated lipids in the intestinal mucosa might have resulted in a reduction of the CCK secretion by intestinal endocrine cells in order to diminish lipase release by the pancreas, and regulate lipid digestion and accumulation in enterocytes (44). The dramatic change in lipase activity in PL5 group at day 37 compared to day 30 could not be attributed to a stimulation of lipase by its substrate, i.e. NL. This change probably reflected an indirect consequence of intraluminal fatty acid concentration resulting from the hydrolysis of dietary PL, which in turn stimulated an hormonal mechanism involving secretin (Sheele, G.A. (1993) Regulation of pancreatic gene expression in response to hormones and nutritional substrates. In: The Pancreas: Biology, Pathobiology, and Disease (Go, V.L.W., Gardner, J.D., Brooks, F.P., Lebenthal, E.P., Di Magno, E.P. and Sheele, G.A., ed.), pp.103-120. Raven Press, New York.).Generally, nutritional studies evaluating lipid digestion and absorption processes in fish are limited to the anterior region of the intestine, which is considered the region involved in the absorption of these nutrients (14, 45). Nevertheless, lipid absorption still continues in the posterior and rectal regions of the intestine, particularly in carnivorous fish with short digestive tracts, such as European sea bass (46). The enterocytes of the pre- and postvalvular intestinal regions showed a larger degree of lipid accumulation than that observed by other authors (47). Such differences were due to the different level and nature of the lipid fraction contained in diets (compound feed vs. live preys). The results of the present study showed that European sea bass larvae have different patterns of lipid absorption and accumulation in the intestinal mucosa depending on the level and class of lipid used in the diet. Thus, we observed an important intracellular and intercellular accumulation of lipid in the anterior intestine of fish fed diets containing high levels of neutral lipid (11%, NL3 diet), while no such accumulation was detected when larvae were fed with low and moderate levels of neutral lipid (PL and NL1 diets). Similarly, the deposition of large lipid vacuoles in the postvalvular intestinal mucosa of larvae fed different experimental diets seemed to be inversely correlated to the level of dietary NL, since PL levels were almost the same among the diets, with the exception of NL3. The different *n*-6/*n*-3 ratio in the diets could have also influenced the accumulation of lipids in the enterocytes (14, 48). Some authors have reported the use of unbalanced feeds in terms of lipid class composition as the cause of high, pathological level of intestinal accumulation of lipids (steatosis) (16, 17). Nevertheless, diet formulation and histological observations in the present study seemed to contradict this hypothesis, even though high levels of lipid deposition were observed in the postvalvular intestine of European sea bass larvae, they cannot be considered to be the cause of any pathological damage, since no signs of epithelial abrasion, cellular necrosis and/or inflammatory reactions were detected along the intestinal mucosa.

The use of the liver as an indicator of the nutritional condition in fish is well-known. Different studies have shown that structural alterations of the liver due to nutritional imbalances can provide useful information on diet quality and metabolism, complementing the information obtained with growth studies to evaluate the nutritional status of the fish. In the present study, levels of lipid accumulation in the liver of the larvae fed high levels of LC-PUFA in the neutral lipid fraction (NL3) were higher than those of fish fed similar or even higher levels of LC-PUFA contained in the diet as phospholipids (PL3 and PL5). These observations indicated that the form of supply dietary LC-PUFA has a direct effect on fat storage in the liver, which could be related to a higher influx of chylomicrons and/or very low density lipoproteins (VLDL) from the intestine due to the differences in lipid exportation capacities of the intestinal mucosa.

The comparative histological study carried out on 37-day-old larvae revealed the existence of a “dietary regime effect” on swimbladder inflation during early stages of development in European sea bass. The high frequency of occurrence of larvae without a functional swimbladder in the NL3 group (90%), contrasted to the rest of the experimental groups (PL and NL1). It is well recognized that inflation of the swimbladder is temporally limited and can only take place during a short window of larval morphogenesis, during which access to the air-water interface is critical for the normal inflation and development of the swimbladder (49). In the present study, the similar rearing conditions and procedures employed for culturing larvae from different experimental groups, coupled with the low incidence of fish with non-inflated swimbladder in larvae fed PL and NL1 diets suggested that anomalies in swimbladder

inflation observed amongst larvae fed NL3 diet might be related to diet quality, rather than problems of larvae in gulping air from the air-water interface. Thus, the high percentage of neutral lipids in the diet might be responsible for a disruption (retardation) of normal fish development, as growth results indicated, resulting in the failure to dilate the swimbladder lumen and the atrophy of this organ (50).

In conclusion, data on larval growth, survival, histological organization of the liver and intestine, and digestive enzyme activities showed that the quantity and form of EPA and DHA (NL or PL) supplied in the diet were determining for European sea bass larval development; fish used EPA and DHA more efficiently when these essential fatty acids were present in the phospholipid rather than in the neutral lipid fraction of the diet. These results must be taken into account in the formulation of compound diet for larvae. Indeed, lipid and LC-PUFA are generally provided by fish oil, which is a neutral lipid, but marine phospholipid would be a nutritionally better source of LC-PUFA.

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Table 1. Composition of the experimental compound microdiets

	Experimental diets				
	PL1	**PL3**	**PL5**	**NL1**	**NL3**
Ingredients[1]	**g/kg dry matter**				
Defatted Fish Meal			510		
Hydrolyzed fish meal (CPSP)			140		
Cod liver oil	0	0	0	7	14
Marine lecithin[2]	7	14	21	0	0
Soy lecithin[3]	14	7	0	14	7
Vitamin mixture[4]			80		
Mineral mixture[5]			40		
Betaine			20		
Proximate composition			**%**		
Proteins (Nx6.25)	59.9	61.6	60.4.	58.7	59.6
Lipids	16.4	16.7	16.7	17.1	18.2
Phospholipids	13.2	13.0	12.1	11.1	8.1
EPA + DHA in PL	1.1	2.3	4.8	0.3	0.4
Neutral lipids	4.8	3.8	3.0	6.2	11.0
EPA + DHA in NL	0.3	0.3	0.3	1.3	2.6
Ash	17.4	17.0	16.8	15.6	15.6
Moisture	6.8	6.3	7.4	6.5	6.4
Energy (kJ/kg)[6]	1618	1658	1638	1624	1681

[1] All dietary ingredients were commercially available. Fish meal (La Lorientaise, Lorient, France), hydrolyzed fish meal (CPSP, Soluble Fish Protein Concentrate, Sopropêche, Boulogne sur Mer, France), cod liver oil (La Lorientaise, Lorient, France), marine lecithine (LC60, Phosphomins ^{TM}, Phosphotech, Saint Herblain, France) and soy lecithin (*Ets Louis François,* St Maur des Fossés, France).

[2] Contains 60% phospholipids (with 45% phosphatidyl choline, 20% ethanolamine, 16% phosphatidyl inositol), 5% triglycerides, 15% cholesterol, 1 mg/g natural tocopherols as antioxydant.

[3] Contains 95% phospholipids (with 26% phosphatidyl choline, 20% ethanolamine, 14% phosphatidyl inositol)

[4] Per kg of vitamin mix: retinyl acetate 1 g; cholecalciferol 2.5 mg; all-*rac*- α-tocopherol acetate 10 g; menadione 1 g; thiamin 1 g; riboflavine 0.4 g; D- calcium pantothenate 2 g; pyridoxine HCl 0.3 g; cyanocobalamin 1 g; niacin 1 g; choline chloride 200 g; ascorbate polyphosphate 20 g; folic acid 0.1 g; biotine 1 g; meso-inositol 30 g, cellulose 732.1 g.

[5] Per kg of mineral mixture: KCl 90 g, KI_4O 40 mg, $CaHPO_4$ $2H_2O$ 500 g, NaCl 40 g, $CuSO_4$ $5H_2O$ 3 g, $ZnSO_4$ $7H_2O$ 4 g, $CoSO_4$ $7H_2O$ 20 mg, $FeSO_4$ $7H_2O$ 20 g, $MnSO_4$ H_2O 3g, $CaCO_3$ 215 g, $MgSO_4$ $7H_2O$ 124 g, NaF 1g.

[6] Calculated as: fat x 37.7 J/kg; protein x 16.7 J/kg.

Table 2. Percentage of the main fatty acids in the phospholipid and neutral lipid fractions of experimental diets, expressed in percentage of total fatty acids in PL and NL.

	Phospholipids					Neutral lipids				
Diets	**PL1**	**PL3**	**PL5**	**NL1**	**NL3**	**PL1**	**PL3**	**PL5**	**NL1**	**NL3**
14:0	0.6	1.4	2.0	0.6	1.6	3.8	4.3	4.7	6.0	6.2
16:0	19.2	23.4	25.6	18.0	18.4	15.7	16.3	17.2	15.8	16.2
18:0	3.9	3.5	2.6	4.4	4.1	4.0	3.8	3.8	2.8	2.8
Σ saturated	24.0	28.5	30.1	23.5	24.4	23.9	24.6	25.9	24.8	25.4
16:1n-7	0.6	1.0	1.2	1.0	2.0	5.3	5.5	5.8	7.0	6.9
18:1n-7	0.0	0.0	0.2	0.0	0.0	0.5	0.5	0.5	1.1	1.1
18:1n-9	6.0	5.0	3.5	7.7	9.6	17.9	15.8	14.7	16.7	16.6
20:1n-9	1.1	2.3	3.7	0.8	1.6	6.3	6.9	8.1	5.0	5.4
18:1n-11	1.3	1.2	1.2	1.6	2.0	3.7	3.7	3.8	3.2	3.2
20:1n-11	0.0	0.0	0.4	0.0	0.0	1.1	1.1	1.4	0.8	0.8
22:1n-11	0.2	0.2	0.2	0.5	1.0	4.5	4.8	5.0	4.8	5.7
Σ monosaturated	9.4	10.0	11.0	11.6	16.7	41.0	40.2	41.3	40.5	41.7
18:2n-6	44.2	25.8	0.3	51.3	42.0	12.8	6.8	1.8	4.8	3.5
20:4n-6	0.8	1.7	3.1	0.3	0.4	0.9	1.4	1.5	0.7	0.6
Σ *n*-6 PUFA[1]	45.4	28.3	4.2	52.1	42.8	14.6	9.4	4.3	6.5	5.1
18:3n-3	6.7	4.9	0.2	7.8	6.6	2.1	1.4	0.7	1.9	1.8
18:4n-3	0.1	0.2	0.2	0.2	0.6	1.4	1.5	1.5	2.6	2.5
20:4n-3	0.1	0.2	0.2	0.2	0.3	0.5	0.6	0.6	1.0	1.0
20:5n-3	3.8	7.5	13.7	1.5	3.0	6.5	8.3	9.3	9.6	9.5
22:6n-3	10.5	21.4	40.0	3.0	5.3	9.1	12.8	15.0	10.1	11.0
Σ *n*-3 PUFA	21.2	33.2	54.6	12.8	16.1	20.4	25.7	28.3	28.2	27.8
n-3 / *n*-6 ratio	0.47	1.17	13.06	0.25	0.38	1.39	2.75	6.63	4.34	5.45

[1] PUFA, polyunsaturated fatty acids.

Figure 1. Growth of European sea bass larvae fed isonitrogenous and isolipidic diets containing different levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Diets are named according to the percentage of dietary EPA and DHA contained in the phospholipid (PL) and neutral lipid (NL) fraction of the diet: PL5 (○), PL3 (■), PL1 (Δ), NL1 (●) and NL3 (□). Results are shown as mean ± S.D. (*n* = 3). Different superscripts for the same day indicate significant differences ($P < 0.05$).

Figure 2. Trypsin (□) and amylase (■) secretion in 37 dph European sea bass larvae fed isonitrogenous and isolipidic diets containing different levels of EPA and DHA. Means ± S.D. (n = 3) with different superscript letters were significantly different ($P < 0.05$).

Figure 3. Lipase specific activity in 30 and 37 dph European sea bass larvae fed isonitrogenous and isolipidic diets containing different levels of EPA and DHA. Means ± S.D. (n = 3) with different superscript letters were significantly different ($P < 0.05$).

Figure 4. Specific activity of alkaline phosphatase (a) and aminopeptidase N (b) of the intestinal brush border in European sea bass at 30 and 37 dph fed isonitrogenous and isolipidic diets containing different levels of EPA and DHA. Means ± S.D. (n = 3) with different superscript letters were significantly different ($P < 0.05$).

Figure 5. Longitudinal paraffin sections of the liver in European sea bass larvae fed isonitrogenous and isolipidic diets containing different levels of EPA and DHA. Liver from fish fed control diet (PL1) and NL1 showing regular-shaped hepatocytes with very few lipid inclusions (a, d). Livers from fish fed PL3 and PL5 with a high (b) and intermediate (c) degree of cytoplasmatic lipid accumulation in hepatocytes. Liver from fish fed NL3 showing the displacement of the nucleus to the periphery of the hepatocytes and the presence of numerous varying-size cytoplasmatic lipid vacuoles (e). Histological sections from larvae fed PLs and NL1 diets were from fish aged 37 dph, while those from the NL3 group were obtained from 30 dph. Scale bar: 25 μm.

Figure 6. Longitudinal paraffin sections of the prevalvular intestine and swim bladder in European sea bass larvae fed isonitrogenous and isolipidic diets containing different levels of EPA and DHA. Prevalvular intestine from 37 days-old larvae fed PL1 showing the mucosa

lined by a simple columnar epithelium with centrally located nuclei and not containing lipid accumulations in cytoplasmatic vacuoles (a, b). Prevalvular intestinal mucosa from larvae aged 30 days post hatch fed PL3 showing the presence of small apical lipid droplets (asterisk), the displacement of nuclei to a basal position (arrow head) and large intercellular lipid vacuoles between enterocytes (arrow) (c, d). Normal inflated swimbladder from larvae fed PL1 (e). Non-inflated swim bladder with hyperplasic gas gland cells (asterisk) from fish fed NL3 (f). Gg, gas gland; pi, prevalvular intestine; rm, rete mirabile. Scale bar: 100 μm.

Figure 7. Longitudinal paraffin sections of the postvalvular intestine in European sea bass larvae fed isonitrogenous and isolipidic diets containing different levels of EPA and DHA showing different levels of intestinal steatosis depending on the experimental diet [PL1 (a), PL3 (b), PL5 (c-d), NL1 (e) and NL3 (F)]. Note the lower accumulation of lipids in the enterocytes of fish fed NL3 in comparison to those fed PL3 diets (b, f). Scale bar: 100 μm.

Figure 1

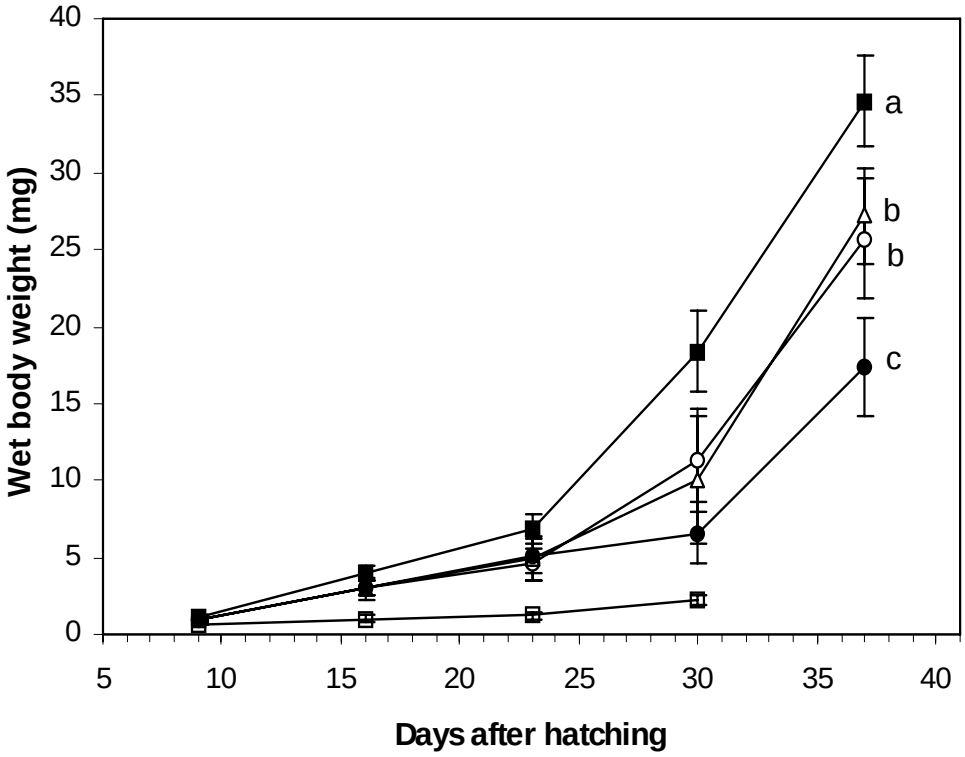

Wet body weight (mg)
Days after hatching
0
5
10
15
20
25
30
35
40
a
b
b
c

Figure 2

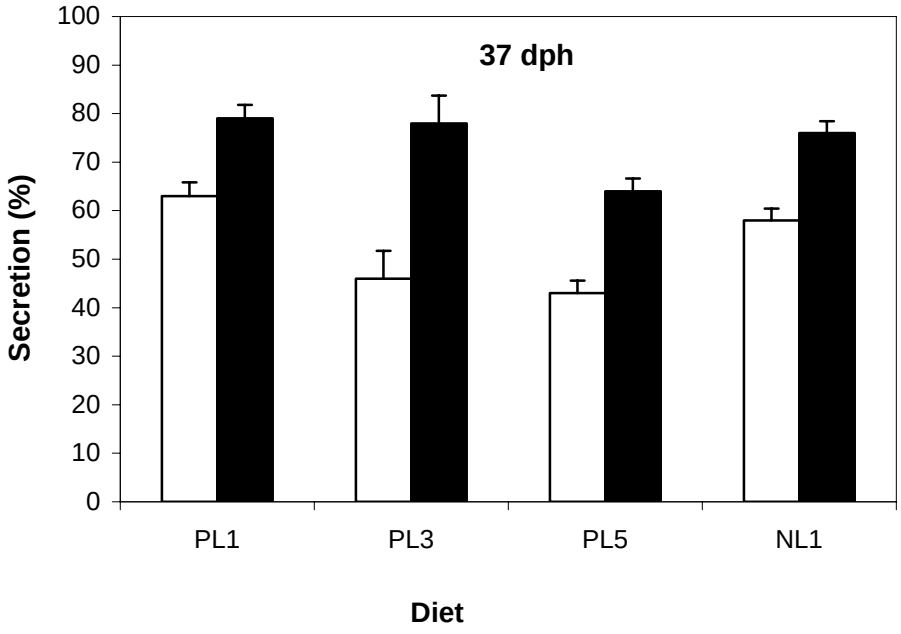

37 dph
Secretion (%)
100
90
80
70
60
50
40
30
20
10
0
PL1
PL3
PL5
NL1
Diet

Figure 3

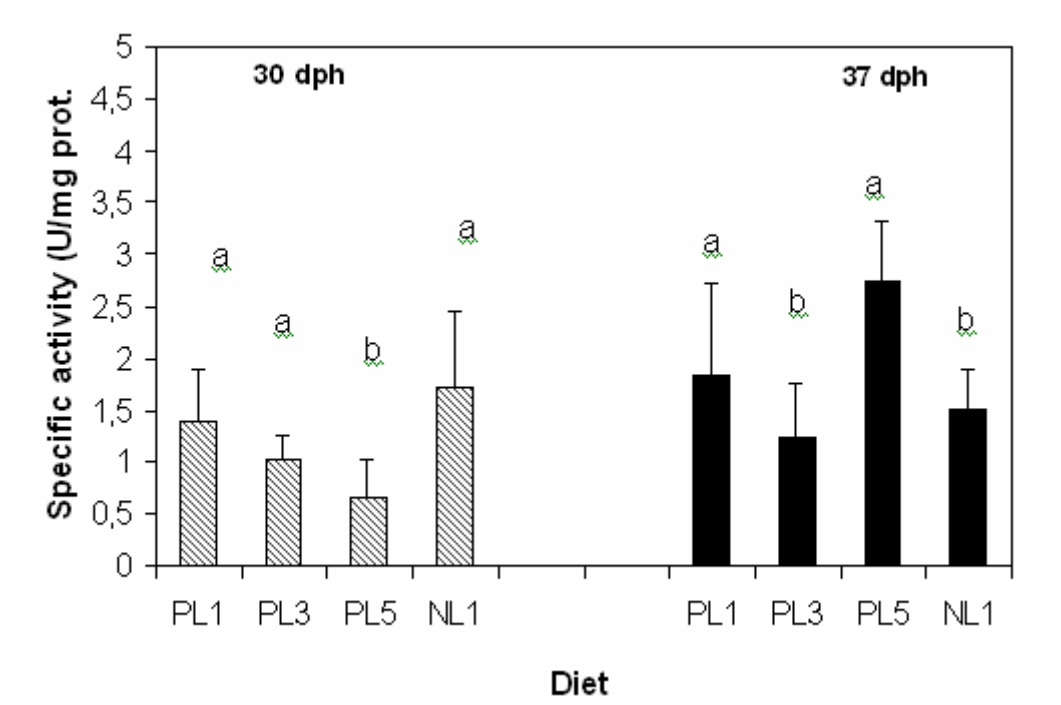

30 dph
37 dph
Specific activity (U/mg prot.
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
a
a
b
a
a
b
a
b
PL1
PL3
PL5
NL1
PL1
PL3
PL5
NL1
Diet

Figure 4

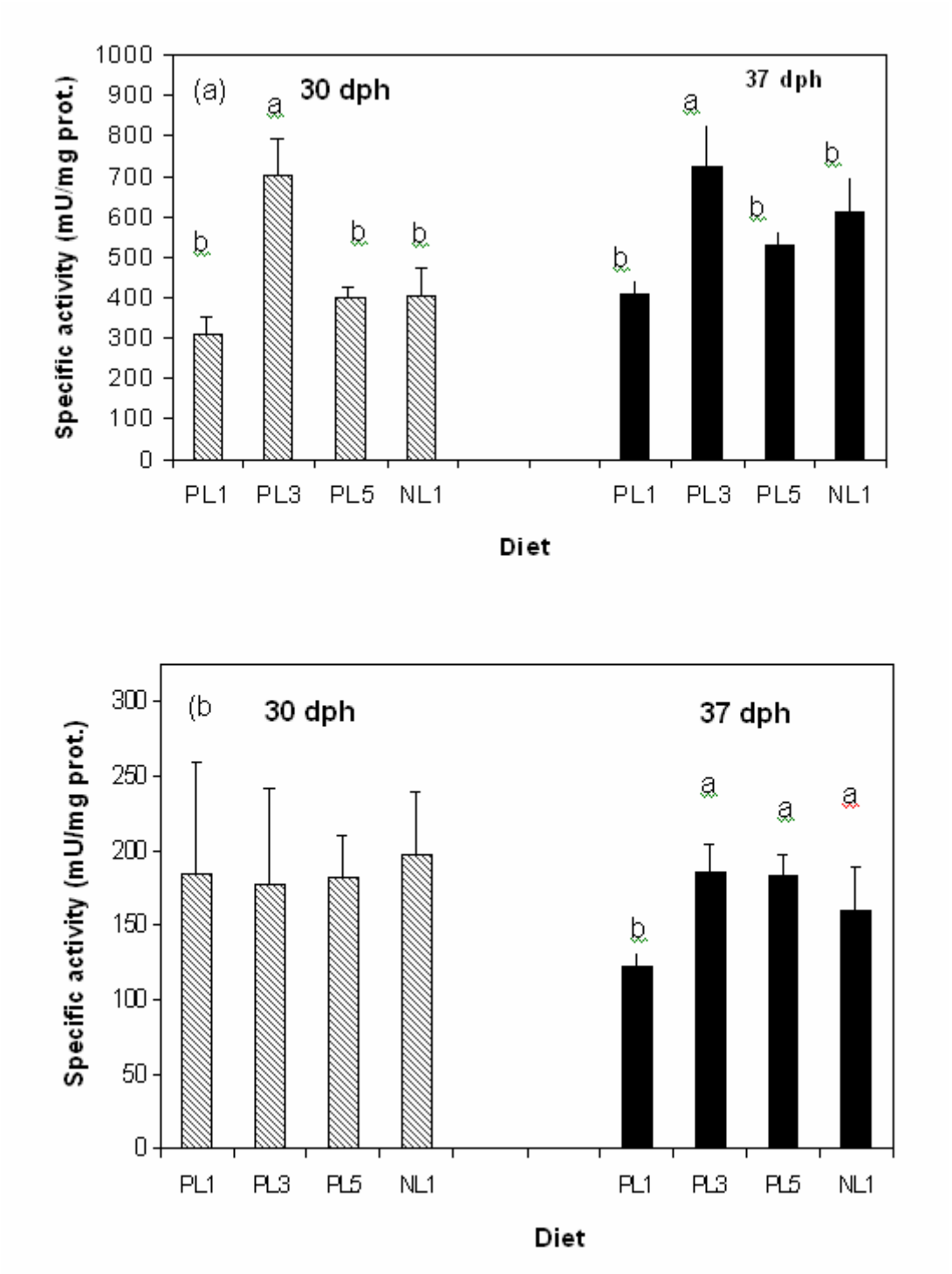

(a)
30 dph
37 dph
Specific activity (mU/mg prot.)
1000
900
800
700
600
500
400
300
200
100
0
a
b
b
b
a
b
b
PL1
PL3
PL5
NL1
PL1
PL3
PL5
NL1
Diet
(b
30 dph
37 dph
Specific activity (mU/mg prot.)
300
250
200
150
100
50
0
b
a
a
a
PL1
PL3
PL5
NL1
PL1
PL3
PL5
NL1
Diet

Figure 5

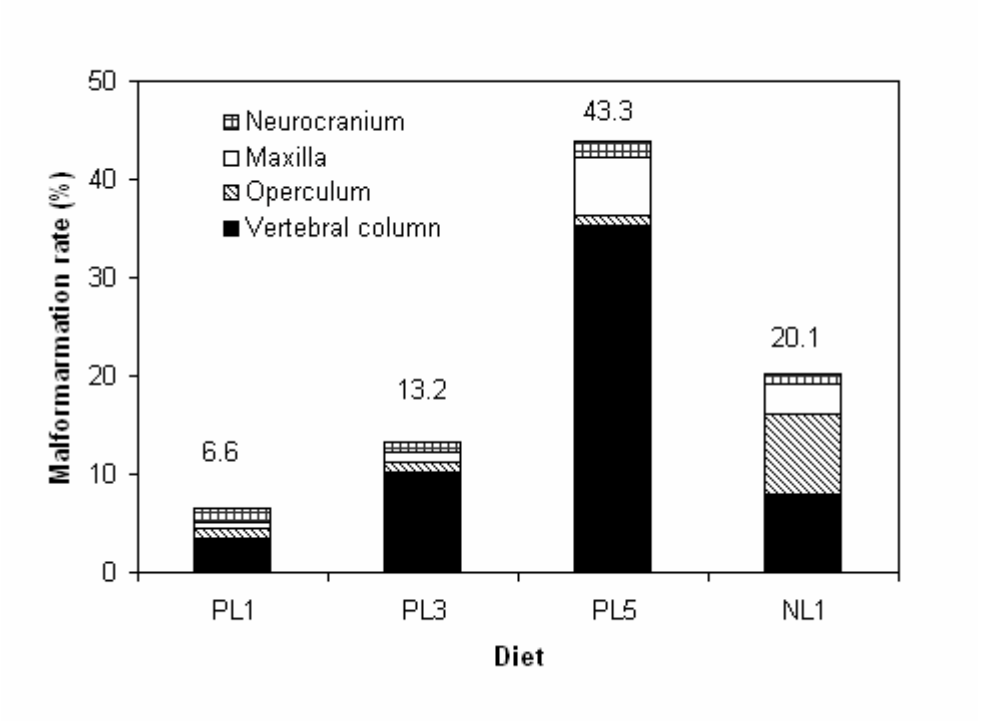

Neurocranium
Maxilla
Operculum
Vertebral column
Malformarmation rate (%)
50
40
30
20
10
0
6.6
13.2
43.3
20.1
PL1
PL3
PL5
NL1
Diet

Figure 6

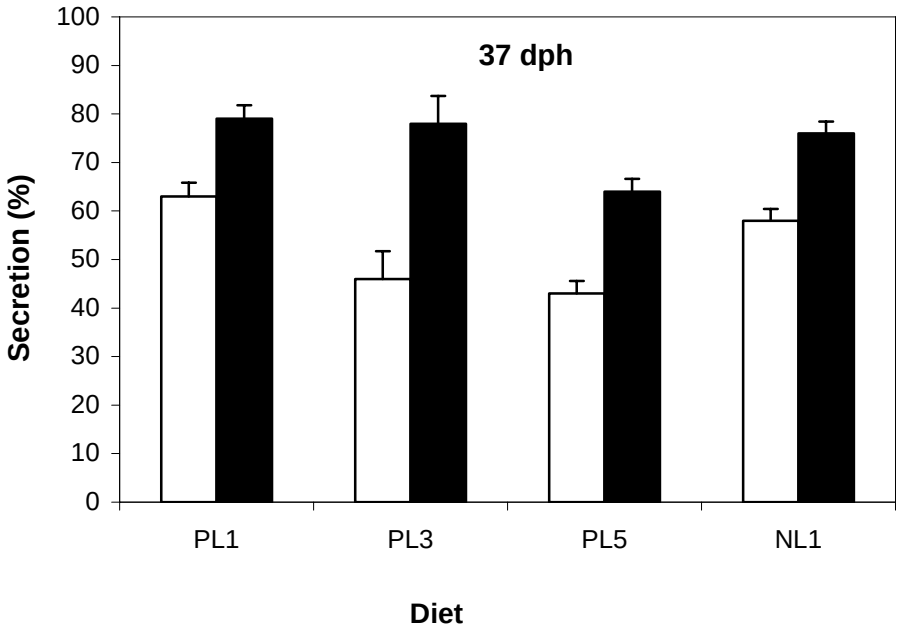

37 dph
Secretion (%)
100
90
80
70
60
50
40
30
20
10
0
PL1
PL3
PL5
NL1
Diet

Figure 7

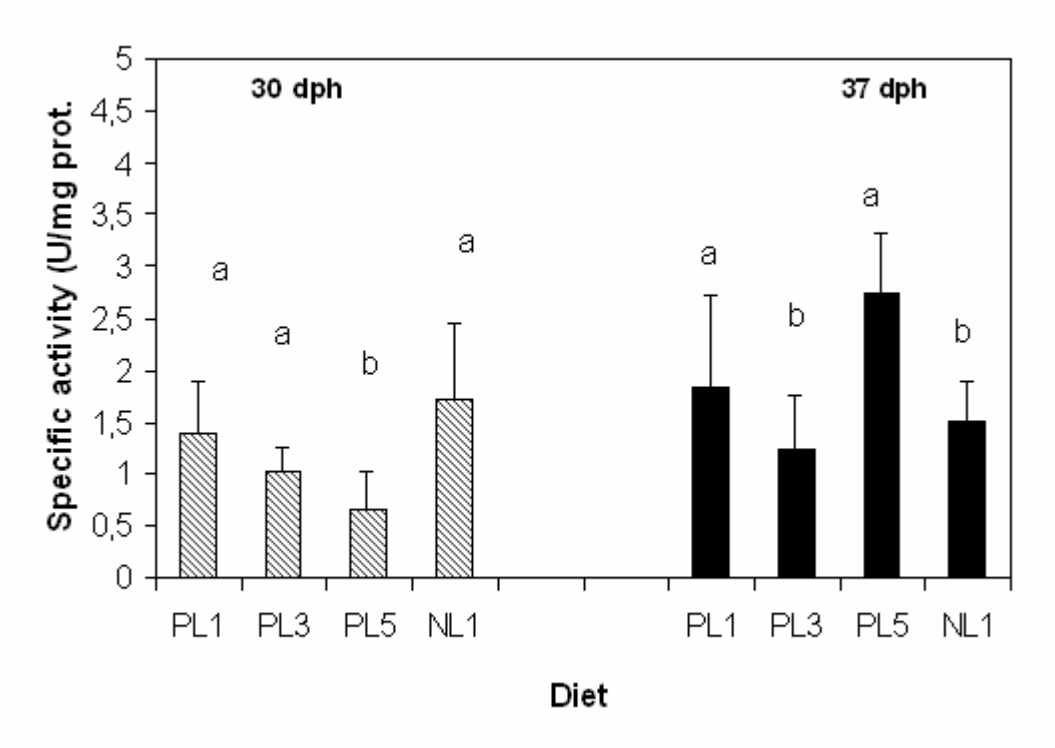

30 dph
37 dph
Specific activity (U/mg prot.
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
a
a
b
a
a
b
a
b
PL1
PL3
PL5
NL1
PL1
PL3
PL5
NL1
Diet

Figure 8

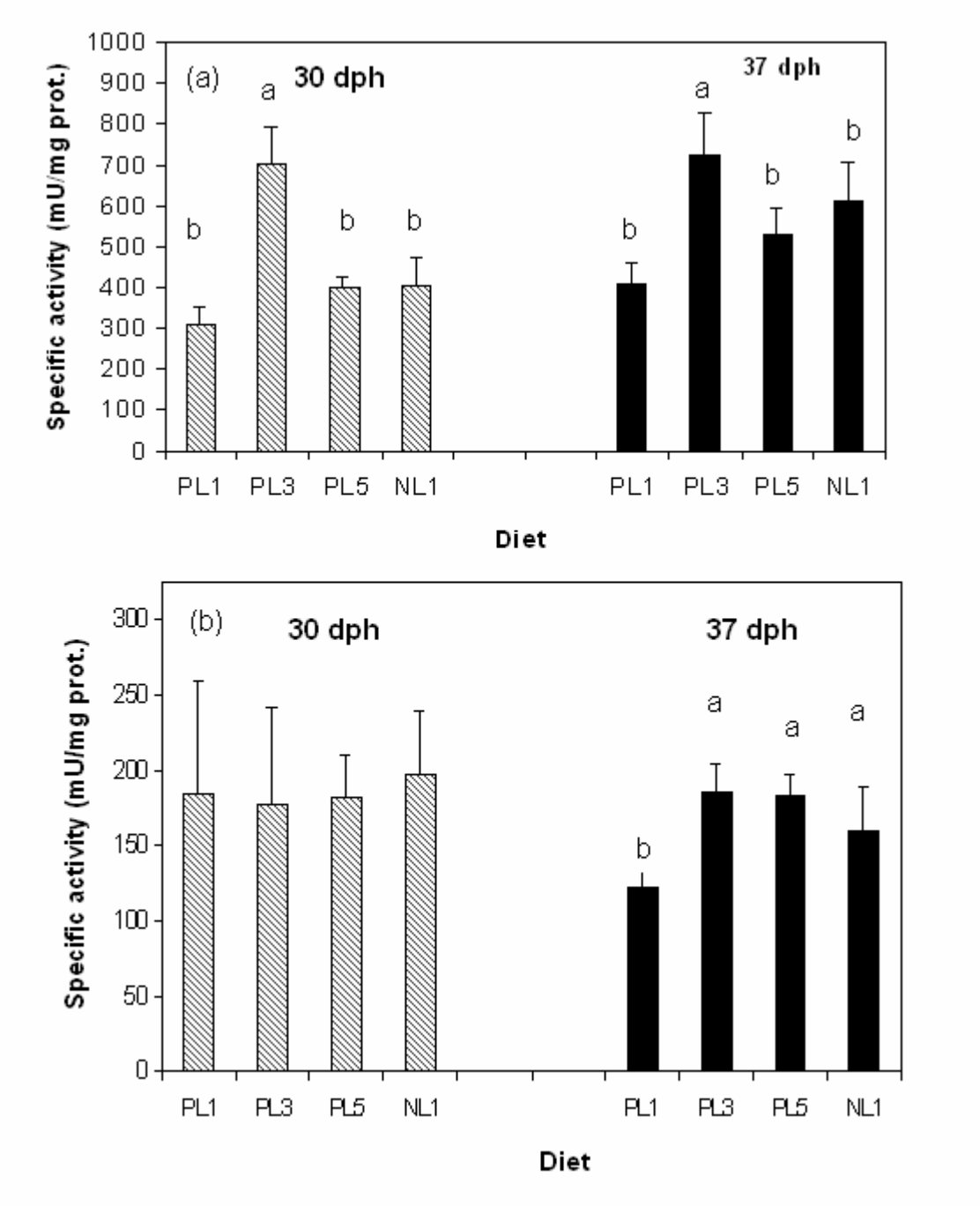
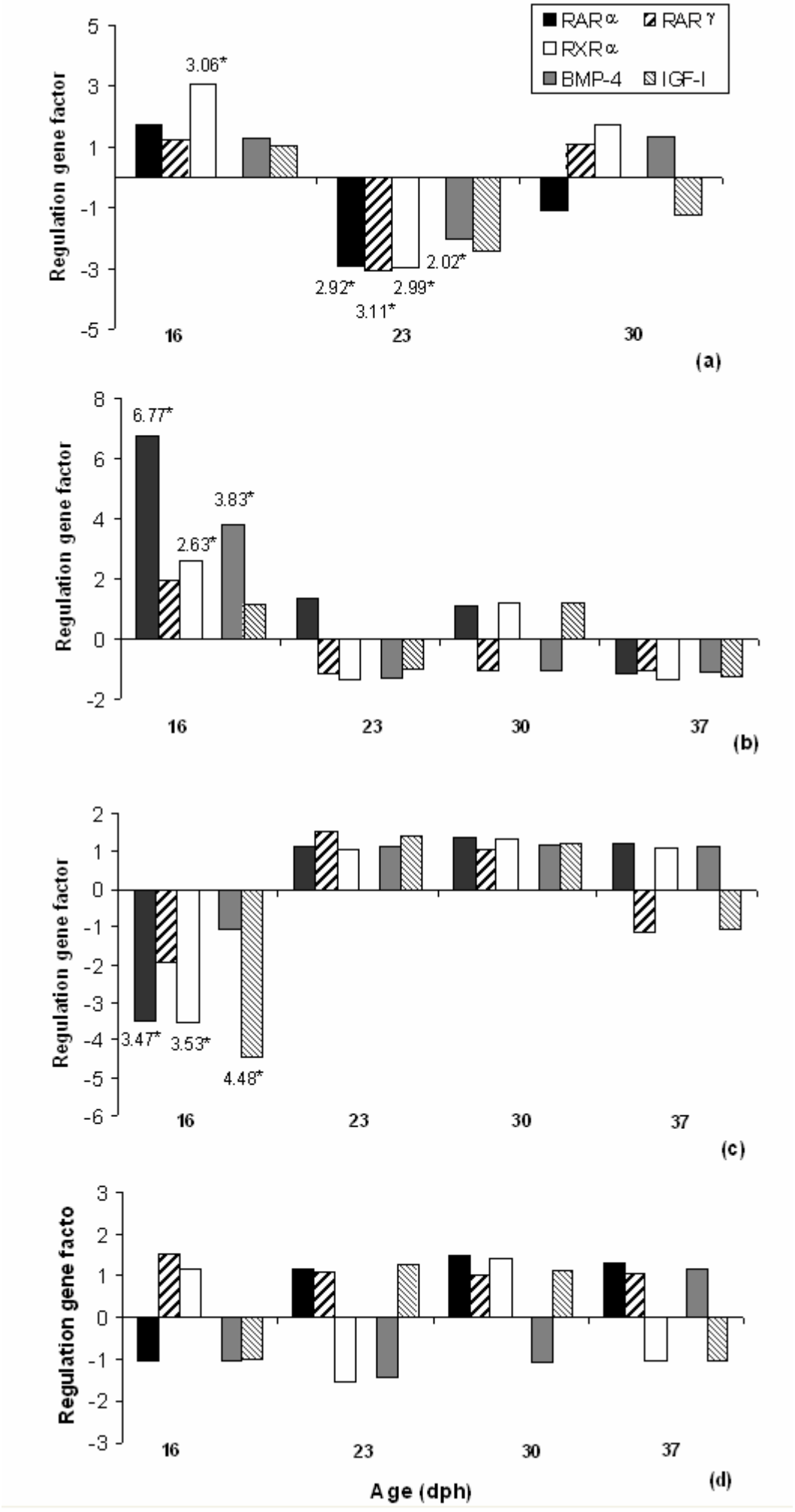

(a)
30 dph
37 dph
Specific activity (mU/mg prot.)
1000
900
800
700
600
500
400
300
200
100
0
a
b
b
b
a
b
b
b
PL1
PL3
PL5
NL1
PL1
PL3
PL5
NL1
Diet
(b)
30 dph
37 dph
Specific activity (mU/mg prot.)
300
250
200
150
100
50
0
b
a
a
a
PL1
PL3
PL5
NL1
PL1
PL3
PL5
NL1
Diet

Figure 9


RARα
RARγ
RXRα
BMP-4
IGF-I
Regulation gene factor
3.06*
2.92*
3.11*
2.99*
2.02*
16
23
30
(a)
6.77*
2.63*
3.83*
16
23
30
37
(b)
3.47*
3.53*
4.48*
16
23
30
37
(c)
Regulation gene facto
16
23
30
37
Age (dph)
(d)