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Influence of dietary lipid composition on yolk protein components in sea bass, *Dicentrarchus labrax**

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SUMMARY: The influence of diet on yolk protein composition and egg survival was studied using the sea bass, *Dicentrarchus labrax*, as an experimental model. Females were fed to satiation with a high essential fatty acid (EFA) diet during four different periods in the annual cycle: from September to February (vitellogenesis, Group A), from February to April (spawning, Group B), from April to September (postvitellogenesis, Group C) and all the year round (Group D). During the remaining months groups were fed with a low EFA diet. The percentage of egg viability and the hatching rates were observed in every group. The two major yolk proteins, lipovitellin (LV) and phosvitin (PV), were isolated from the ovary and their changes were studied by SDS-gel electrophoresis. In fish fed the diet enriched with EFA only for two months (group B) a decrease was observed of the 8 kDa PV components associated with a diminished egg viability and hatching. However, no modification of vitellogenin (VTG) plasma levels and no improvement in fish growth were observed under the dietary conditions used.

Key words: fish, vitellogenin, lipovitellin, phosvitin, fatty acids, yolk components, diet, egg quality.

INTRODUCTION

The sea bass, *Dicentrarchus labrax*, spawns naturally in the Mediterranean area from December to March, but the period of spawning can be delayed and restricted in length in more northern latitudes (Carrillo *et al*., 1995). It also may be shifted by maintaining the animal under modified photoperiods (Carrillo *et al*., 1989; 1995). Moreover, it has been found that exposure of female fish to modified photoperiod affects the rate of maturation and timing of spawning (Carrillo *et al*., 1989; Barnabè and Paris, 1984), while little loss in egg quality in terms of survival of eggs and fry or of fecundity was observed in photoperiodically advanced broodstock (Devauchelle *et al*., 1987; Carrillo *et al*., 1995). In contrast, poorer fecundity and egg survival have been found in photoperiodically delayed fish (Zanuy *et al*., 1986; Devauchelle and Coves 1988; Carrillo *et al*., 1991). Currently, one of the major constraints to continued expansion of farmed marine species is poor egg qual-

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ity leading to low and variable survival of eggs In sea bass, survival rates during incubation reach only 15-20% of total eggs spawned.

Together with poor or inappropriate husbandry or other culture conditions is the widespread belief that various constituents of the egg, including genetic composition and those materials which are sequestered and stored during ovary maturation (Bromage *et al.*, 1992), may be the cause of low survival rates. The effects of broodstock nutrition on reproductive performance and egg survival have received little attention and the results on the effects of experimental alterations in diet formulation are conflicting. For instance, a high dietary protein level in trout increased fecundity and egg diameter (Smith *et al.*, 1979; Roley, 1983), while other authors did not detect differences in egg hatchability and fry survival in trout on a low protein diet (Takeuchi *et al.*, 1981; Watanabe *et al.*, 1984d). In marine fish species, a low protein diet seems not to induce alteration in egg production and hatching rates (Watanabe *et al.*, 1984b; 1984c). However, other studies have shown low fecundity and lower percentages of buoyant eggs and hatching when the red seabream was fed diets with low protein levels (Watanabe *et al.*, 1984a). Recently, Cerdà *et al.* (1994) found that dietary protein levels in sea bass, *Dicentrarchus labrax*, greatly affect reproduction and progeny survival. In the same species, Cerdà *et al.* (1995) found that long-term dietary deficiency of PUFA (polyunsaturated fatty acids), especially n=3 fatty acids, may affect the patterns of plasma lipids and induce early gonadal atresia, and that, this follicular atresia may reduce the production of gonadal steroids, fecundity, and egg survival. In addition, it was found that the fatty acids docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) and their phospholipid derivatives phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol (Sargent *et al.*, 1989), and lastly the carotenoid micronutrient astaxanthin (Watanabe *et al.*, 1991), exert the most significant influence on survival rate.

It is now accepted that the size of the egg is not related to its quality, while, in contrast, the integrity of the proteins in eggs might be. Most proteins in eggs have as precursor a large lipoglycophosphoprotein produced in the liver under the influence of estradiol (Bergink and Wallace, 1974; Tata, 1976). This precursor is released in the bloodstream and sequestered by developing oocytes through a receptor-mediated uptake; once in the oocytes, this protein, called vitellogenin (VTG), is cleaved into two smaller proteins: lipovitellin (LV) and phosvitin (PV), and stored as yolk (Mommsen and Walsh 1988; Tyler *et al.*, 1988a,b; 1990). In *X. leavis* (Ohlendorf *et al.*, 1977), native lipovitelin contains about 20% lipid of which 75% is phospholipid. The phospholipid fraction consists of mostly phosphatidylcholine and phosphatidylethanolamine, the neutral lipid fraction contains mainly triglyceride. The phosphoprotein, PV, contains 10% of P and one-half of its residues are phosphoserine. Recently, it was demonstrated in the chicken that the catalyst for VTG processing is cathepsin D (Retzek *et al.*, 1992).

In the species studied here, Carnevali *et al.* (1993) found that yolk components change during oocyte maturation, with a secondary proteolitic process occurring when the oocytes reach 0.7 mm in diameter; it was hypothesized that such secondary proteolysis may generate part of the osmotic gradient associated with resumption of oocyte meiotic maturation before ovulation. During embryo development, there is a rapid degradation of both LV and PV, which produces free amino acid, fatty acids, phosphorus, calcium, etc, and these yields will be utilized by the embryo during its development.

The purpose of the present study was to assess whether in *Dicentrarchus labrax* different diet conditions could be related to yolk components, and in turn, could affect egg viability and hatching.

MATERIALS AND METHODS

Broodstock and diets

Groups of 35-45, 1.5-year-old broodstock sea bass (413±117 g in weight and 32.9±11 cm in length) were reared in 8000-L glass-fiber tanks and maintained in aereated running sea water in natural conditions of ambient temperature and photoperiod (40ºN and 0ºE) at the Instituto de Acuicultura de Torre de la Sal, Spain. Animals came from the installations of this Institute and the mean weight of the fish and the proportion of males to females in each tank were similar. The experiment began in June 1992. During two consecutive years, groups were fed to satiation with high essential fatty acid (EFA) diets at various times coinciding with different phases of the reproductive cycle: group A received the high EFA diet during vitellogenesis and maturation (September to February); Group B during spawning (February to April); Group C during

TABLE 1. – Source of raw materials of the pelleted diet used to produce the two experimental diets and proximate composition (g/100 g dry diet) of the experimental diets. The total content of lipids in the base diet was 10%. This percentage was increased to 22% by soaking the pellets in fish oil or maize oil.

Source of raw materials	% of total weight
Fish meal	66
Fish oil	8
Vegetable protein	15
Cereal	5
By-products of sugar	5
Vitamins and minerals	1
Approximate composition	**g/100 g dry diet**
Protein	52
Total lipids	22
NFE*	15
Ash	10

* Nitrogen Free Extract.

post-spawning (April to September); Group D, the control group, throughout the year. Those groups not receiving the high EFA diet for the whole year (Groups A, B, and C) were fed a low EFA diet for the remainder of the year. Both high and low EFA diets were produced by soaking a commercially available pelleted feed (9 mm pellet, BOCM Pauls) in fish oil or maize oil, immediately prior to feeding, with the total lipid content of the diets being raised from 9% to 20%. The origin of the components and proximate composition of the pelleted diet used appear in Table 1, while the fatty acid profiles of the diets are shown in Table 2.

Data Collection

In December of the second year, after all groups had received the mentioned treatments for a complete experimental period, at least six females per group were sacrificed and the ovaries dissected and used for yolk protein preparation. The remaining females from each group (14 in Group A, 13 in Group B, 15 in Group C, and 10 in Group D) began to spawn after the others were sacrificed. During the course of the spawning season, naturally fertilized eggs were collected each day to determine the proportion of viable (floating) to unviable (sinking) eggs, as described by Carrillo *et al*. (1989). Further aliquots of viable eggs were transferred to incubation facilities for assessment of survival to hatching (Carrillo *et al*., 1989).

TABLE 2. – Percentage fatty acid composition of the total lipid extracted from the experimental diet.

Fatty acid	Low EFA diet	High EFA diet
20:4 n-6	0.3	0.5
20:5 n-3 (EPA)	4.9	8.8
22:6 n-3 (DHA)	5.7	8.1
Total Saturates	19.3	21.7
Total Monoenes	40.2	47.1
Total Dienes	27.9	4.0
Total PUFA	42.5	27.1
Total n-3	14.2	22.3
Total n-6	28.3	4.8
n-3:n-6 Ratio	0.5	4.6
DHA:EPA Ratio	1.2	0.9

Yolk proteins preparation

Ovaries from individual fish were collected in F.O. modified solution (178 mM NaCl, 1mM KCl, 2 mM $CaCl_2$, 5 mM Hepes, pH 7.5; following Wallace and Selman, 1978), briefly rinsed with cooled water and homogenized in 4 vol 0.1M sodium phosphate buffer (monobasic:dibasic = 1:1, pH 6.8) containing 2 mM PMSF, this step being performed on ice. The homogenates were spun at 13,000 g for 90 min at 4 ºC to remove debris and fatty materials. Coomassie blue staining proteins were separated from the highly charged Stains-all staining proteins by $(NH_4)_2\ SO_4$ (4 M) precipitation following Carnevali *et al*. (1993).

The $(NH_4)_2\ SO_4$ supernatant from whole ovary, remaining after Coomassie blue staining protein precipitation, was desalted with Bio Gel P6-DG column (Bio-Rad) and lyophilized. The resulting Stains-all staining fractions were subjected to electrophoresis.

Gel electrophoresis

Coomassie blue staining fractions, and highly charged Stains-all staining proteins from ovary were analyzed on linear gradient slab gels (6.93-20.44% polyacrylamide) prepared as previously described by Wallace and Selman (1985).

Samples and heated Bio-Rad high plus low molecular weight standards were placed in wells and electrophoresed at constant current as in Wallace and Selman (1985). Certain highly-phosphorylated proteins do not stain well with Coomassie blue (Wallace and Begovac, 1985); therefore, in order to indicate highly charged peptides, fixed gels were transferred to 50% methanol for 30 min and then to a freshly made solution of Stains-all (1-ethyl-2-[3 -(1-ethylnaphtho [1,2-

d]thiazolin-2-ylidene)-2-methylpropenyl] naphtho [1,2-d] thiazolium bromide) and left on a rotor platform in the dark overnight following Wallace and Begovac (1985).

Molecular masses of the protein bands were determined by the log-log method of Lambin (1978).

Chromatography

All chromatographic manipulations were performed on a Pharmacia FPLC system. A size exclusion column (Superose HR 12, Pharmacia) was applied to FPLC. Protein samples (200 µg) were injected onto the column through 0.45-µm-pore nylon syringe filters, and then eluted with 0.05M $(NH_4)HCO_3$. This solution was initially filtered through a 0.22-µm-pore filter and degassed by sonication. The flow rate was 0.5 ml/min, and elution was monitored at 214 nm. Sequential fractions eluted from the column (1 ml/tube) were dried in a vacuum centrifuge and prepared for gel electrophoresis.

Western blot analysis

LV and PV were electrophoresed on homogeneous SDS 10% (w/v) polyacrylamide gels using a discontinuous system previously described (Wiley *et al*., 1979), and electroblotted onto a Bio-Rad filter using a Bio-Rad mini trans-blot electrophoretic transfer cell. The transfer was carried out at 7 V/cm^2, overnight at 4ºC using a 25 mM Tris base, 192 mM glycine and 20% Methanol as electrode solution. The nitrocellulose membrane was soaked in 5% Nonidet P-40 for 1 h to remove SDS, and incubated with 2% BSA in PBS buffer. The antibody (anti-VTG) used for this analysis was the same as that used by Mañanós *et al*. (1994a); it was diluted 1:2000 in a solution containing 2% BSA , 0.01% NaN_3 in PBS, and was incubated for 2 h at room temperature. The filter was rinsed several times with PBS plus 0.05% Tween 20 (10 min X 3 times, shaking). The second antibody solution (HRP-êconjugated anti-rabbit IgG; Bio-Rad) diluted 1:2000 in 2% BSA, 0.01 Thimerosal in PBS solution, was incubated for 2 h. The filter was rinsed as above, and in addition once more with PBS but without Tween 20.

As substrate, freshly made 0.025% Diaminobenzidine, 0.01% H_2O_2 20 mM Tris Cl at pH 7.5 was used. Distilled water was added to stop the reaction and the membrane was dried by filter paper.

Densitometric analysis

PV samples run on SDS gradient gel and stained by Stains-all were analyzed on a CGA Cellomatic 2 densitometer at 555 nm, to quantify the amount of the single bands with respect to the total PV components.

Vitellogenin (VTG) assay

Plasma VTG titers were assayed using the method of Mañanós *et al*. (1994a). The sensitivity was calculated at 50% of binding, the intra-assay variation was 5.3% (N=5) and the inter-assay variation 9.8% (N=9).

Statistical analysis

For calculating hatching, the Kruskal-Wallis non-parametric analysis of variance followed by multiple comparisons as described by Connover (1980), was used.

The plasma VTG levels, quantitative analyses of PV components, and difference between weight were tested using ANOVA, and differences between groups were assessed through the Student-Newman-Kewls multiple composition test. Results were considered significant at $p<0.05$.

RESULTS

Animals

Enrichment of the diet with EFA did not alter the plasma VTG levels (3-4 mg/ ml) in any of the experimental groups compared with controls. Moreover, no significant differences in body weight (ca 2 kg/animal) were observed (data not shown).

Yolk components

In Western blot analysis, an antiserum raised against sea bass VTG recognized all components stainable by Coomassie blue indicating that all these components have VTG as precursor and correspond to LV. In contrast, the Stains-all staining proteins such as PV were not recognized (Fig.1).

The major proteins present in sea bass ovary during the reproductive period in the four groups studied and examined by SDS-PAGE showed that the LV components, stained by Coomassie blue, are localized

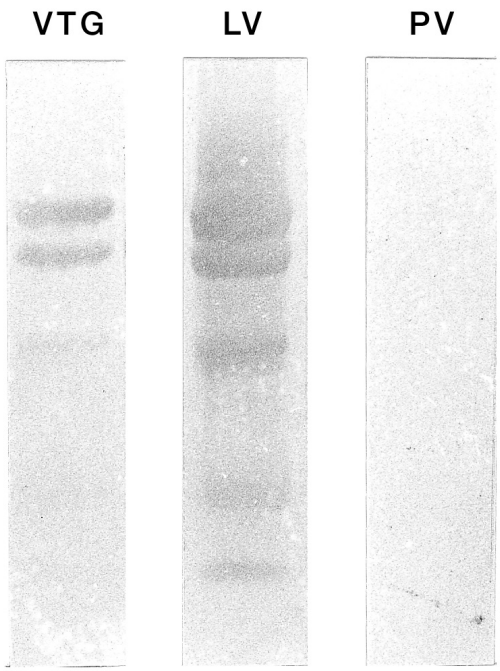

FIG. 1. – Western blot analysis of *Dicentrarchus labrax* L. vitellogenin (VTG), lipovitellin (LV) and phosvitin (PV). Homologous anti-vitellogenin antiserum was used as primary antibody.

at apparent molecular masses of 110, 96, 75, 30, 19, and 13 kDa. Comparing the electrophoretic pattern of the LVs from the four groups studied, slight differences were observed between the groups (Fig. 2a).

The supernatant containing PV after Bio-Gel P6DG desalting column, was subjected to SDS-gel electrophoresis and stained by a cationic stain, Stains-all, specific for highly charged peptides, and showed three major components with apparent molecular masses of 21, 17, and 8 kDa in all groups (Fig. 2b). The amount of each single band with respect to the total PV was determined by densitometer. This analysis was performed in the four experimental groups showing that in Groups A, C, and D the amount of the band at the lowest molecular weight (8 kDa) was higher (22.64, 28.40, and 25.70% of the total proteins, respectively) than that in Group B (10.08% of total proteins), as shown in Fig. 3. Moreover, in the fishes receiving extra EFA from September to February (Group A), a significative difference in the amount of the band at 17 kDa was observed, it being lowest in this group (Fig. 3).

In gel filtration on Superose HR12 (Bio-Rad) column applied to FPLC system, PV was resolved

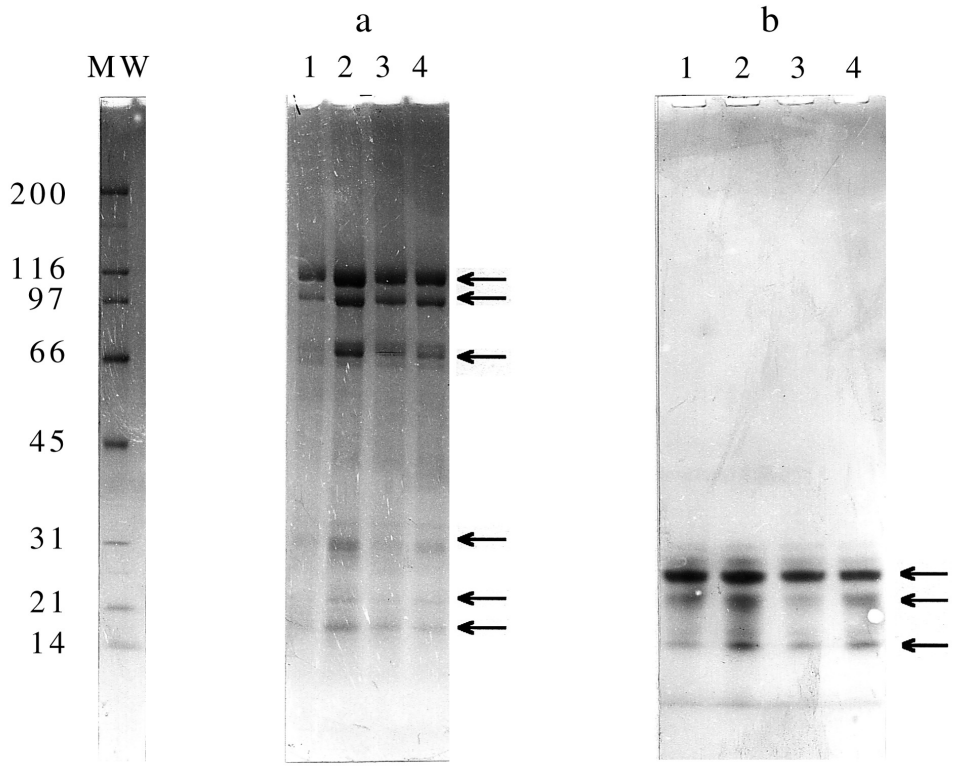

FIG. 2. – (a) SDS gel gradient electrophoresis (6.93-20.44% polyacrylamide) of lipovitellins from (1) Group B, (2) Group C, (3) Group A, and (4) Group D revealed by Coomassie blue.(b) SDS gel gradient electrophoresis (7-20% acrylamide) of phosvitin from (1) Group B, (2) Group C, (3) Group A, and (4) Group D revealed by Stains-all. Group B was fed with the high EFA diet from February to April; Group C from April to September; Group A from September to February; and Group D, all around the year MW= molecular weight.

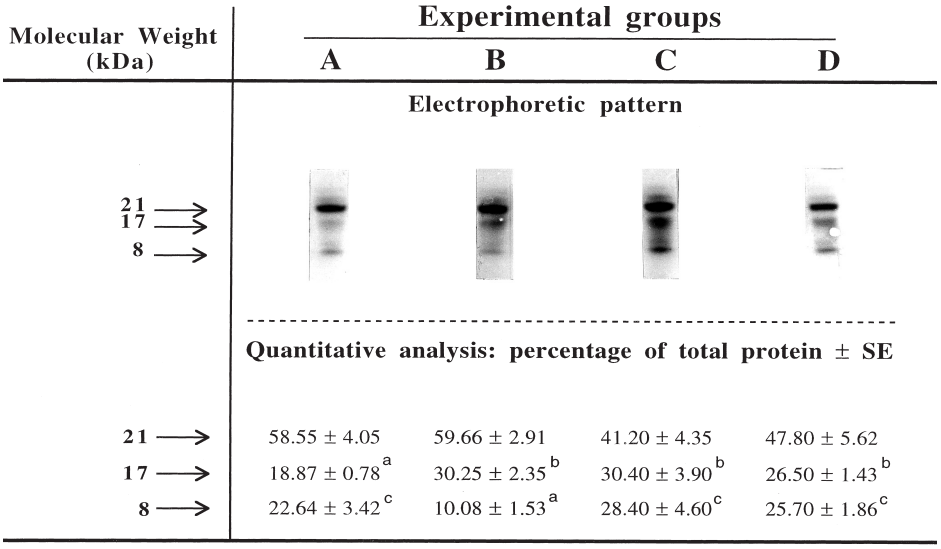

FIG. 3. – Quantitative analysis performed by densitometer on PV components isolated by SDS-PAGE showed a different abundance of the three PV components. The results are expressed as means ± SE of the percentages of total proteins. The insets represent a typical electrophoretic pattern of phosvitin components from each experimental group. Group A was fed with the high EFA diet from September to February; Group B from February to April; Group C from April to September; and Group D, all around the year. OD= ?

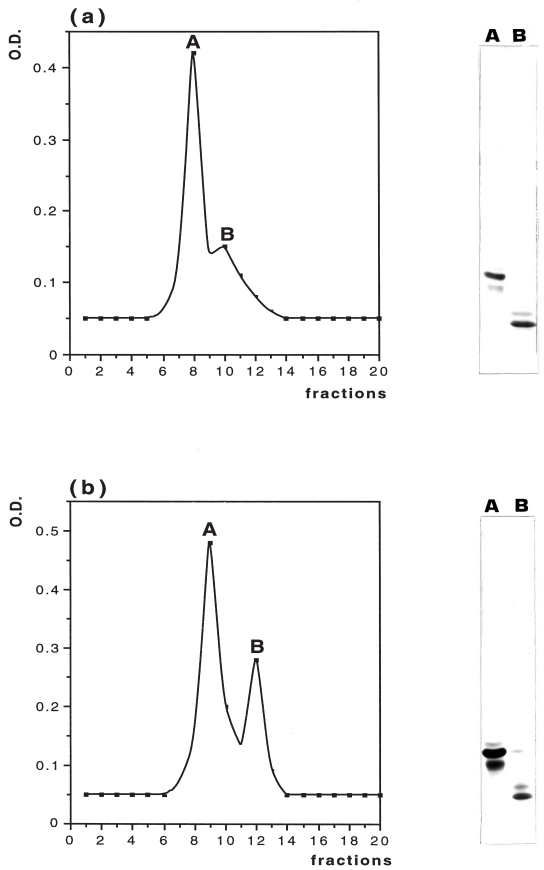

FIG. 4. – Resolution of the crude *Dicentrarchus labrax* phosvitin by size-exclusion chromatography of 200 µg of the $(NH_4)_2SO_4$- soluble fraction from yolk platelets from ovaries of Group B (a) and Group D (b). Group A was fed with the high EFA diet from September to February; group B from February to April; Group C from April to September; and Group D, throughoutthe year.

as two peaks. Groups A, C, and D, receiving EFA during vitellogenesis-maturation, post-êspawning, and all the year respectively (Fig. 4b), the area of the first peak represents about 65% of the total, and the second about 35%. In Group B (Fig. 4a), the area of the first peak represents about 78% of the total, and that of the second ca 22%.

The fractions obtained by gel filtration run on SDS-PAGE showed that the components eluted in the first peak were those with higher molecular weight, while 8 kDa was eluted in the second one.

Effect of diet on percentage of viable eggs and of hatching

Table 3 shows that both the percentage of egg viability and of hatching of Group B, receiving EFA for the shortest time and during the spawning peri-

TABLE 3. – Percentage of viable eggs and hatching, with respect to the total egg production of groups of sea bass receiving diets supplied with essential fatty acids at different times of the year. The results are presented as means ± SEM. Means in the same column with different letters as suffix are significantly different (P<0.05).

GROUP	% Viable egg	% Hatching
A (Sep-Feb)	$38\pm5.27_{a}$	$19\pm4.38_{a}$
B (Feb-Mar)	$17\pm3.89_{b}$	$4\pm1.73_{b}$
C (Apr-Sep)	$28\pm4.82_{ab}$	$13\pm3.74_{ab}$
D (All Year)	$47\pm5.91_{a}$	$23\pm4.14_{a}$

od, is significantly ($p<0.05$) lower than that of groups receiving EFA for at least six months. The highest percentage of viable eggs, as well as that of hatching, was found in Group D, fed on diets with EFA throughout the year.

DISCUSSION

In oviparous vertebrates, VTG is the precursor of yolk proteins. During embryo development, there is a rapid degradation of the yolk which produces free amino acids, fatty acids, phosphorus, calcium, etc..., and these yields will be utilized by the embryo during its development (Wallace, 1985; Wallace and Selman, 1985; Mommsen and Walsh, 1988; Selman and Wallace, 1989). In this study we first identified the major yolk protein components, the LVs and PVs. Multiple molecular weight forms of yolk proteins were found and by Western blot analysis, using a specific antibody raised against seabass VTG, we demonstrated that the major oocyte yolk components have VTG as precursor. Since only LV are recognized by the antibodies raised against VTG, we can speculate that most of the VTG immunodomains are located in the region of the molecule that after cleavage gives rise to LV subunits. The lack of PV reaction was not surprising; in fact, it seemed that PV is not immunogenic (Francis, 1952), which may be due to its very high negative surface charge. In denaturation condition, sea bass VTG breakdown was found as previously observed in this species by Mañanós *et al.* (1994b).

Afterwards, we addressed our attention to the effect of diet improved with EFA on: the size of the fishes; the levels of VTG in the plasma; the yolk proteins composition; and the egg viability and hatching. Apparently, diet improved with EFA for different times and during different periods of the reproductive cycle, does not play any important role either in fish growth or in plasma VTG levels. The improvement of EFA level in the diet influences yolk protein composition. Surprisingly, lipids in the diet do not affect LV components which contains all the lipidic part of the precursor, the VTG. Instead differences of the PV components were clearly demonstrated by densitometric analysis and also confirmed by gel filtration chromatography study. It is possible that the EFA composition of the diet influences the phosphorylation process during vitellogenesis. However, from our data we cannot state this because we have not analyzed the VTG phosphate content in the groups.

In previously published works (Ramos *et al.*, 1993; Navas *et al.*, 1997) it has been reported that the administration of diets with high EFA levels to seabass broodstock during five months, in vitellogenesis (September to February, Group A) or during post-spawning (April to September, Group C), has a beneficial effect on egg viability and hatching rates. At the same time the fatty acid composition of the eggs was clearly influenced by the fatty acid composition of the diet (Navas *et al.*, 1997). The animals under the short time EFA treatment were fed with the high EFA diet only during two months at the end of the spawnings. During this period the hepatic VTG production is diminished and the fatty acids of the diet cannot arrive to the developing oocytes. In fact, the short-time EFA treatment did not provoke any change in the fatty acid composition of the eggs (Navas *et al.*, 1997). The high levels of VTG previously detected in the sea bass during this period (Mañanós *et al.*, 1994 b; Carrillo *et al.*, 1995; Mañanós *et al.*, 1997) are probably provoked by the cross reactivity of reabsorbed yolk proteins in the inmunoassay used to measure VTG (Mañanós *et al.*, 1997).

Taken together, these findings suggest that the lipid composition of diet can improve oocyte composition, and in turn egg development. However, to understand the role played by EFA components during yolk protein formation, further deeper studies are necessary. In the present study we found that PV components undergo most of the modifications. Since PV is a very acidic protein, about half of whose aminoacids are phosphoserine, it will be interesting to study the possible role of EFA components on the phosphorylation process.

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