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Substitution of live food by formulated diets in marine fish larvae

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Abstract:

Until recently, it was considered impossible to feed newly hatched marine fish species with a compound diet. Substituting a compound diet for live prey was performed several weeks after hatching, depending on the species. Compound diets were well ingested at the early stage but larvae died with a gut full of food, suggesting that larvae were unable to digest the compound diet. The hypothesis was that younger larvae have insufficient digestive enzymes to thrive on compound diets, and that exogenous enzymes, provided from live prey, are necessary for early stages.

The organogenesis of marine fish larvae is not completely achieved at hatching and histological studies have revealed that the anatomy of the digestive tract undergoes developmental changes over some weeks. Nevertheless, biochemical studies over 20 years have shown that most of the digestive enzymes are present in young larvae. Recent studies have provided better understanding of digestion mechanisms in larvae and have led to proposed dietary compositions meeting larvae nutritional requirements. Pancreatic digestive enzymes are detected before mouth opening. Their synthesis is not induced by diet ingestion, but secretory mechanisms in the pancreas, and so enzymatic action, become efficient chronologically after those of synthesis. Inadequate diets can delay the onset of secretion mechanisms. The ratio of secreted enzymes to total enzymes indicates the nutritional value of the diet ingested by the larvae.

At the intestinal level, cytosolic enzymes, which are peptidases, exhibit high activity in the early stages, suggesting a high capacity in larvae to digest protein hydrolysate. Indeed, larvae growth and survival is improved by the incorporation of a moderate concentration of peptide or hydrolysate in the diet. Peptidase activity abruptly decreases around day 25 in sea bass, concurrent with an increase in enzymes of the brush border membranes. This corresponds to a normal maturation process of enterocytes. Compound diets can slightly delay the onset of this maturation process, and inadequate diet can prevent it, leading to near death of the larvae. A proper onset of the maturation process has been associated with high larvae survival.

The early developmental stage larvae exhibit high hydrolytic capacity, related to their weight. Enzyme activity pattern is age-dependent, but can be modulated by diet composition. Thus, larvae have the ability to digest and thrive on compound diet, if this diet is well adapted. Larvae have different specificities in digestion and nutritional requirements when compared to juveniles. Taking these specificities into consideration, recent research has led to the formulation of a compound diet that was well adapted for larvae from mouth opening, and could totally replace live prey.

Keywords: Fish larvae; Gut maturation; Protein hydrolysate; Intestinal enzymes; Microparticulate diet; Pancreatic enzymes; Phospholipid; Protein

1. Introduction

Production of marine fish juveniles in commercial hatcheries still depends on supply of live preys, such as rotifers and Artemia. Compound diet substitution for live prey is crucial for lowering production cost and for sustaining production of high and constant quality juveniles. Until now, compound diet substitution for live preys, known as weaning, is only performed after some weeks of life in marine fish in hatchery, when fresh water species can be fed compound diet as early as mouth opening.

During the last decade, the pre-weaning period has been greatly reduced thanks to conclusive results obtained in laboratory. As an example, Person Le Ruyet et al. (1993) formulated a diet adequate to sustain good growth and survival in European sea bass (Dicentrarchus labrax) from day 40 (40 days after hatching), when the weaning of this species was conducted at day 55 in hatchery. In 1997, a significant growth and a good survival (close to that obtained with live prey feeding) were obtained in sea bass fed only compound diet from day 20 (Zambonino Infante et al.). Recently, it was reported that 35% of sea bass larvae, fed exclusively compound diet from mouth opening, survived at day 28 (Cahu et al., 1998). The survival rate can be attributed to the efficiency of the compound diet, since unfed sea bass larvae do not survive after day 15, and cannibalism is not observed in young Dicentrarchus labrax stages. Concurrently, some survival were obtained in other marine species fed compound diet from mouth opening, such as sea bream Sparus aurata (Fernandez-Diaz and Yufera, 1997) or red sea bream Pagrus major (Takeuchi et al., 1998).

Formulation of compound diet adequate for fish larvae is not easy to achieve because the estimation of nutritional requirements of fish larvae cannot be conducted by traditional nutritional approaches, since, for the moment, commercially formulated diets do not support the growth of larvae. Moreover, as already pointed by Dabrowski in 1984, data obtained in juvenile fish are of little help when studying the requirements of larval stages, since

mechanisms of digestion and absorption change during larval development, nutritional requirements also change.

This review attempts to explain some physical particularities of the diet for larvae and to describe the onset of digestion mechanisms during larvae development and the specific nutritional requirements of early stages.

2. Compound diets for marine fish larvae: physical aspects

2.1. Size

Diet must be prepared as microparticles, whose size must be adapted to the size of the larvae mouth. As an example, size of the microparticulated diets used in our experiments was 50 to 125µm for sea bass at first feeding, then 125-200 µm from Day 14 to Day 25, then 200-400 µm to D 40 (Cahu and Zambonino Infante, 1994). The size of commercial microparticles used in hatchery for sea bass or sea bream weaning, used from Day 40, is generally 400 to 600 µm. Microparticles must be well calibrated to minimize waste.

Accurate size of the microparticles is essential. Particularly small microparticles (less than 50 µm diameter) cannot be easily detected by larvae, whereas large ones are difficult to ingest and may even promote a blockage of the digestive valve (Walford et al., 1991). Fernandez Diaz et al. (1994) showed clearly that sea bream larvae select the size of ingested microcapsules, in relationship to their size and their mouth width. Larvae below 4.5 mm in total length selected particles between 50 and 150 µm in diameter; at greater than this length, larvae selected particles in the range 151 to 250 µm, and from 6 mm total length, larvae ingested particles larger than 250 µm. This experiment was conducted with soft and deformable wall. But selected size was always lower with microparticles with hard and rigid wall.

The composition of microparticles must be homogenous, so, ingredients must be incorporated as very fine meal. The size of meal particles must be much smaller than the size of the final dietary microparticle. Some type of meal, such as fish meal, must be ground and sieved before being included in microparticles.

2.2. Manufacture techniques

Nutrient leaching is one of the problems in developing suitable diets for fish larvae. Particles must be water-stable, palatable and digestible. Diets used for late weaning (after Day 40) in the hatchery can be crumbled, prepared by grinding and sieving pellets, but diets of smaller size must be prepared in microbound, microcoated, or microencapsulated form.

In microbound diets, the powdered ingredients are microbound with a water stable matrix such as agar, carrageenan or calcium alginate (Lopez-Alvarado et al., 1994) or by a protein such as casein or zein (Person-Le Ruyet et al., 1993). Larvae of fresh water fish species, such as sturgeon, may have a weak ability to break and digest carrageenan matrix, limiting the utilization of the nutritional content of carrageenan microbound diet (Gawlicka et al, 1996). Conversely, sea water species possess in their gut bacteria synthesizing carrageenases and this may explain their ability to digest carrageenan microbound diets. Indeed, Kanazawa and Teshima (1988) reported good growth and survival in starry flounder and red sea bream fed carrageenaan microbound diet. Microcoated diets are produced by using a glucidic (carrageenan, alginate) or proteic (gelatin, zein) binder. Microencapsulated diets are prepared with a cross-linking agent (Yufera et al., 1999). Microencapsulation produces regular shape and water stable microparticles, but the microcapsules can be difficult to digest. Fernandez Diaz and Yufera (1995) showed that the ability of larvae to break microcapsules depends on the thickness of the capsule coating. Microcapsules isolated using alcohol as dispersant have hard shell, when the use of gelatin as dispersant resulted in soft shell. Yufera et al. (1999)

reported efficient growth of sea bream larvae fed from day 8 to day 15 post-hatching with a microcapsule manufactured using gelatin as dispersant. Table 1 presents some formulations of microdiets prepared using these different processes.

2.3. Distribution and ingestion of the diet

Dietary microparticles must be distributed in large excess. Indeed, early stage larvae have a limited movement and microparticles must be caught during their fall in the water column. Yufera et al. (1999) reported good results with low density microcapsules (400-600 g/L), sinking at about 25 cm/h average. Ingestion is triggered by visual and chemical stimuli. Light intensity, the color of the microparticles and of the tank are essential for ingestion. Some pigments, such as asthaxanthin, have been incorporated in microparticles, more for improving the visibility of the particle by larvae than for their nutritional value. Free amino acids, such alanine, glycine and arginine and the compound betaine, have been identified as efficient chemical stimulator for microdiet in gilthead sea bream larvae (Kolkovski et al., 1997).

In most of the species, larvae exhibit a catching behavior and do ingest inert microparticles from the first feeding. It is particularly easy to check the ingestion of particles by larvae by examination of the gut content under a light microscope. Yufera et al. (1995) proposed a method, based on the number of microcapsules counted in the gut, to quantify the ingestion rate in fish larvae. Ingestion rates in sea bream larvae increased from 0.5-3 µg.larva^{-1}.h^{-1} at first feeding to 18-25µg.larva^{-1}.h^{-1} for 20-day-old larvae. The authors concluded that microcapsules are ingested at similar rates than living prey.

Thus, ingestion is not a limiting factor for compound diet feeding in larvae. It can be wondered if larvae can really digest compound diets, and if the knowledge in larvae nutritional requirement is sufficient to formulate an adequate diet.

3. Digestion in fish larvae

3. 1. The onset of digestive enzymes

Different authors have hypothesized a deficiency in digestive enzymes in very young larvae to explain the unsuccessful results in compound diet feeding (Dabrowski and Glogowski, 1977; Lauff and Hofer, 1984). A potential role of the enzymes of prey organisms in larval digestion has often been argued, but without conclusive results. We have estimated the trypsin activity contribution of Artemia at maximum 5% of the total assayed activity in 20-day-old sea bass larvae (Cahu and Zambonino Infante, 1995). Recently, Kurokawa et al. (1998) estimated the protease activity derived from rotifers to 0.6% of the total protease activity in the intestine of two-day-old Japanese sardine. It seems that the enzymes of ingested live prey are not a substantial contribution to the digestion in young larvae. Moreover, the addition of pancreatic enzymes in microparticles has been tested for improving compound diet digestion by fish larvae. This exogenous enzyme supplementation led to an enhanced diet assimilation and growth improvement in gilthead sea bream larvae (Kolkovski et al., 1993) but was ineffective in sea bass larvae (Kolkovski et al., 1996). This last result is consistent with results obtained in mammals: Officier (1995) showed that enzyme supplement failed to improve growth in piglets during pre and post-weaning periods.

Fish larvae do not lack digestive enzymes. The onset of digestive functions, associated to morphological transformations, follows a sequential chronology in developing fish like that in developing mammals. The digestive tract of fish larvae is not achieved at hatching, but undergoes major developmental changes over several weeks (Vu, 1983; Cousin et al., 1985; Boulhic and Gabaudan, 1992). Particularly, the stomach is not differentiated at hatching. The development of this organ occurs several weeks later in marine fish. In sea bass, for example, the stomach develops from Day 25 to 30 (Vu, 1983). Pepsin activity is detected from this date, but the lack of stomach do not hinder protein digestion, since protein hydrolysis is

ensured by several pancreatic and intestinal enzymes (Zambonino Infante and Cahu, 1994a). Recent studies have more particularly focused on the functional changes in the digestive tract, during larval development, by studying the onset and the variation of pancreatic and intestinal digestive enzymes and the response of these enzymes to diet concentration and composition.

3.2. Variation of pancreatic enzymes during development and effect of diet composition

Results obtained by Pedersen et al. (1987) on Clupea harengus, Zambonino Infante and Cahu (1994b) on Dicentrarchus labrax and Ribeiro et al. (1999) on Solea senegalensis confirmed that the synthesis process of pancreatic enzymes is not induced by food ingestion. The specific activity of the main pancreatic enzymes follows a similar pattern during development. This activity, expressed as the activity of the enzyme related to protein concentration of the larvae, increases from hatching to Day 20 in sea bass, then decreases until Day 25, and remains at a same level during post-larval development (Zambonino Infante and Cahu, 1994b). This pattern demonstrates that digestive capacity of young larvae is very high, related to their weight. It also reveals that the synthesis process is linked to age. Amylase is a good example of the influence of age on the expression of pancreatic enzymes. Specific activity of amylase is very high during young larval stages and this activity decreases during the development of larvae. Higher amylase mRNA levels are found in young larvae than in old larvae: 20 day old sea bass larvae exhibited amylase mRNA levels two times higher than those found at day 29. The coordinated decrease between specific activity and mRNA levels of amylase suggests a transcriptional regulation of amylase expression during larval development (Fig. 1). The decrease in amylase is observed irrespective of the dietary glucide concentration. This suggests that the decrease in amylase specific activity during larvae development may be genetically programmed. Variation in amylase expression during fish larvae development can be compared to the lactase decline during mammals

development. Amylase, as lactase in mammals, can be considered as a characteristic enzyme of the post-natal period. Nevertheless, amylase expression can be modulated by dietary glucide concentration, since the decrease of amylase activity was slower in larvae fed diet incorporating 25% glucides than in larvae fed diet including only 5% glucides. The ontogeny of digestive enzymes is primarily 'preprogrammed' and only subtly modified by the diet composition (Henning et al., 1994).

This modification in activity of pancreatic enzymes in response to diet changes is efficient from the earlier stages. The higher amylase specific activity in sea bass larvae fed diet incorporating high starch level was obtained for 18-day-old larvae as well for 35-day-old larvae. This observation reveals an adaptation of the enzyme activity (amylase) to the concentration of its substrate (glucides). Péres et al. (1998) demonstrated that the regulation of amylase by dietary glucides occurs mainly at a translational level (Fig.2). In the same way, feeding sea bass larvae with diets containing different protein concentrations (from 30% to 60% / dry matter) led to an increase in trypsin activity in 35-day-old larvae, but failed to modify trypsin activity in 18-day-old or 28-day-old larvae. We assumed that mechanisms involved in amylase regulation are efficient since early stages, while those related to trypsin become efficient later. Experiment conducted by feeding larvae with two different protein sources showed that protein nature modulated trypsin mRNA transcription. This finding shows the importance of the protein nature in compound diet for larvae.

In sea bass larvae, the mechanisms controlling the dietary adaptation of trypsin and amylase are independently regulated and age-dependent. The phenomenon of age-dependent regulation of enzyme synthesis has been described in mammals (Henning et al., 1994).

The presence of lipolytic enzymes in young larvae was debated for a long time (Cousin et al., 1987; Koven et al., 1993), but recent studies reported strong evidences of activity of phospholipase A2 and lipase in young larvae of different species, such as red drum, striped

bass or Atlantic halibut (Holt and Sun, 1991; Ozkizilcik et al., 1996; Evans et al., 1998). Particularly, activity of lipase and phospholipase A2 were revealed in 15-day-old sea bass larvae (Zambonino Infante and Cahu, 1999). A direct response of phospholipase to the dietary concentration of phospholipid was shown in 38-day-old sea bass larvae fed diets containing 2.3 to 6.6% phospholipid. Phospholipase would be regulated at a post-transcriptional level in sea bass larvae: PLA2 activity continued to increase in groups fed diets containing between 4.5% to 6.6% phospholipids when mRNA levels remained constant. Direct response of lipase to its substrate, triglycerides, also appeared, but a plateau observed in the activity of this enzyme suggested that maximal capacity of lipase synthesis was reached for 15% triglycerides in diet. Therefore, regulation of lipolytic enzyme synthesis seems to be efficient in young larvae, explaining why larval growth is so dependent on dietary lipid levels.
The mRNA studies conducted on digestive enzymes of sea bass larvae showed that the molecular mechanisms which control the dietary adaptation of pancreatic enzymes are independently regulated, age-dependant and influenced by the composition and the quantity of the diet (Pérès et al., 1998). Fish larvae have, to a certain extent, capacity to adapt their enzymatic activity to diet composition, but formulation of a larvae diet must take into consideration the genetically programmed pattern of enzymes.

3.3. Variation of intestinal enzymes during development and effect of diet composition

After digestion by pancreatic enzymes, the hydrolysis of dietary components proceeds with intestinal enzymes. Some intestinal enzymes, such as peptidases, are located in cytosol of enterocytes. We showed that leucine-alanine peptidase exhibits high activity in young sea bass stages, then this activity markedly decreases around Day 25. Most of the intestinal enzymes involved in digestive process are mainly located in brush border membranes of enterocytes. These enzymes, such as alkaline phosphatase, γ glutamyl transpeptidase, N

aminopeptidase, maltase are detected very early during the larval development. Indeed, aminopeptidase activity was detected at hatching in Solea solea (Alliot 1979) and Scophthalmus maximus (Cousin et al., 1987) by histochemistry and in Paralichtys olivaceus by immunological reactivity (Kurokawa and Suzuki, 1998). It has been shown that activity of the brush border membrane enzymes abruptly increase around the third week of life in several species, including sea bass (Cahu and Zambonino Infante, 1997), sole (Ribeiro et al., 1999) or red drum (Buchet et al., 1997). The marked decrease with age of cytosolic enzymes and the concurrent and abrupt increase in alkaline phosphatase and aninopeptidase N in intestinal brush border membranes (Fig. 3) characterize the normal maturation of the enterocytes in developing animals (Henning, 1987). Brush border membrane digestion represents an adult mode of digestion.

Ingestion of inadequate diet may delay or prevent the genetically programmed sequence of intestinal maturation, leading to larvae death. Hydrolyzed proteins incorporated in diet facilitate the maturation processes. Indeed, it has been shown that increase in brush border membrane enzyme activity and decrease in leucine alanine peptidase occurs earlier in larvae fed diet containing protein hydrolysate than in larvae fed diet containing native protein (Cahu et al., 1999). Good growth and survival in larvae experimental rearing has often been related to early maturation of enterocytes (Zambonino Infante et al., 1997). The effect of others factors, such as epidermal growth factor, insulin growth factor, polyamines or the amino-acid L-glutamine on intestinal maturation has been evidenced in mammals and is worth investigating in fish. Among the polyamines, a positive effect of spermine on intestinal tract was shown (Péres et al., 1997).

Taken together, these results show that digestive functions of the different digestive organs (the pancreas and intestine, but not the stomach) are efficient before the onset of exogenous feeding, but a formulated microdiet has to meet specific nutritional requirements of the larvae.

4. Nutritional requirements

The changes in larval digestive equipment during larval development let suppose that the nutritional requirement are not similar between larvae and juveniles. Indeed, a dietary formulation sustaining good growth in juveniles induces poor results in larval growth and survival. Recent studies have revealed specificities in larvae nutritional requirements.

Most of the works conducted on nutritional requirements in fish have focused on lipid requirements (for review, see Watanabe and Kiron, 1994; Sargent et al. 1999). Indeed, as no compound diets for larvae were available, studies on lipid requirements were easier to conduct, because total lipid content or fatty acid profile can be modified in live prey, while it is quite impossible to change the amino acid profile of an organism. Nevertheless, growth is essentially protein deposition, and adequate proteins must be supplied to sustain optimal growth.

The use of formulated diet allowed investigating protein requirement in larvae, to obtain the most conclusive data on lipid requirements, and to consider the glucidic and vitaminic nutrition.

4.1. Lipid requirements

Eggs of marine fish exhibit a high lipid content (around 20% in sea bream). Lipids are, with free amino acids, the most important energy reserve in marine fish embryos (Vetter et al., 1983; Ronnestad et al., 1999). In a recent review, Sargent et al. (1999) argued that fish egg lipid composition square with the lipid requirement for marine fish larvae.

Many experiments have been conducted to determine the optimal lipid composition in diet formulated for marine fish larvae and particular attention has been paid to phospholipid and highly unsaturated fatty acid (HUFA) requirements.

4.1.1. Lipid sources and total lipid

Lipids included in microparticulated diets come, in part, from fish meal or other meals mainly incorporated as protein source. They generally derived from marine products. Other lipids, such as cod liver oil, roe oil or menhaden oil are added as triglycerides, and phospholipid come from soy lecithin or marine (fish) phospholipid (Geurden et al., 1995).

In a diet, the lipid level determines the energy level. Lipids are the main energy source in developing larvae. Lipid levels in larval diets are generally high, 18% in compound diet for sea bream larvae (Salhi et al., 1999), 29 to 37% in Artemia for sea bream larvae (Koven et al., 1992) or 25% in Artemia for Paralichtys olivaceus (Furuita et al., 1998). Brinkmeyer and Holt (1995) tested the response of red drum larvae to graded levels of menhaden oil with a semi-purified diet. The best growth was obtained in larvae fed the diet containing 18% lipid, compared to diet containing 13, 23 or 27% lipid. In sea bass larvae, growth and survival were directly related to the lipid content of the diet (Fig. 4). Best results were obtained with the diet containing 30% lipid, brought as cod liver oil and soy bean lecithin (Zambonino Infante and Cahu, 1999).

4.1.2. Phospholipid requirements

A beneficial effect due to phospholipid (PL) incorporation in diet was reported as early as 1981 (Kanazawa et al.) and since this date, phospholipid requirements in fish larvae have been extensively studied (for review, see Coutteau et al., 1997). Nevertheless, fish have been shown to be able to synthesize *de novo* phospholipids (Sargent et al., 1993). For explaining the necessity of phospholipid for larval stages, Kanazawa (1993) and Geurden et al. (1995) suggested that larvae were incapable of synthesizing PL at a sufficient rate to meet larval

requirement during a period of high cell multiplication. Indeed, PL is a major component in cell membrane.

Studies have been conducted to clarify the role of dietary phospholipids and had the benefit of previous studies conducted on shrimp. The role of phospholipid as energy supply has been mentioned by Olsen et al. (1991), as larvae have a limited digestibility of neutral lipids. It has been also suggested that PL are more efficient than neutral lipid for HUFA source (Koven et al., 1993, Salhi et al., 1999). The role of phospholipid in facilitating lipid emulsification and digestion has also been hypothesized, but has led to controversial conclusions. A role in enhancing lipid transport between the various tissues and organs has been demonstrated for PL, particularly in crustaceans. Lipids are transported as lipoproteins. Phosphatidylcholine is the main polar lipid in fish lipoprotein (Sheridan, 1988).

The main phospholipid source used in experiments is soybean lecithin. Good growth and survival have been obtained by feeding sea bass larvae with a diet containing 6.6% phospholipid from soybean lecithin (Zambonino Infante and Cahu, 1999). Sargent et al. (1999) assume that the ideal diet for marine fish larvae would include 10% marine fish phospholipid, since egg or yolk sac larvae exhibit 10% phospholipid concentration. Nevertheless, Geurden et al. (1997) obtained similar growth in sea bass post-larvae fed either vegetable or animal -including marine- PL sources.

An indirect role of PL must be also considered for interpreting the beneficial effect of PL incorporation in compound diets. PL would improve the cohesiveness of dietary microparticles and therefore, reduce the leaching of soluble nutrients.

Studies are now conducted to determine the specific role of the different phospholipid classes, mainly phosphatidylcholine and phosphatidylinositol.

4.1.3. Essential fatty acid requirements

The n-3 highly unsaturated fatty acids (HUFA) have been identified as essential dietary components for marine fish since a long time, since marine fish cannot synthesize them. A special attention was paid to eicosapentaenoic (EPA= C20:5n-3) and docosahexaenoic acid (DHA= C22:6n-3), which are in large amount in fish cell membranes.

Experiments conducted using live prey (Izquierdo et al, 1989, Watanabe et Kiron, 1994) or a compound diet (Zambonino Infante and Cahu, 1999) have shown that the optimal level of EPA+DHA in diet for marine fish larvae is around 3% of dry matter. It has been shown in several species that DHA is more efficient than EPA for improving growth and survival in Seriola quinqueradiata and Paralichtys olivaceus (Watanabe and Kiron, 1994) (Fig.5) and stress tolerance, such as salinity stress or reduced dissolved oxygen. Sargent et al. (1999) investigated the role of DHA in neural and visual development of larvae and the importance of the ration of EPA, DHA and AA (arachidonic acid C20:4n-6) in flatfish pigmentation.

4.2. Protein requirements

4.2.1. Protein sources

The first compound diets formulated for fish larvae included mixture of protein sources, with the hypothesis that some nutrient deficiency in a protein source can be compensated by an other one. Person Le Ruyet et al. (1989) weaned 23-day-old sea bass, Dicentrarchus labrax, with a compound diet including squid, shrimp and hen eggs. A mixture of fish meal, shrimp meal, squid meal, lactic yeast was used in a diet given to 25-day-old sea bass larvae (Zambonino Infante and Cahu, 1994b). Kanazawa and Teshima (1988), using a mixture of different fish meal, yeast powder, krill meal, crab meal, and gluten meal obtained significant growth and survival in flounder, Paralichthys olivaceus. Then the authors moved toward a formula simplification essential for understanding the nutritional requirements of larvae. Protein sources were selected following their amino acid profile and were incorporated in

microdiet as the only protein source. Squid meal was chosen for the larvae diet of sea bream, Sparus aurata, and incorporated up to 40% of dry matter in one study (Kolkovski et al., 1993) or 64% in another (Salhi et al., 1994). Fish meal has been used as the main protein source in diet formulated for sea bass (Zambonino Infante et al., 1997) and was incorporated up to 65% in the diet used from Day 20.

Then, following the experimental approach conducted on freshwater species larvae, goldfish (Slaminska et al., 1993) or carp (Radünz-Neto et al., 1994), nutritional studies were conducted using semi-purified or purified based casein diet. Brinkmeyer and Holt (1998) obtained a significant growth of red drum (Sciaenops ocellatus) larvae weaned at Day 8 by using a semi-purified casein based diet, though this growth was greatly inferior to that obtained with a compound diet. A semi-purified diet induced also a significant growth in sea bass larvae weaned at Day 25, while a totally purified diet including 57% of casein mixture (casein, hydrolysate of casein with a chin length of 10 to 40 amino acids, Dl methionine and L tryptophan) induced a poor growth (Cahu and Zambonino Infante, 1995).

4.2.2. Protein level

The optimum protein level for freshwater species larvae has been extensively studied. It is higher in larvae and juveniles than in adults for the same species. This difference was attributed to the high growth rate and high utilization of protein as energy in larvae (Dabrowski, 1986). On the opposite, data concerning the protein requirement of marine fish larvae are lacking and protein level was generally adjusted in diet around 50-70%, based on data obtained on juveniles. Salhi et al. (1994) used compound diet including up to 73% protein in diet for sea bream, while Brinkmeyer and Holt (1995) formulated a diet containing 50% protein for red drum. The first attempt to determine optimal dietary protein concentrations for sea bass at very young stages was conducted by Péres et al. (1996) by

feeding larvae from Day 15 to Day 35 with isoenergetic compound diets incorporating a gradient in protein level (fish meal plus casein hydrolysate). The best growth was observed with 50% of protein. A slightly slower growth was induced by 60% protein. Very poor growth was observed with 30 and 40% protein (Fig. 6).

4.2.3. Amino acid requirements

Some results have been obtained in freshwater species, by using semi-purified diets. Fiogbé and Kestemont (1995) have shown that essential amino-acid requirements for goldfish (Carassius auratus) larvae are much higher compared to juveniles. Some data on amino acid requirement specific to fish larvae have been obtained by feeding larvae with live prey having different amino acid profiles, such as Artemia and copepods. By this way, Conceiçao et al. (1997) showed that taurine, and its precursors methionine and cysteine, and leucine may limit growth in turbot larvae. But to our knowledge, no experiment have been conducted with experimental formulated diets to determine the essential amino acids for marine fish larvae and their optimal level in a diet.

We can assume that the essential amino acids are the same for developing larvae than for juveniles. The profile of essential amino acids of fish body is generally considered as a good indicator of their amino acid requirements. Nevertheless, nothing is known about the functionality of the amino acid intestinal transporters in the very early stages of development of marine fish. It can be supposed, as demonstrated in other vertebrates and particularly in mammals that transport of the different amino acids appears at different moments of the perinatal development (Buddington and Diamond, 1989). Fish larvae can be compared to an organism in fetal stage. It can be supposed that some amino acid transporters are not functional in fish larvae during early development. Thus, it is crucial to determine the

essential amino acid requirements in fish larvae and the onset of their transporters during development.

4.2.4. Molecular form of the protein fraction

The role of dietary free amino acids and short peptides on larvae development has been investigated by several authors. As early as 1989, Fyhn suggested that free amino acids constitute a substrate for energy production in marine fish larvae during early larval stages and that larvae during young stages need an exogenous supply of free amino acid. Indeed, stomach is not differentiated in early stages of marine fish larvae. Ronnestad et al. (1999) suggested that, in absence of HCL and pepsin secretion, ingested protein cannot be denaturated and then free amino acid would be more efficiently absorbed than protein. Naess et al. (1995) reported that 7% of free amino acid (dry weight basis) included in *Artemia* is sufficient to sustain growth of Atlantic Halibut *(Hippoglossus hippoglossus*) at early developmental stages. Conversely, Watanabe and Kiron (1994) considered that it is not clear if fish larvae have a sufficient ability to digest food protein or whether free amino acid must be provided by diet. In the same way, the incorporation of 10% essential amino acid mixture in fish meal based diet failed to improve growth and survival in sea bass larvae, compared with larvae fed diet with the same nitrogenous level brought as whole protein (Cahu and Zambonino Infante, 1995). Nevertheless, the dietary incorporation of free amino acids induced an increase in trypsin secretion in early larvae stages, suggesting that pancreatic digestion would be improved.

Beside their nutritional function, free amino acids may play a very important role in first feeding by acting as chemo-attractant.

Protein hydrolysate has been since a long time considered as an advantageous protein form for fish larvae (Gabaudan et al., 1980) and the product was incorporated in most of the larval

diets, at least for improving microparticle physical properties. Recent experiments have shown evidence of the high nutritional value of protein hydrolysate and its role in larval nutrition. First, experiments conducted with a semi-purified diet have shown that the incorporation of casein hydrolysate led to a better survival in carp (Szlaminska et al., 1993) as in sea bass larvae (Cahu and Zambonino Infante, 1995). Then, Berge and Storebakken (1996) demonstrated that growth of salmon fry was enhanced by replacing 5 or 8% of the amino acid nitrogen in a fish meal based diet by fish protein hydrolysate (CPSP). Growth of carp larvae also improved when fish protein hydrolysate accounted for 50% of the total nitrogen supply (Carvalho et al., 1997). Nevertheless, a replacement of 75% protein by hydrolysate adversely affected growth. These experiments were conducted by using peptide chains of 10 to 20 amino acids (Cahu et al., 1999). Recently, Zambonino Infante et al. (1997) showed that a 20% replacement of fish meal by di-et tripeptides (obtained from fish meal hydrolyze) in diet resulted in an improvement of the main biological parameters in sea bass larval rearing: growth, survival and skeletal formation (Fig.7).

Incorporating di- and tri-peptides to the diet led to a growth improvement when an amino acid mixture failed to induce the same effect. Hydrolysates are beneficial to larvae, while they do not affect, or in some cases, depress juvenile growth. These results suggest that fish larvae have specific nutritional requirements, which can be understood by the analysis of larval digestion. The beneficial effect of dietary peptides can be explain by the existence of specific transmembrane transporters and the high cytosolic peptidase activities we have observed in young larvae. In juveniles, the specific activity of cytosolic peptidases decreases, and hydrolysates become less efficient for growth.

5. Conclusion

Nutritional requirements of larvae appear perceptibly different of those of juvenile fish. In particular, larvae require dietary phospholipids, while this component is not essential in juveniles. Larval growth is promoted by diets containing high energy levels, supplied as neutral lipid and phospholipid mixtures. Incorporation of protein hydrolysate into the feed is determining for growth and survival, when hydrolysate does not induce beneficial effect in juveniles. The specific role of these nutritional components for developing larvae is becoming clear. Knowledge recently acquired on nutritional requirements constitutes a platform for formulating experimental diets. These diets will allow researchers to perform proper nutrition experiments and to accurately determine larvae requirements. Some current experimental diets can now sustain larvae growth and survival. The interspecific variability in the ontogeny pattern, feeding physiology, nutrition and behavior, and, consequently, in feeding requirement can be studied by using compounds diets.

Future diets will have to improve larvae quality. For example, the pigmentation default frequently observed in flat fish may by caused, in part, by a perturbation of the preprogrammed pigmentation process, due to an inadequate dietary supply in phospholipid, HUFA and retinoic acid. In the same way, spinal malformation (scoliosis, lordosis, coiled vertebral column) could be originated from inadequate vitamin or mineral supply, or an excess, or a deficiency. The study of pigmentation or calcification processes will allow identifying the nutritional components involved. The dietary optimal concentration and form of supply of the identified components will be determined using formulated diets. The use of improved diets would sustain the production of constant high quality fingerling in hatchery.

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Table 1. Composition of experimental microparticles (g /100g dry diet) for marine fish larvae: I, microbound diet (Person Le Ruyet, in Guillaume et al, 1999); II, crumbles (Zambonino Infante and Cahu, 1999); III microcapsulated diet (Yufera et al., 1999).

Ingredients	I	II	III
Fish meal	-	55	-
Fish protein hydrolysate	4	11	12
Casein	4	-	50
Squid meal	14	-	10
Mussel meal	4	-	-
Crayfish meal	14	-	-
Fish roe	19	-	-
Egg yolk	14	-	-
Lactic yeast	4	-	-
Fish oil	5	9	12
Soy Lecithin	3	7	3
Starch		5	
Dextrin	-	-	6
Vitamin mixture	5	8	7
mineral mixture	4	4	-
Betaine	-	1	-
Zein	6		

Figure 1. Variation of specific activity and mRNA level of amylase during sea bass (Dicentrarchus labrax) development.

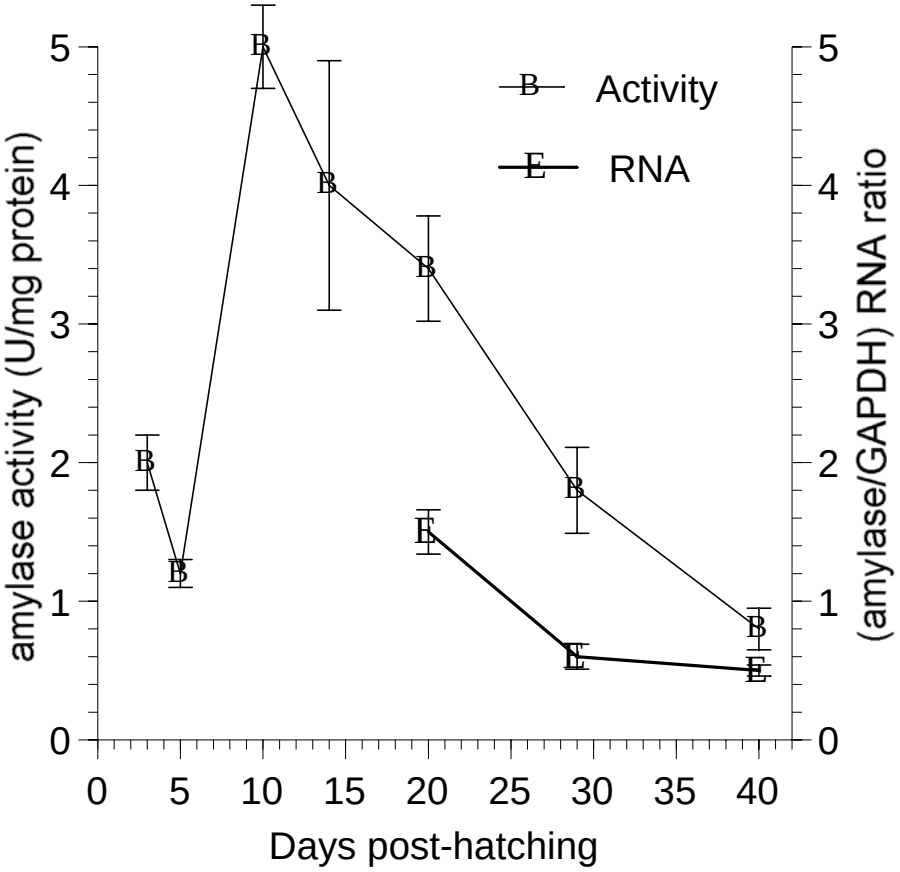

Figure 2. Specific activity and mRNA level of amylase in 29 day old sea bass (Dicentrarchus labrax) fed diets containing 5% (S5) or 25% (S25) starch. Means ± SEM (n=4) with different superscripts, independently for RNA and activity, are significantly different ($p<0.05$).

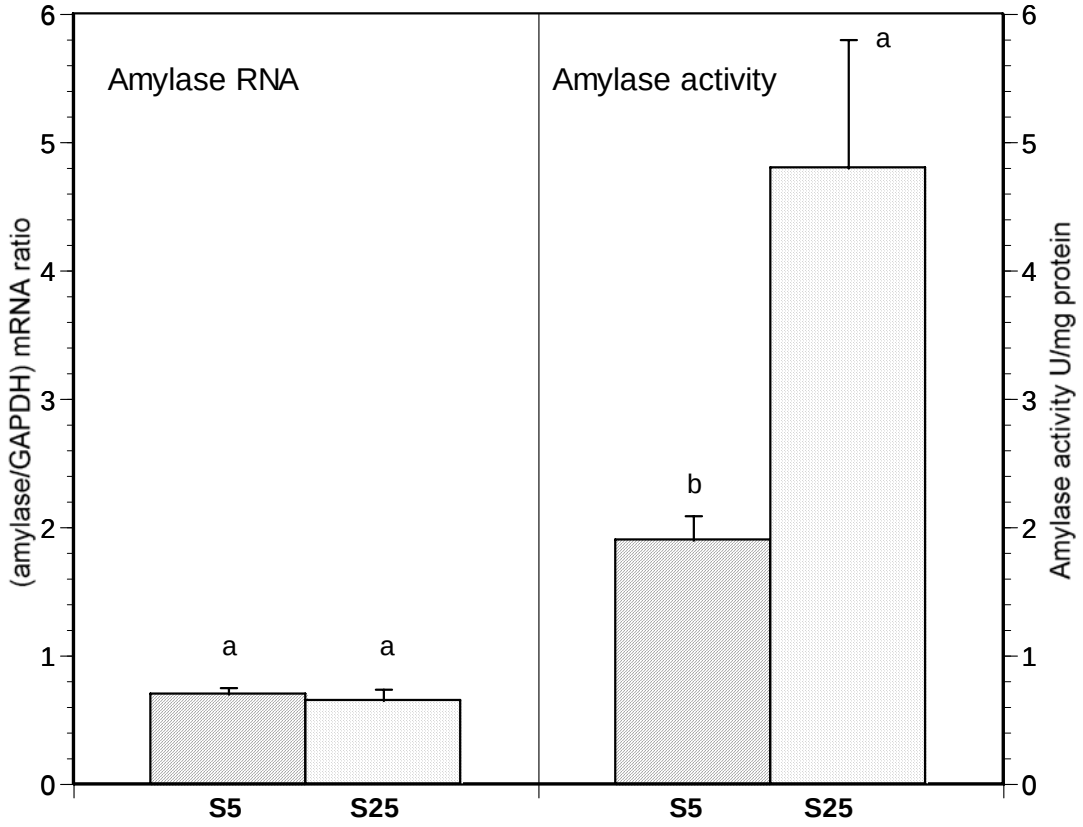

Figure 3. Variation in specific activity of enterocyte enzymes during fish larvae (Dicentrarchus labrax) development: increase in a brush border membrane enzyme (AP: alkaline phosphatase) and decrease in a cytosolic enzyme (leu ala: leucine alanine peptidase) in larvae fed live preys (filled line) or weaned with a conventional diet (dotted line).

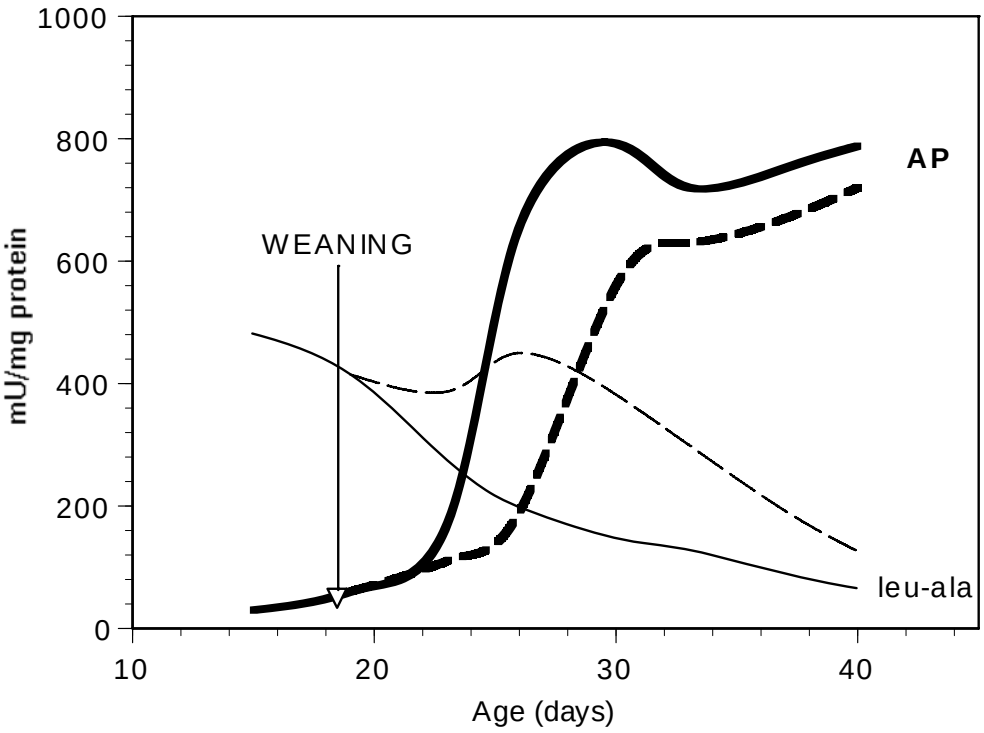

Figure 4. Growth (wet weight) of sea bass (Dicentrarchus labrax) larvae fed from day 14 isonitrogenous compound diet containing 10, 15 20, 25 or 30% lipids. Survival rate, from hatching to Day 39, is in brackets (from Zambonino Infante and Cahu, 1999). Means ± SEM (n=4) with different superscripts are significantly different ($p<0.05$).

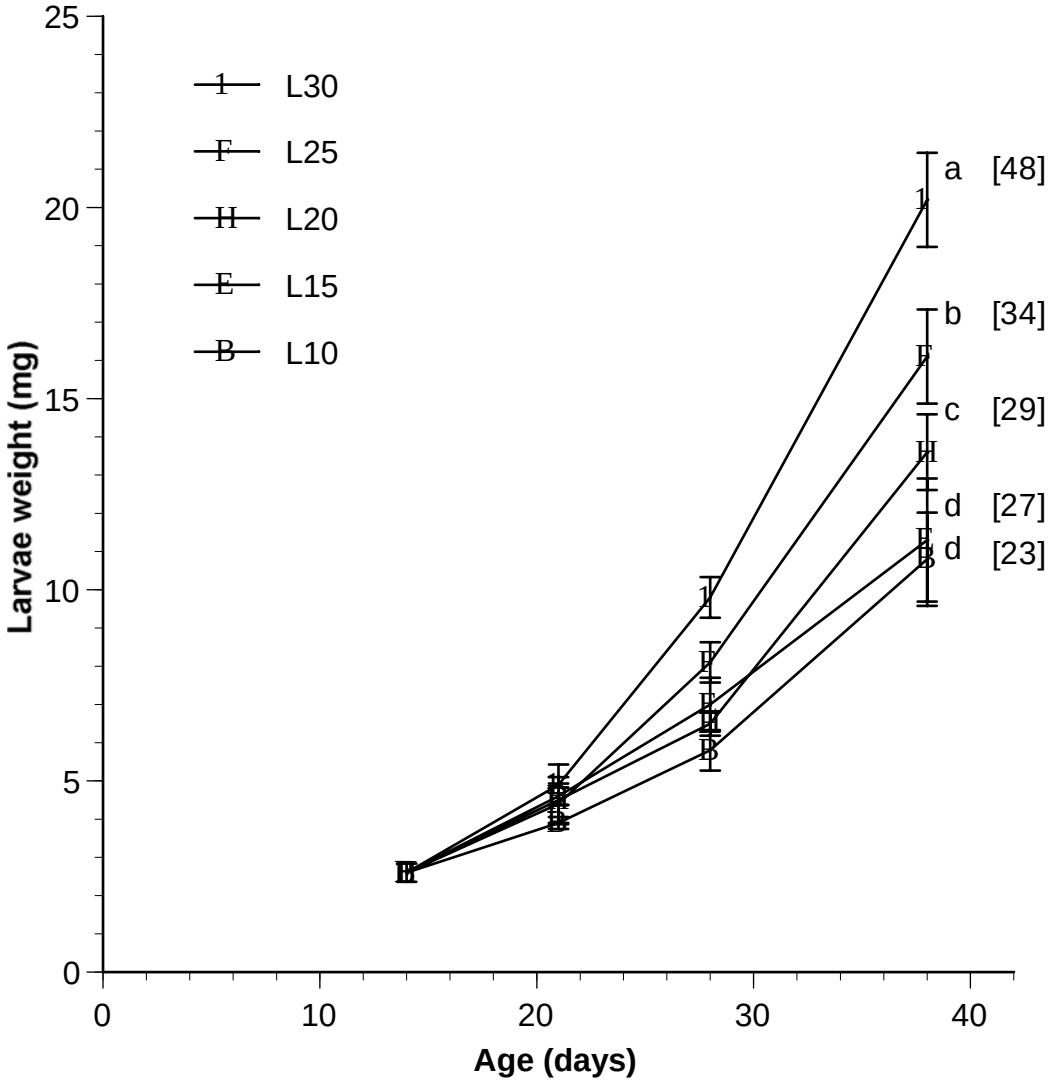

Figure 5. Growth of *Paralichtys olivaceus* larvae fed different levels fed eicosapentaenoic acid (EPA) and docohexaenoic acid (DHA) (from Kanazawa et al., 1989 in Watanabe and Kiron, 1994).

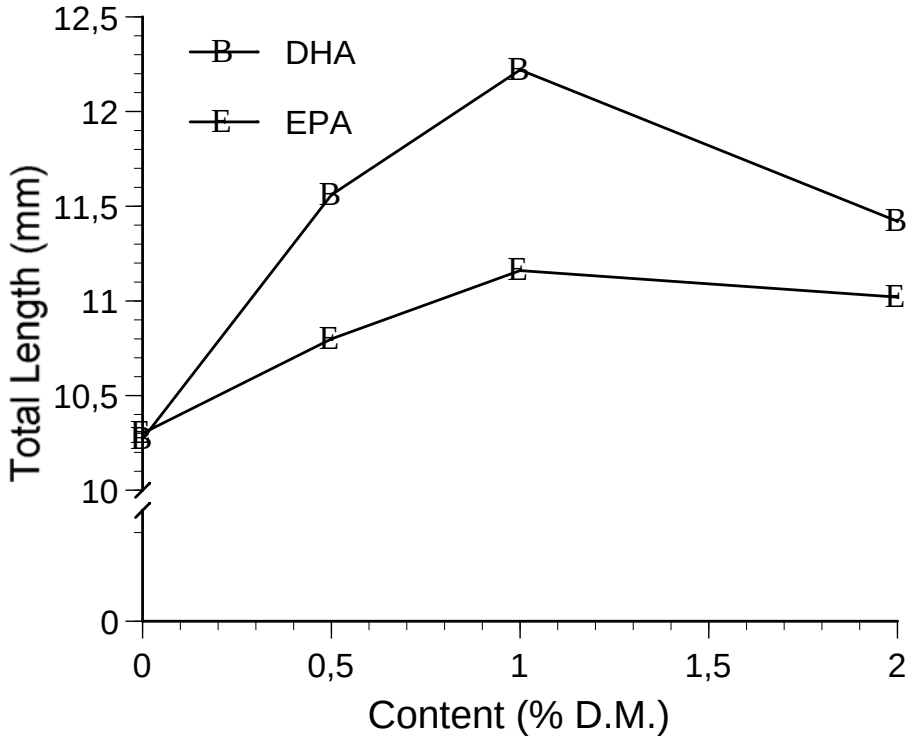

Figure 6. Growth (wet weight) of sea bass larvae (Dicentrarchus labrax) fed from day 14 with isoenergetic compound diets incorporating 30, 40, 50 or 60% proteins. Survival rate, from hatching to Day 36, is in brackets (from Péres et al., 1996). Means ± SEM (n=4) with different superscripts are significantly different ($p<0.05$).

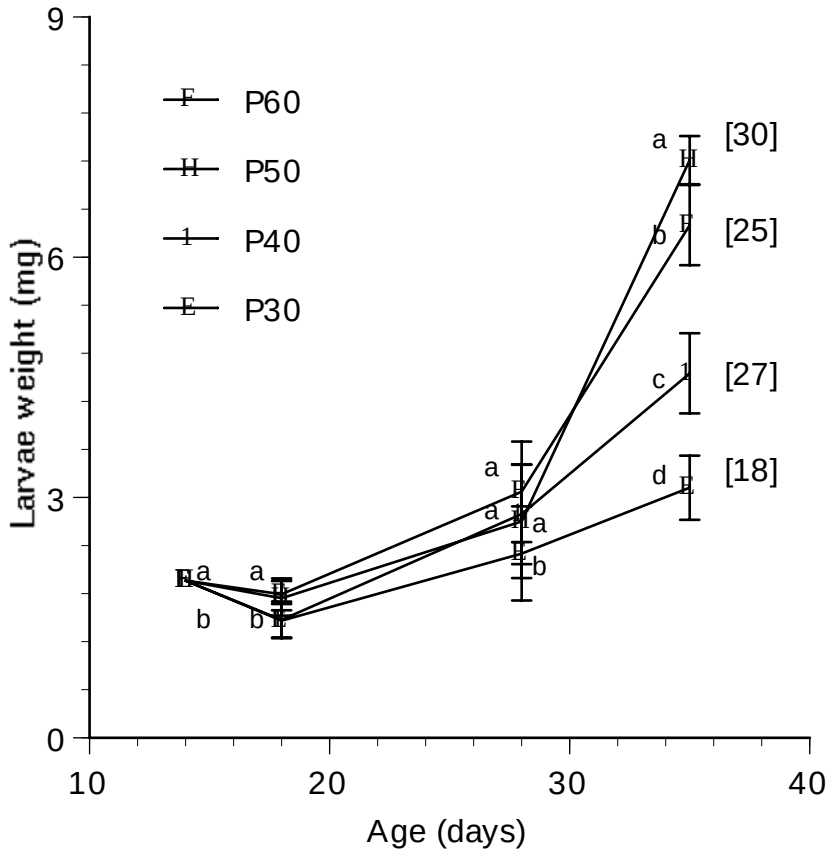

Figure 7. Growth (wet weight) of fish larvae fed diets incorporating different protein hydrolysate level in replacement of protein. 7a. Goldfish (Carassius auratus) larvae fed diets incorporating 0% (H0), 50% (H50) and 100% (H100) hydrolysate (from Slaminska et al., 1993). 7b. Sea bass (Dicentrarchus labrax) larvae fed diets