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Effect of dietary phospholipid level on the development of gilthead sea bream (Sparus aurata) larvae fed a compound diet

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Abstract:

The aim of the study was to determine the influence of dietary phospholipid (PL) levels on survival and development of first feeding gilthead sea bream (*Sparus aurata*) larvae. Larvae were fed from day 4 to 23 posthatching with an isoproteic and isolipidic formulated diet with graded levels of PL from 90–150 g kg^{-1} dry matter (DM). A dietary PL content of more than 90 g kg^{-1} DM seems to be necessary for sustaining growth of first feeding sea bream larvae. The survival rates of larvae fed the formulated diets (31–40% at day 23) were similar to those generally observed in marine aquaculture hatcheries with live prey feeding sequence. However, this high survival rate was not associated with high growth and the larvae showed, at the end of the study, a high proportion of individuals with abnormal liver and calculi in the urinary bladder. It is concluded that although the diets used here cannot be used in total replacement of live preys, they constitute a solid starting point for further nutritional studies with first feeding gilthead sea bream larvae.

Keywords: Sparus aurata ; larvae ; phospholipids ; compound diet ; first feeding; development

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Running title: Compound diet for first feeding sea bream larvae

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ABSTRACT

The aim of the study was to determine the influence of dietary phospholipid (PL) levels on survival and development of first feeding gilthead sea bream (*Sparus aurata*) larvae. Larvae were fed from day 4 to day 23 post-hatching with an isoproteic and isolipidic formulated diet with graded levels of PL from 9 to 15 % dry matter (DM). A dietary PL content of more than 9 % DM seems to be necessary for sustaining growth of first feeding sea bream larvae. The survival rates of larvae fed the formulated diets (31-40% at day 23) were similar to those generally observed in marine aquaculture hatcheries with live prey feeding sequence. However, this high survival rate was not associated with high growth and the larvae showed, at the end of the study, a high proportion of individuals with abnormal liver and calculi in the urinary bladder. It is concluded that although the diets used here cannot be used in total replacement of live preys, they constitute a solid starting point for further nutritional studies with first feeding gilthead sea bream larvae.

INTRODUCTION

A high increase in farmed fish production is expected during the next few years. But fry availability is still a bottleneck for the development of marine aquaculture industry. For the moment, fry production in marine fish hatcheries relies on a live prey feeding sequence. The use of live prey presents several disadvantages such as high cost, low reliability in production and variable nutritional quality. Therefore, the main objective in fish larval nutrition is to formulate a compound diet that can be substituted for live prey as early as possible during larval development (Watanabe & Kiron, 1994).

Recently, sustained good growth and survival (higher than that generally observed with live prey feeding) were obtained in European sea bass (*Dicentrarchus labrax*) fed exclusively compound diet from mouth opening (Cahu *et al*., 2003). However, for the gilthead sea bream *Sparus aurata,* in spite of research efforts devoted to the development of microdiets, it is still not possible to completely replace live prey during the first larval rearing phase (Fernandez-Diaz & Yufera, 1997; Yufera *et al*., 2000; Robin & Vincent, 2003; Robin & Peron, 2004).

The suitability of an inert diet for larval fish depends on the characteristics of both diet and larvae, as well as the rearing system used. More specifically, recent studies have yielded some progress in understanding the behaviour, physiology and nutrition of larvae when fed inert diets (Yufera *et al*., 2000; Cahu & Zambonino Infante, 2001). In this regard, the importance of dietary phospholipids (PL) for growth and survival of larvae has been demonstrated by various studies (for review Coutteau *et al*., 1997). Initial studies of Geurden *et al*. (1995) showed that larval development is variably affected by dietary PL sources and levels. Recently, Cahu *et al*. (2003) showed that

high dietary phospholipid level improved sea bass development and demonstrated that larvae have a high capacity to utilize phospholipids.

To explain the nature of the PL requirement and the role of these lipids during larval stage, several authors suggested the participation of PL in the intestinal absorption of neutral lipids (Coutteau *et al.,* 1997; Fontagné *et al.,* 1998; Salhi *et al.,* 1999). Thus, by histological analysis of the anterior intestine of larval carp, it was demonstrated that lipid accumulation in enterocytes of fish fed PL-free diet was prevented by adding Phosphatidylcholine (PC) to the diet (Fontagné *et al.,* 1998). The higher liver and hepatocyte volume of PC-fed fish confirmed the more important lipid export from the intestine when feeding PC. In analogy, feeding PC-rich diets to turbot resulted in higher levels of DHA in their lipid classes, as compared to fish fed the PC-poor and PL-deficient diets with the same amount of ethyl esters of polyunsaturated fatty acids (PUFA) (Geurden *et al.,* 1998). In addition to a higher DHA content, the lipid of turbot and sea bass fed dietary PC also consisted of significantly higher amounts of triglycerides, in agreement with a higher neutral lipid uptake. All together, these findings confirm a specific role of PC shown before in an *in vitro* study on rat intestine, which indicated that PC stimulated the synthesis and secretion of the apoB48 protein of triacylglycerol-rich lipoproteins (Field and Mathur, 1995). The observed growth-promoting effect of PL can thus, at least partially, be explained by an increased energy flux from the intestinal mucosa towards the blood. A promoting effect of Phosphatidylinositol (PI) on initial development of fish larvae has also been observed but the role of this lipid class seems to be different to those of PC in that larvae fed PI-rich and PC-poor diets display high survival rate but lipid accumulation in enterocytes, indicating a poor neutral lipid uptake (Geurden *et al* 1998). Although it is now well accepted that PI and its metabolically active derivatives (namely inositol

triphosphate and diacylglycerol) may regulate the protein kinase C activity and the maintenance of salt balances (Bell and Sargent, 1987), the exact nature of PI requirement is still not understood.

All together and in regard to the importance of PL for the initial development of fish larvae, the aim of the present study was to determine the influence of soy lecithin as a rich source of PC and PI (22% and 14%, respectively) on the survival and development of first feeding gilthead sea bream larvae. For this purpose we took advantage of the progress made on inert diet development for European sea bass larvae (Cahu & Zambonino Infante, 2001; Cahu *et al*., 2003).

EXPERIMENTAL PROCEDURES

Fish rearing and diets

Eggs were obtained from natural spawning of gilthead sea bream (*Sparus aurata*) broodstock maintained at the Ferme Marine de Douhet fish farm (La Brée-les-Bains, France). Larval rearing was conducted at the Ifremer – Station de Brest and lasted 23 days. Newly hatched larvae were transferred from incubators to conical fibreglass tanks (35 litres) with black walls (initial stocking density of 120 larvae.L^{-1}, i.e. 4200 larvae per tank). They were supplied with running sea water, which had been 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 g.L^{-1} respectively. Dissolved oxygen level was maintained above 6 mg.L^{-1} by setting the water exchange up to 30 % per hour (flow rate 0.18 litres.min^{-1}). The light intensity was 9 W/m^2 maximum at the surface. All animal procedures and handling were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1985).

Larvae were distributed into eleven tanks before mouth opening (day 1 post-hatching). Tank randomization was conducted at the start of the experiment; two tanks with larvae keep starved (negative control) and three tanks per dietary group. Larvae were fed from day 4 to day 23 post-hatching with dry microdiets (Table 1) formulated to contain 840 g.kg^{-1} of a basal mixture developed from a diet giving, in sea bass larvae, as good growth and survival results as live prey (patent WO0064273, 2000). In order to determine the influence of dietary phospholipid level without affecting fatty acids composition, diets of the three groups incorporated increasing levels of soybean lecithin (SDA, Marne la Vallée, France) and decreasing

levels of soybean oil (Bouton d'Or, France) and were designated as PL8, PL12 and PL16 (Table 1). Microdiets were processed as follows: dietary ingredients were mechanically mixed with water, pelleted and dried at 50°C for 20 min. The pellets were ground and sieved to obtain two sizes of particles; 75–150 μm used during the first 10 days, then 150–250 μm until the end of the experiment. Fish were continuously fed in excess for 18 h per day using a belt feeder.

Assayed composition of the diets was ($g.kg^{-1}$ DM): Crude protein 509 – 524; Crude fat 204 – 222, differing only by the lipid source used (Table 1).

Sampling and data analysis

To monitor growth, thirty larvae per tank were taken once weekly from each tank. Larval body length was measured under a binocular microscope. Ten larvae per tank were also sampled on day 5, 7, 10, 13, 16 and 19 and examined under a binocular microscope to determine several nutritional and physiological parameters such as the number of larvae having food in the digestive tract, the ingestion rate (IR) corresponding to the quantity of diet in the digestive tract, the liver morphology which become round and brown when degenerating, the presence of urinary calculi and spinal malformations, i.e. scoliosis, lordosis and coiled vertebral column. For monitoring IR, the following formula was applied for each tank: $IR = (\Sigma q / (3 \times N)) \times 100$ with q = quantity of diet in the digestive tract estimated with an arbitrary scale from 0 (no food) to 3 (maximum filling) and N = number of observations. The other mentioned criteria were determined in each tank as follows: (number of larvae with considered parameter / N) × 100 with N = number of observations. Finally, at the end of the study (day 23), larval survival rates were determined by counting individuals.

Statistical analyses

Results are given as mean ± standard deviations. Percentage data were arcsin ($X^{1/2}$) transformed before analysis. Diet-related differences were analyzed using one-way ANOVA followed by the Newman-Keuls multiple range test at the 0.05 significance level with the computing program STAT-ITCF (ITCF, 1988).

RESULTS

Larval survival and growth

All the unfed larvae died by day 9-10 after hatching (data not shown). In contrast, 31 to 40% of larvae fed micro-diets survived to day 23, but did not show statistical difference between diet treatments (Table 2). In contrast, the larval body length monitoring revealed differences between the three groups of fed larvae. At day 17, larvae fed PL8 were significantly smaller than those fed PL12 and PL16. However, the beneficial effect of high dietary phospholipid content on larval growth grew blurred thereafter and was not significant any more at day 23.

Feeding incidence and physiological status

The larvae ingested the diets from first feeding and continued to accept them throughout the experimental period irrespective of the diet (Fig. 1A). As soon as day 5, 90% of fed larvae showed food in the gut. This percentage ranged from 90 to 100% up to day 10 and decreased in the second week of the experiment to reach 70% at day 19. No significant difference was found between the three groups of fed larvae. The IR also reached a maximum level between day 5 and day 10 (ca 65%) and decreased progressively in the second week of feeding to reach 38% at day 19, with no statistical difference between the different fed groups (Fig. 1B). In contrast and as expected, the unfed group showed very low percentage of larvae with food in the gut and very low IR value (Figs. 1A and 1B). The presence of food in the digestive track of unfed larvae is somewhat surprising and could be due to cannibalism between larvae as already observed in previous studies. However, all

the unfed larvae died by day 9-10 after hatching indicating that this phenomenon has no incidence on larval development.

The morphology of liver, which is triangular and transparent in normal larvae and become round and brown when degenerating, was also monitored (Fig. 2). As expected, the percentage of unfed larvae showing abnormal liver was high as early as day 5 and reached 90% by day 7. In contrast, in the fed groups, between days 5 and 16, the percentage of larvae with degenerated liver did not exceed 20%. Nevertheless, the last sampled point (day 19) showed an increase of this percentage up to 30% revealing an aggravation of the physiological state of fed larvae irrespective of the diet at the end of the experiment.

It has been shown that the presence of urinary calculi induces severe mortality of sea bream larvae (Modica *et al*., 1993). The causes of calculi formation are not well understood but it seems that an in-adapted diet could contribute to their appearance. This physiological parameter was also monitored here (Fig. 3). As expected, the unfed larvae showed a high percentage of individuals with urinary calculi that reach 90% at day 10 (Fig. 3C). In the fed groups, the percentage of larvae with urinary calculi increased progressively, not exceeding 10% at day 5 and reaching values higher than 40% at day 19. However, no significant difference was found between the three different fed groups.

Finally, spinal malformations such as scoliosis, lordosis or coiled vertebral column were monitored and irrespective of the group of larvae considered (fed or unfed) the percentage of larvae with skeletal malformation did not exceed 10% (data not shown).

DISCUSSION

The present study described a feeding experiment of gilthead sea bream *Sparus aurata* larvae using exclusively dry microdiets. The previous attempts of compound diet feeding of gilthead sea bream larvae right from mouth opening have reported high mortality and poor growth (Yufera *et al*., 1999; Robin & Vincent, 2003; Robin & Peron, 2004). The determination of dietary requirement and the formulation of a diet for marine fish larvae represented for several years a major objective of many laboratories. In contrast, diet formulation has been easily achieved for some freshwater species larvae, which are bigger at hatching.

The tested diets, only differing by their lipid mixture composition, induced a survival rate (31-40% at day 23) much higher than those obtained previously with exclusive dry microdiet feeding (Yufera *et al*., 1999; Robin & Peron, 2004) and similar to that generally observed in marine aquaculture hatcheries with live prey feeding sequence. However this rather high survival rate was not associated with a high growth result. Indeed, sea bream larvae fed live prey can reach around 7 mm at day 17 (Chatain, 1994). This poor growth performance remains the main bottleneck for the use of dry microdiet for sea bream larvae feeding from mouth opening. Yufera *et al*. (2000) failed to obtain effective growth by using microcapsules as first food whereas they obtained high growth rate when microcapsules were fed to larvae after a few days of being fed *Brachionus plicatilis*. Similarly, two recent studies on sea bream larvae fed microdiets from mouth opening showed some but significantly lower growth compared to what could be obtained with live food (Robin & Vincent, 2003; Robin & Peron, 2004). Put together, these results indicate that the diets tested in this study as those of previous studies do not meet fully the nutritional requirement of first feeding

sea bream larvae. In this regard, at the end of the present experiment, the larvae showed an increasing percentage of individuals with an abnormal liver and calculi in the urinary bladder (Figs. 2 and 3).

Several studies conducted on different fish species (Kanazawa *et al*., 1981; Shields *et al*., 1999) showed that fish do require dietary phospholipids, and that this requirement is higher in larvae than in juveniles (Kanazawa, 1993). In the present study, a significant difference appeared at day 17 in larval growth among the experimental groups. Indeed, PL8 fed larvae were significantly smaller than those fed PL12 and PL16. However, the beneficial effect of high soy lecithin contents on early larval growth was not significant any more at day 23. This was probably due to an aggravation of general state of larvae at the end of this period, also revealed by the high proportion of individuals with an abnormal liver and with calculi in the urinary bladder and by the decreasing percentage of larvae with food in the gut (Figs. 1-3). Therefore, the phospholipid content of PL8 diet seems to be limiting for the growth of first feeding sea bream larvae. This result is in agreement with those obtained in the European sea bass, showing that an amount of 11-12% of dietary phospholipid is needed and sufficient for a good development of larvae fed dry diet right from first feeding onwards (Grangier *et al*., 2001; Cahu *et al*., 2003). However, our results as those on sea bass were obtained with the use of phospholipid from vegetable sources (soy lecithin) and could be somewhat different with other PL sources especially those of marine origin rich in n-3 PUFA. Indeed, according to Sargent *et al*. (1999), some developmental promoting effect of PL are modulated by their fatty acid composition and marine PL appeared to be essential for fish larvae development. In the case of sea bass, the small amount of marine PL contained in fishmeal and fishmeal hydrolysate of the experimental diets was sufficient to allow good larval

growth and survival (Cahu *et al.*, 2003). Further studies will be necessary to demonstrate whether gilthead sea bream responds differently to different PL sources.

In conclusion, a dry microdiet sustaining a survival rate of 31-40%, similar to that generally observed in marine aquaculture hatcheries with live prey feeding, has been formulated for the first time. This diet is efficiently accepted from the beginning of exogenous feeding. However, the larvae fed with these microdiets exhibited poor growth performance and increasing abnormalities in the end of the experiment. Therefore, although this diet cannot be used in total replacement of live preys, it constitutes a solid starting point for further nutritional studies. Besides the development of practical diets for juvenile production (Watanabe & Kiron, 1994; Cahu & Zambonino Infante, 2001), compound diets, and especially semi-purified diets, are useful tools to study the nutritional requirements in larval stages. Such an approach was successfully used in the nutrition of larvae of some fresh water species (Radünz-Neto *et al*., 1994; Radünz-Neto *et al*., 1996) and since few years of marine fish species (Cahu *et al*., 2003).

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Fig. 1. Diet ingestion monitoring. (A) Percentage of larvae having food in the digestive tract at day 5, 7, 10, 13, 16 and 19. (B) Ingestion rate (IR) of formulated diets at day 5, 7, 10, 13, 16 and 19 calculated as described in experimental procedures. (C-F) Quantity of diet in the digestive tract estimated with an arbitrary scale from 0 (no food) to 3 (maximum filling).

Fig. 2. Evolution of the percentage of larvae with abnormal liver. The liver of sea bream larvae (red star), which is triangular and transparent in normal larvae (A) and become round and brown when degenerating (B), was monitored at day 5, 7, 10, 13, 16 and 19 (C).

Fig. 3. Evolution of the percentage of larvae with calculi in the urinary bladder. (A) urinary bladder (red star) without calculi. (B) urinary bladder with calculi. (C) Increase of the percentage of larvae with urinary calculi during the course of the experiment.

Table 1. Diet formulation and composition

Diet	PL8	PL12	PL16
Formulation ($g.kg^{-1}$)			
Basal diet[a]	840	840	840
Soyabean lecithin[b]	80	120	160
Soyabean oil[c]	80	40	-
Composition			
Dry matter[d] ($g.kg^{-1}$ DM)	958	952	938
Crude protein[e] ($g.kg^{-1}$ DM)	509	522	524
Total lipids[f] ($g.kg^{-1}$ DM)	222	208	204
Phospholipids[g] ($g.kg^{-1}$ DM)	89	110	149

[a] Basal diet (per kg diet): 400g fish meal, Sopropêche, France; 160g yeast, Fromagerie Bel, France; 150g soluble fish protein concentrate, CPSP G, Sopropêche, France; 80g vitamin premix; 50g mineral premix. Vitamin mixture ($g.kg^{-1}$ vitamin mix): retinyl acetate, 1; cholecalciferol, 2,5; dl--tocopherol acetate, 10; menadione, 1; thiamin-HCl, 0.1; riboflavin, 0.4; d-calcium panthothenate, 2; pyridoxine-HCl, 0.3; cyanocobalamin, 1; niacin, 1; choline, 200; ascorbic acid (l-ascorbyl-2-polyphosphate), 20; folic acid, 0.1; d-biotin, 1; meso-inositol, 30. All ingredients were diluted with a-cellulose. Mineral mixture ($g.kg^{-1}$ mineral mix): KCl, 90; KI, 0.04; $CaHPO_4 \cdot 2H_2O$, 500; NaCl, 40; $CuSO_4 \cdot 5H_2O$, 3; $ZnSO_4 \cdot 7H_2O$, 4; $CoSO_4$, 0.02; $FeSO_4 \cdot 7H_2O$, 20; $MnSO_4 \cdot H_2O$, 3; $CaCO_3$, 215; MgOH, 124; Na_2SeO_3, 0.03; NaF, 1.

[b] Soybean lecithin, DAFA LPR, SDA, France.

[c] Soybean oil, Bouton d'Or, France.

[d] After drying at 105°C, 24h.

[e] By the Kjeldahl method (N $\times$ 6.25).

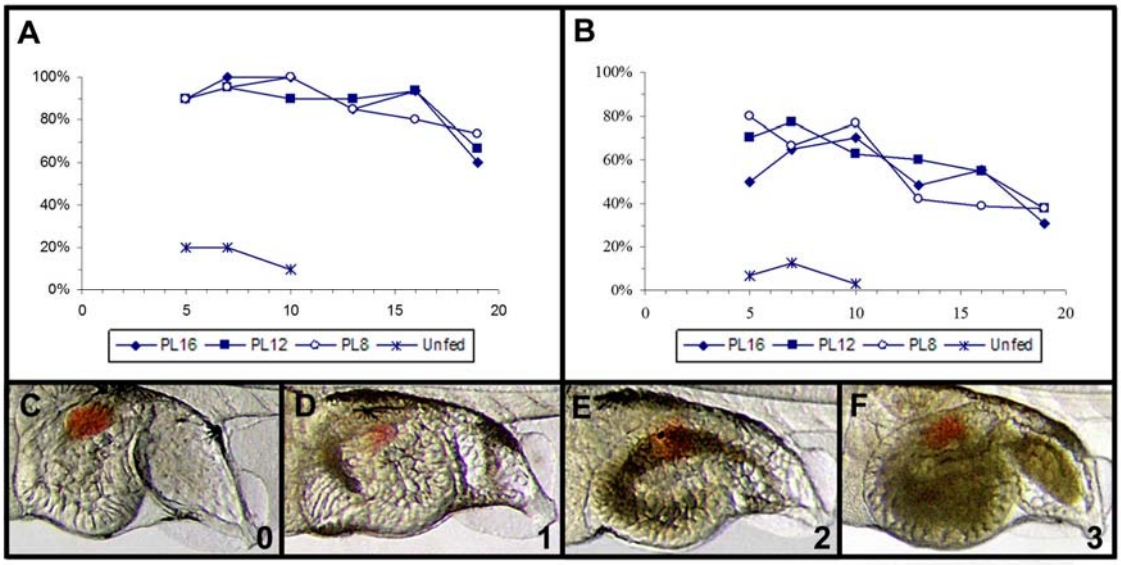
[f] Extracted according to Folch et al. (1957).

[g] By the Juaneda and Roquelin method (1985).

Table 2. Survival and growth rates of gilthead sea bream larvae fed the experimental diets.

Diet	PL8	PL12	PL16
Survival (%)			
day 23	38 ± 5	40 ± 2	31 ± 5
Total length (mm)			
day 4	3.5 ± 0.2	3.5 ± 0.2	3.5 ± 0.2
day 10	4.0 ± 0.1	4.2 ± 0.2	4.1 ± 0.2
day 17	4.7 ± 0.0[a]	4.9 ± 0.2[b]	5.0 ± 0.1[b]
day 21	4.7 ± 0.1[a]	5.0 ± 0.2[a]	5.2 ± 0.1[b]
day 23	5.0 ± 0.2	5.1 ± 0.2	5.3 ± 0.1

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).



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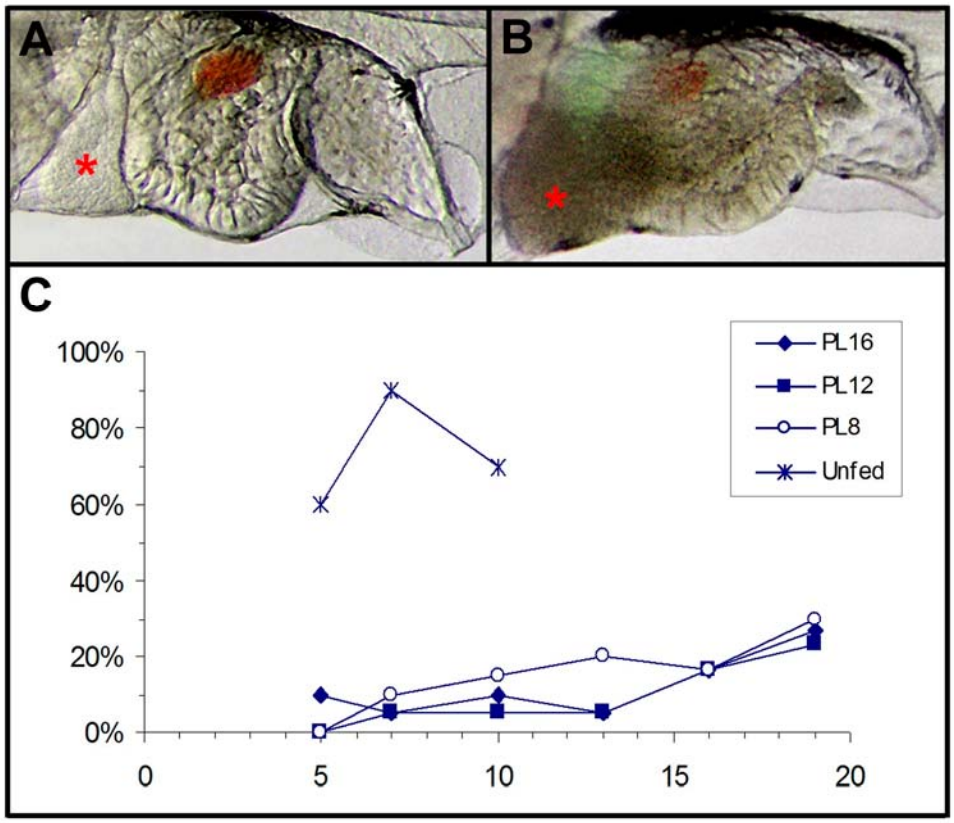

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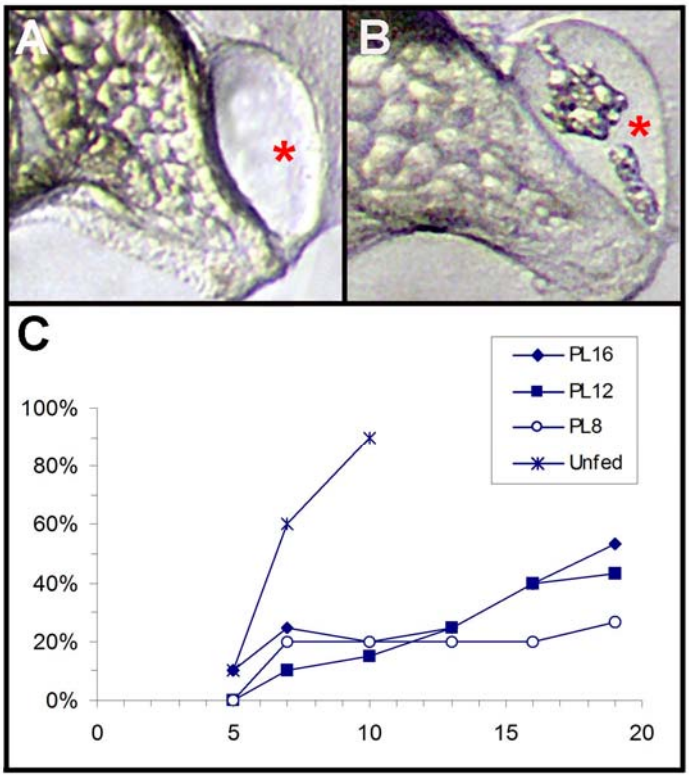

Seiliez et al., 3