The effect of dietary lecithin and lipase, as a function of age, on n-9 fatty acid incorporation in the tissue-lipids of Sparus aurata larvae

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Abstract

The present study tested the effect of dietary lecithin and exogenous lipase on the incorporation of oleic acid in the tissue lipids of gilthead seabream larvae (Sparus aurata). Two of four microdiets were prepared by the addition of [14C]oleic acid as free fatty acid (FFA) to diets containing either 5% cuttlefish liver oil (CLO) or 5% soybean lecithin. Glycerol tri[1-14C]oleate was similarly incorporated in two other diets identical in lipid (4% cuttlefish liver oil, 1% soybean lecithin) and non-lipid composition but differed in that one contained a supplement of 0.05% porcine lipase. The effect of these diets was tested by following the incorporation of the label (dpm/mg larvae DBW) in the neutral and phospholipid fractions of seabream larvae at four different ages (21, 27, 32 and 45 days after hatching).

A significant (p<0.05) effect of dietary lecithin on the incorporation of labelled FFA in both larval neutral and phospholipid fractions was demonstrated at all ages. This was particularly pronounced during early development (day 21) where fish fed the lecithin supplement incorporated 6.75 times more label than the diet containing [14C]oleic acid in CLO. The dietary lecithin enhancing effect diminished with age but was still significant at day 45 (2.17 times more label). In addition, the label was considerably higher in the phospholipid fraction compared to the neutral lipid, reflecting the high demand for membrane synthesis during rapid growth. Lecithin-fed larvae demonstrated a higher consumption rate and efficiency of incorporation than fish consuming the cuttlefish liver oil diet, suggesting an emulsifying function for dietary phospholipid.

In contrast, the supplementation with lipase showed a clear effect only in older fish where 45 day old larvae fed the lipase diet demonstrated a 3.42 times increase in radioactivity in their tissue lipids. This late lipase response may be the result of an insufficient level of dietary lecithin (1%) and a short intestinal length being ineffective, in the early larval stages, in incorporating labelled free fatty acid from dietary glycerol tri[1-14C]oleate breakdown.

Introduction

It is now widely believed that many species of marine fish larvae have a requirement for the n-3 highly unsaturated fatty acids; eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) (Watanabe 1982; Sargent et al. 1989). However, it should be emphasized that the majority of studies determining essential fatty acid requirements were carried out using enrichment techniques on zooplankton such as rotifers (Brachionus plicatilis) and artemia (Artemia salina). Conclusions using these animals as true test diets are limited since their body composition is not completely defined and may provide substances that have not been considered in the experimental design. In fact, this may be particularly relevant during the early stages of larval life where the digestive tract, in many species, is still developing and may not be totally functional (Dabrowski 1984; Hofer 1985). There is accumulating
evidence that larvae are utilizing enzymes from the prey they consume to facilitate the process of digestion and incorporation until their own alimentary systems are fully differentiated and developed (Dabrowski and Głogowski 1977a,b; Lauff and Hofer 1984). In support of this, Kolkovski et al. (1991) found in gilthead seabream that dietary pancreaticin significantly enhanced protein and neutral lipid incorporation.

Dietary phospholipids may also be necessary for the digestive process. Studies have shown that supplementation of phospholipids such as lecithin, unlike in adult stages, improved growth in larval ayu, Plecoglossus altivelis (Kanazawa et al. 1981, 1983a, 1985), red seabream, Chrysophrys major, knifejaw, Oplegnathus fasciatus (Kanazawa et al. 1983b) and starry flounder, Paralichthys olivaceus (Teshima et al. 1987). These authors suggested that dietary lecithin may (1) enhance the absorption of ingested fats such as triglycerides in the undeveloped digestive tract or (2) be utilized in the production of lipoproteins and cellular components when larvae possess a limited ability for phospholipid synthesis. Interestingly, in nature young marine larvae preferentially consume the smaller earlier instar stages of zooplankton such as copepods which are considerably richer in phospholipid than the adult forms (Sargent et al. 1989).

These findings have important implications for larval microdiets which incorporate marine oils rich in the essential eicosapentaenoic and docosahexaenoic acids. The omission of dietary supplements such as lipases and phospholipids that may increase the efficiency of the digestion process could result in poor fatty acid incorporation and lower growth rate.

The aim of the present experiment was to test, as a function of age, the effect of dietary lecithin and exogenous lipase on fatty acid incorporation in the tissue lipids of Sparus aurata larvae.

Materials and methods

Experiment 1

The effect of dietary lecithin and exogenous lipases on fatty acid incorporation was tested in 21, 27, 32 and 45 day old gilthead seabream larvae by feeding four microdiets identical in the non-lipid fraction but which differed in lipid composition and the form of their labelled oleic acid (18:1n-9). Two of the four gelatin bound microdiets, designed to test the effect of dietary lecithin, were prepared by the addition of [1-14C]oleic acid as a free fatty acid (FFA) to diets containing 5% cuttlefish liver oil (CLO), diet A, or 5% soybean lecithin, diet B. The triglyceride glycerol tri[1-14C]oleate was similarly incorporated in the other two diets (C and D) which were identical in lipid composition (4% cuttlefish liver oil, 1% soybean lecithin) but differed in that one (diet D) was supplemented with 0.5% porcine lipase. These latter diets were designed to test the effect of exogenous lipase. The theoretical lipid fraction composition of the microdiets is detailed in Table 1.

Table 1. The composition of the lipid fraction of the four microdiets (A, B, C and D). These treatments tested the effect of dietary lecithin (A,B) and lipase (C,D) on the incorporation of fatty acids into the tissue lipids of gilthead seabream larvae

<table>
<thead>
<tr>
<th>Microdiets</th>
<th>[14C] labelled Lipid Lipase1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fraction2 fraction3</td>
</tr>
<tr>
<td>A</td>
<td>Oleyic acid (FFA) 5% CLO no</td>
</tr>
<tr>
<td>B</td>
<td>Oleyic acid (FFA) 5% Lecithin no</td>
</tr>
<tr>
<td>C</td>
<td>Glycerol 4% CLO, oleic trioleate 1% Lecithin no</td>
</tr>
<tr>
<td>D</td>
<td>Glycerol 4% CLO, oleic trioleate 1% Lecithin yes</td>
</tr>
</tbody>
</table>

1The basal composition, except for the lipid fraction, was identical in all diets and is a closed formula. The proximate composition was: moisture 2.5%, protein 87.4%, lipid 5% and ash 5.2%; 2250 μCi of [14C]oleic acid (FFA) or glycerol tri[1-14C]oleate (Amersham, UK) was added to three grams of the appropriate diet; 3CLO is cuttlefish liver oil. Lecithin is a commercial soybean lecithin (Eiz hazit, Tel Aviv) that is approximately 60-65% phospholipid with the following composition: phosphatidylcholine 48%, phosphatidylethanolamine 33%, sphingomyelin 15%; 4Porcine lipase (Sigma).

The diets were prepared by first extracting lipid from fat containing components, four times with chloroform:methanol (2:1, v/v) (Folch et al. 1957) at a sample:solvent ratio of 1:10. This was followed by drying the filtrate at 50°C, then grinding it to a fine powder with mortar and pestle before use.

Dry ingredients for 3 g of each of the experimental diets were first weighed (including lipase for diet D) and combined. A fixed amount (250 μCi) of [14C]oleic FFA or glycerol tri[1-14C]oleate in toluene was evenly distributed to their corresponding diet mixtures along with the appropriate oils.
and/or lecithin and mixed well. Finally, gelatin binder was dissolved in a small volume of heated distilled water equal to double its weight and mixed thoroughly with each of the treatments to form a paste. These slurries were dried at \(50^\circ\)C for 48 hrs before being ground and sieved into three particle sizes: 150 \(\mu\)m, 150-250 \(\mu\)m and >250 \(\mu\)m which gave radioactive levels ranging from 80 to 100 dpm/\(\mu\)g. The effect of heating (\(50^\circ\)C) was previously tested and was found not to affect enzyme activity (Kolkovski 1990). A similar procedure was followed to prepare sufficient amounts of the four diets A, B, C and D without labelled compounds.

The experimental flow through system consisted of twenty-four 600 ml polycarbonate beakers supplied with filtered seawater of 40-41 ppt salinity, \(0.1-0.2 \mu\)M \(\text{NH}_3\) and a pH of about 8.2 which entered at the surface and exited near the base of the beaker through a 350 \(\mu\)m filter at a rate of 150±50 ml/min. Water temperature was increased with larval age and ranged from 20.5 to 24.5°C which corresponded to the normal larval rearing regime used at the National Center for Mariculture (NCM) facility (Tandler et al. 1989). The experimental system was kept under a fume hood and illuminated with fluorescent lighting (daylight 40W) with an intensity of 800 lux at the water surface.

In all larval age classes the four microdiet treatments were tested twice in the 24 beaker experimental system giving two blocks of 6 replicates/treatment. Larvae used in these trials were previously reared in 600 l conical tanks according to the procedure of Tandler et al. (1989) and sampled so that all age classes tested from a block originated from the same larval population. The fish were stocked, unfed, in the experimental beakers (23±5 larvae/beaker) for at least 12-18 hours prior to the beginning of each trial to acclimate them to the test conditions. Dead fish were removed during this period although survival was generally high (ca. 85.0%).

In each trial, larvae were offered, \textit{ad libitum}, up to a maximum of 3.5 mg portions of the labelled diets every 15 minutes so that an excess of food was always available. This was continued for one hour as previous ingestion rate experiments showed that similar age larvae filled their digestive tracts within 60 minutes (Kolkovski 1990). At the end of this period the fish were fed their respective diets without labelled FFA or triglyceride for a further 8 hours to ensure that all labelled diet was absorbed or eliminated from the digestive tract (Kolkovski 1990).

After feeding, larvae were killed with quinaldine (Sigma), individually siphoned out and washed twice in distilled water, then blotted dry and counted on filter paper before being stored at -70°C for further analysis.

The dry weight of these larvae was determined in the following way. In each sampling from the 600 l conical tanks used to stock a block of an experimental run, an additional 200 larvae were taken. These fish were washed in distilled water and then dried at 60°C for 72h before being weighed (4 groups of 50 larvae) on an electronic balance (± 0.1 \(\mu\)g, Cahn 25). The average weight for 21, 27, 32 and 45 day old larvae were 0.20, 0.51, 1.60 and 8.19 mg, respectively. The average dry weight per larva in these samples was used for the diet trials and larval biomass was calculated by multiplying the average by the number of fish removed for analysis from each of the beakers.

The radioactivity of polar and neutral lipid fractions was determined by extracting total lipid from all larvae in each of the experimental beakers in a 2:1 solvent mixture of chloroform:methanol (Folch et al. 1957) and evaporating to dryness under nitrogen. The total lipid was then separated into polar and neutral fractions using silicic column chromatography (Christie 1982). After separation and drying under nitrogen, the lipid fractions were transferred to scintillation vials (20 ml) with two washings of 2 ml of the appropriate solvent (chloroform for neutral lipid and methanol for phospholipid) and 15 ml of scintillation fluid (Packard scintillator 299) and radioactivity determined in a Packard Tri-carb 4530 Scintillation counter.

**Experiment 2**

An inverse relationship exists in fish between ration size and efficiency of absorption (Elliott 1976a,b). After observing the results from the above experiment, a second experiment was carried out to measure the consumption rates of larvae fed the CLO diet A and lecithin diet B in 21 day old fish of approximately the same DBW. This was to examine to what degree the high fatty acid incorporation in
the lecithin fed larvae was due to dietary phospholipid effect or increased absorption from consuming a smaller ration.

The twenty-four beaker experimental system was stocked with 21 day old larvae which were fed, in replicates of 12, the labelled diets A and B as mentioned in experiment 1. This larval age was selected since the strongest lecithin effect was observed at this stage of development. At 20 minute intervals up to and including 60 minutes, larvae from four replicates each of diets A and B were killed and sampled as in the first experiment. This was followed by digesting the samples in 1 ml of Soluene 100 for 2h at 50°C which were subsequently read in a scintillation counter. The rate of increase of label between time intervals was considered as a measure of food consumption rate (CR).

The final readings for the larval neutral and phospholipid fractions as well as whole fish digest samples were expressed as dpm/mg dry body weight (DBW) larvae after correcting for quench. Larval total lipid dpm/mg DBW was the sum of dpm in neutral and phospholipid and replicate results from treatments were tested for homogeneity of variance (Fmax-test). Differences in total lipid dpm/mg DBW between treatments as a function of age were analyzed by one-way ANOVA and Student-Neuman-Keuls (SNK) test.

Results

In each age class tested, larvae fed the lecithin diet B demonstrated significantly (p<0.05) higher incorporation of labelled oleic acid in their lipids than larvae from the CLO diet A (Fig. 1). However, the enhancing effect of fatty acid incorporation by dietary lecithin diminished with larval age until 32 days, then did not decrease further. Twenty-one day old larvae fed diet B incorporated 6.75 times more label into their tissues than those fed diet A while forty-five day old lecithin fed larvae incorporated...
Fig. 1. The effect of feeding duration on accumulation of [14C]oleic acid in 21 day old gilthead seabream (gut and tissues) fed cuttlefish liver oil diet A or lecithin diet B. Consumption rates (dpm/min) for diets A (eRA) and B (eRB) for every 20 minute period and for the total 60 minute (60T) period are shown. Standard error (SE) bars are shown for 20, 40 and 60 minute periods. Treatment values having the same letter within a feeding period were not significantly different (p>0.05).

In Figure 3 larvae fed diet B demonstrated higher dpm consumption rates after 20 and 40 minutes (4.1 dpm/min and 17.9 dpm/min, respectively) than fish consuming the CLO diet A (3.1 dpm/min and 2.7 dpm/min, respectively). This rate decreased dramatically during the last 20 minutes (2.8 dpm/min), in fish fed diet B possibly indicating a full gut after 1 hour of feeding. In contrast, larvae fed diet A had not filled their digestive tract during this period and still showed an increased consumption rate (4.7 dpm/min).

Although larvae fed the lecithin diet were con-

![Graph showing consumption rates](image)

### Table 2. The percent efficiency of absorption of dietary [14C]oleic acid in larvae fed the cuttlefish liver oil (CLO) diet A and lecithin diet B after one hour of feeding

<table>
<thead>
<tr>
<th>Microdiet</th>
<th>Dpm/mg larva in total lipid (TL)</th>
<th>Dpm/mg larva in TL and gut</th>
<th>% Efficiency of absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLO diet A</td>
<td>30.7</td>
<td>209.7</td>
<td>14.6</td>
</tr>
<tr>
<td>Lecithin diet B</td>
<td>207.3</td>
<td>496.2</td>
<td>41.8</td>
</tr>
</tbody>
</table>

1Data from Fig. 1; 2Data from Fig. 3; 3Calculated by: \( \frac{\text{dpm/mg larva in total lipid}}{\text{dpm/mg larva in total lipid and gut}} \times 100 \)
Fig. 4. Comparison between the effects of dietary lecithin and lipase on larval \(^{14}C\) oleic acid incorporation with age. Effect was measured as the times (x) increase in fatty acid incorporation in fish fed the lecithin diet B over fish fed the cuttlefish liver oil diet A and fish fed the lipase diet D over fish fed the non-lipase diet C.

In contrast to diets A and B, a marked effect (p<0.05) of dietary lipase (diet D) on fatty acid incorporation in larval lipids was not apparent prior to day 32 (Fig. 2). Interestingly, fish fed the lipase diet D actually showed a lower accumulation of the label than the lipase deficient diet C in 21 and 27 day old larvae.

The association between age and fatty acid incorporation in fish fed the lecithin diet B and the lipase diet D appeared to be inversely related (Fig. 4). The time increase in fatty acid incorporation in lecithin fed larvae decreased from 6.75 in the youngest larvae (21 days) to 2.17 in older fish (45 days) while the effect on the level of fatty acid incorporation from exogenous lipase increased from -0.28 to 3.42 over the same age range.

Discussion

In this study, a lecithin containing diet significantly increased the incorporation of \(^{14}C\) oleic acid in the tissue lipids of larvae, particularly the phospholipid fraction. Possibly a similar increased membrane deposition would occur with other dietary fatty acids such as the essential n-3 highly unsaturated fatty acids (HUFA) although, in general, these fatty acids may be more selectively incorporated than less unsaturated fatty acid groups (Linares and Henderson 1991). Nevertheless, an enhanced effect of fatty acid incorporation might explain the improved growth and survival observed in larval ayu (Kanazawa et al. 1983a), red seabream, knife jaw (Kanazawa et al. 1983b), and starry flounder (Teshima et al. 1987) fed this dietary phospholipid.

The predominance of the label in the polar fraction most likely signifies a period of rapid membrane synthesis and larval growth. Gilthead seabream larvae between ages 21 to 36 days have demonstrated relative daily growth rates in excess of 85%/day (Koven et al. 1992). The mechanism, however, by which dietary lecithin increases fatty acid incorporation in the larval lipids has not been defined. Some authors (Kanazawa et al. 1985; Teshima et al. 1987) have postulated that larval fish have limited ability to synthesize phospholipid, used in lipoprotein production and consequently require an abundance of dietary phospholipid to provide these serum carriers during rapid growth. Alternatively, higher incorporation of the label may have been a function of increased absorption of free oleic acid from the gut into the mucosal layer of the larval intestine. It is conceivable that dietary lecithin (particularly in its lysophospholipid form) is acting as a supplemental emulsifier in the absence of sufficient levels of larval bile salts. This may come about from inadequate bile acid synthesis and/or their poor recovery in the hindgut. During early larval development the intestine, in many species, is short relative to body length, and therefore the time allowed for digestion and nutrient absorption as well as the reabsorption of digestive enzymes and bile salts is severely reduced (Hofer 1985). However, with larval development the absorptive ability and intestinal length increases, possibly accompanied by an increase in bile acid production. This may explain the decreasing effect.
of dietary lecithin on labelled oleic acid incorporation with increasing age of larval gilthead seabream. This hypothesis is further strengthened by recent findings (Poston 1990a,b) which showed dietary lecithin, supplemented at 4%, increased growth, survival, food conversion and deposition of body fat in rainbow trout and Atlantic salmon fry. Furthermore, the marked effect of lecithin on 0.18 g salmon decreased with fish size and was not apparent in fry larger than 1.7 g (Poston 1990b). This may explain why previous lecithin studies which focused on older fish (Stickney and Andrews 1972; Watanabe and Takeuchi 1976; Hung et al. 1987; Hung and Lutes 1988) were unable to demonstrate a dietary requirement for this phospholipid.

The enhancing effect of dietary lecithin may have been due to increased absorption of the label as a result of diet B fish consuming a smaller ration size than those fish fed the cuttlefish liver oil diet. This is suggested from amino acid studies showing digestible energy, or that part of the food energy absorbed by the fish, to decrease with amount consumed (Elliott 1976b; Hudon and Noue 1985). In fact, lecithin fed larvae demonstrated a higher consumption rate than fish consuming the cuttlefish liver oil diet and contrary to expectation exhibited a distinctly higher efficiency of absorption than diet A larvae, giving further credence to the emulsion hypothesis. However, it is unclear whether enhanced feeding by the lecithin diet was due to the attraction of dietary phospholipid or the faster absorption and processing of the lecithin diet sustaining high appetite and increase food consumption.

The present study suggests a dietary effect of exogenous lipase in Sparus aurata larvae. This is in agreement with the lack of detectable lipase activity reported in larval turbot, Scophthalmus maximus, (Cousin et al. 1987) and Coregonus lavaretus (Segner et al. 1989). In general, the undeveloped state of the alimentary system in larvae has been related to the relatively low activity of its digestive enzymes (Dabrowski 1984; Lauff and Hofer 1984). In many fish species such as gilthead seabream (Tandler 1986), seabass, Dicentrarchus labrax, (Vu 1983) and Coregonus schinzii palaea (Dabrowski and Kaushik 1985), which have no functional stomach during the early larval stages, there is characteristically low acid proteolytic activity until the later development of the digestive organs. There is accumulating evidence suggesting that larvae depend on a supply of exogenous enzymes in the prey they consume to complete the digestive process (Dabrowski and Glogowski 1977a,b; Lauff and Hofer 1984).

It is unclear, however, why a significant effect of dietary lipase was apparent in older larvae (32 and 45 days old) but not observed in younger fish (21 and 27 days old). A possible interpretation is implied from the relationship between the decreasing effect of dietary lecithin on fatty acid incorporation with larval age and the reverse pattern which appears to be true with supplemental lipase. Presumably in 21 day old larvae there is a large excess of [14C] breakdown products (free fatty acids, monoglycerides and diglycerides) from the lysis of dietary glycerol tri[1-14C]oleate by the high lipase content in diet D. However, the low levels (1%) of dietary lecithin (whether acting as an emulsifier or contributor to lipoprotein production) combined with the short intestinal length may not effectively absorb the excess label. Lipid absorption in fish is exceedingly slow where fatty acids enter the enterocyte by a diffusion process (Bergot 1981; Sargent et al. 1989). In addition, the gut evacuation rate is generally faster and the incorporation efficiency rate lower in larvae compared to older fish (Govoni et al. 1986). A sudden excess of triglyceride lysis products would quickly saturate the limited area for lipid absorption resulting in a significant amount of the label being passed out of the fish. In contrast, a more gradual fatty acid absorption would be expected from the lower endogenous lipolytic activity in the non-lipase diet C. This may also explain why diet D larvae demonstrated a net loss of radioactivity at days 21 and 27. Nevertheless, as larval age progresses, intestinal absorption and length increased to the point where older larvae may be capable of assimilating the excess [14C] fatty acid breakdown products, thereby demonstrating a dietary lipase effect on fatty acid incorporation.

In summary, the results demonstrated an enhancing effect of dietary lecithin on the incorporation of fatty acids in gilthead seabream larvae, particularly in the phospholipid fraction. This suggests that lecithin supplementation in microdiets containing oils rich in n-3 HUFA may increase the presence of these acids in the membrane phospholipids which has been shown to be positively correlated with larval growth. A marked effect on fatty acid incor-
poration was shown in older larvae by the supplementation of exogenous lipase in the microdiet. Additional study is necessary to determine if a further lipase effect can be shown in younger larvae by increasing the level of dietary lecithin to enhance the incorporation of excess lipolytic products.

References cited


