IMPORTANCE OF DIETARY N-3 HUFA FOR EYE DEVELOPMENT AND CONE FORMATION ALONG GILTHEAD SEABREAM *Sparus aurata* LARVAL DEVELOPMENT.

Roo, F. J, Izquierdo, M. S. , Socorro, J, Hernández-Cruz,C.M. Valencia, A.

Grupo de Investigación en Acuicultura, ULPGC & ICCM, PO Box 56, E-35200 Telde, Las Palmas, Canary Islands, Spain.

INTRODUCTION

Being fish larvae visual feeders, vision plays an important role in larval orientation at first feeding (Blaxter, 1986). Larval trophic behaviour is closely related with the development of the visual capacity, which directly depends on retina organogenesis. In sparids, such as *Pagrus major* (Kawamura, 1984) and *Pagrus auratus* (Pankhurst, 1996), the most important changes in the eye structure occur along the lecitotrophic stage as a preparation for prey capture.

Neuringer *et al.*,(1988) has established a critical role for n-3 polyunsaturated fatty acids and, particularly docosahexaenoic acid (DHA) in neural and retinal tissue functions in mammals. Similarly, in larval fish there is a high demand of DHA to form nervous membranes. Bell and Dick (1993) found photoreceptors in the eye, rods and cones accumulate and selectively retain DHA in external segments. Bell *et al.* (1995) found that feeding juvenile herring a DHA poor *Artemia* diet during the period of rod development resulted in impaired vision at low light intensities, when rod vision is essential.

OBJECTIVES

The present study aimed to obtain information about the effect of dietary n-3 HUFA and EPA/DHA ratio combined with light quality and intensity on eye histological development and the biochemical composition of the neural tissue.

MATERIALS AND METHODS

Eggs obtained from a gilthead seabream (*Sparus aurata)* stock by spontaneous spawning were distributed into fifteen 100-litre cylinder-conical fibreglass tanks (125 eggs/l) filled with 50µm filtered sea water provided with constant aeration and water flow (350-500 ml/min).

Four-day-old larval gilthead seabream (*Sparus aurata*), were fed once a day during 11 days with rotifers (*Brachionus plicatilis*). In order to feed the larvae with different levels of essential fatty acids three types of rotifers were used , A rotifers fed only baker´s yeast, B rotifers, fed a n-3 HUFA rich oil emulsions (DHA27, EPA28 Nippai Co. Ltd., Japan) respectively, C rotifers having similar DHA and higher EPA contains than B rotifers. Estimations of the rotifers in larval tanks were performed daily, keeping a concentration of 5 rotifers/ml until day 8 and 10 rotifers/ml in the next days. Larval rearing was carried out under three different light conditions (Table I).White light, low intensity white light and total spectral light treatments were tested in triplicate.

Larval growth was assessed by measuring total length, miotomo height and eye diameter of 20 larvae at days 0, 3 and 10 after hatching by a profile projector (Nikon V-12A, Nikon, Tokyo, Japan).

During the experiment three samples of each enrich rotifers type together with three samples of no enrich A rotifers and larvae's eyes from light treatment 1 were taken for analysis of their total lipids content and fatty acid composition.

Light conditions	DIET A	DIET B	DIET C
T_1(White light (1800-2000 Lux))	NL-A	NL-B	NL-C
T_2(Low intensity white light (500-700 Lux))			LI-C
T_3(Total espectral light (1800-2000 Lux))		TE-B	

Table I: Experimental design.

Lipid extraction and fatty acid analysis.

Total lipid extraction from A rotifers and larvae was carried out using a chloroform/methanol (2:1, v/v) mixture as described by Folch *et al.* (1957). Polar and neutral lipids from larvae eyes were separated by adsorption chromatography on silica cartridges (Sep-pack, Waters S.A., Milford, Massachusetts) as described by Juaneda and Rocquelin (1985).Fatty acid methyl esters FAMEs were analysed in a Shimadzu GC-14A gas Chromatograph (Shimadzu, Kyoto, Japan).Inyector temp 250ºC, detector temp 250ºC, column temp 180ºC, temp rate 2.5ºC/min, final temp 215ºC.

Cell counts and light microscopy

At days 2,3,10 and 15 after hatching, twenty-five gilthead seabream larvae were fixed in 10% buffered formaldehyde for histological studies. All fish were sacrificed during the photophase so that the eyes were adapted to light at the time of fixation. Larvae were then dehydrated in an ethanol series, embedded in paraffin, serially sectioned at 4-5µm, stained with hematoxylin and eosin (H&E) and Periodic Acid Shift Reactive-Haematoxylin (PAS-Hx) (García del Moral, 1993). An image analysis program was used (Image–pro 2.0),on histological preparations of the visual system. Photoreceptors (cones) contained in two segments of 0.05mm of the external nuclear layer of retina, were counted in three larvae from each age and treatment.

Statistical analysis

The statistical analysis was performed on the obtained data by one-way analysis of variance (ANOVA) and Duncan test for comparison of means (P<95%). T-student test for two samples means comparison (P < 95%).

RESULTS

As expected, lipid composition of rotifers showed the lowest lipid content in A rotifers fed with baker's yeast, being these levels similar for both types of enriched rotifers, B and C (Table II). A rotifers also showed the lowest n-3 HUFA content and high levels of monoenoic fatty acids, particularly palmitoleic acid. B rotifers and C were equal in DHA content, but C showed the highest EPA content and, subsequently, higher n-3 HUFA content and EPA/DHA ratio. The reduced n-3 fatty acid content in B rotifers in comparison with C rotifers were matched with increased saturated (about 20% higher than in the other rotifers) and n-6 fatty acids (30% higher).

	Rotifers Fatty Acids Diet A	Diet B	Diet C
Total Lipids	15,40	26,46	25,90
20:5n-3*	0,60	1,58	3,29
22:6n-3*	0,95	1,85	1,87
Saturated	16,72	25,08	19,94
Monoenes	58,16	44,99	46,26
n-3	11,36	14,34	21,25
n-6	6,53	9,95	6,98
n-9	22,17	20,54	21,23
n-3/n-6	1,74	1,44	3,05
n-3HUFA*	1,55	3,43	5,16
EPA/DHA*	0,70	0,87	1,76

Table II. Total lipid and fatty acid composition of rotifers in the experimental treatments(% of total fatty accids and (*) % dry weight) .

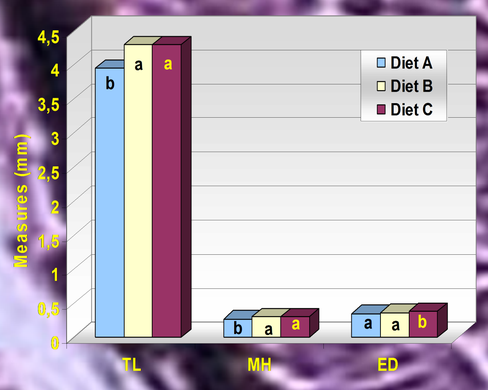

Figure 1: Morphometric measures of 10 day larvae, rearing under white light conditions and fed with different diets.

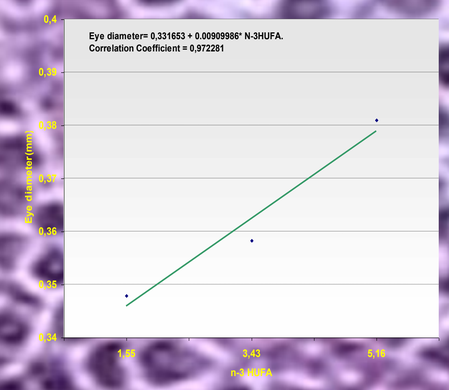

Figure 2.The results of fitting a linear model to describe the relationship between eye diameter and n-3 HUFA content in the diets

After 10 days of feeding seabream larvae under white light conditions T_1, fish fed rotifers A showed the lowest growth in terms of total lenght (TL) and miotomo height (MH), no significant differences were found between larvae fed with rotifers B and C but eye diameter was significantly higher in larvae fed rotifers C, richer in n-3 HUFA (figure 1). Thus, a positive linear correlation was found between larval eye diameter and rotifer n-3 HUFA content (figure 2). Larval growth in terms of dry body weight was also highly correlated with n-3 HUFA content of rotifers.

Despite affecting eye diameter, n-3 HUFA dietary levels did not affect photoreceptor density under white light conditions.

Regarding larval fatty acid composition of eye lipids after 14 days of feeding, highest n-3 HUFA, EPA and DHA content in the neutral lipids were found in larvae fed with A rotifers and lowest in larvae fed rotifers C. EPA/DHA ratios in the neutral lipids were similar among the different larvae and close to 1, regardless their dietary values (table III).

On the contrary, in the polar lipids highest n-3 HUFA, EPA and DHA were found both in A rotifers and C. Polar lipids from eyes of larvae fed B rotifers showed the lowest n-3 HUFA content mainly due to the low EPA levels. This larvae also showed a double content in saturated fatty acids than the other larvae, and low n-3/n-6 and EPA/DHA ratios (table IV).

Larval head neutral lipids fatty acids

	Diet A	Diet B	Diet C
20:4n-6	0.85	1.16	1.56
20:5n-3	19.21	14.20	8.58
22:6n-3	17.48	13.70	8.08
Saturated	25.29	23.69	27.78
Monoenes	16.45	23.56	27.99
n-3	47.31	37.13	26.32
n-6	9.49	14.99	16.09
n-9	8.20	12.29	14.27
n-3HUFA	23.51	18.89	13.65
n-3/n-6	4.98	2.48	1.64
EPA/DHA	1.10	1.04	1.06

Table III. Fatty acid composition of neutral lipids of the larval head (% of total fatty acids).

Larval head polar lipids fatty acids

	Diet A	Diet B	Diet C
20:4n-6	1.22	1.43	1.05
20:5n-3	19.50	3.16	21.84
22:6n-3	24.29	14.51	18.91
Saturated	20.42	48.47	22.81
Monoenes	17.39	17.91	16.10
n-3 series	49.59	21.74	49.50
n-6 series	10.22	10.86	10.73
n-9 series	8.65	10.10	9.23
n-3HUFA	47.47	19.96	46.13
n-3/n-6	4.85	2.00	4.61
EPA/DHA	0.80	0.22	1.16

Table IV. Fatty acid composition of total polar lipids of the larval head (% of total fatty acids).

Light Intensity

Larvae fed C rotifers, with highest n-3 HUFA content, showed an improved growth rate in terms of TL , MH and ED, when they were reared under low light intensity in comparison with normal light intensity conditions (figure 3). Photoreceptors density were affected by light intensity (Table V).

Treatment	Photoreceptors number / 0.05mm of retina — Larval age (days) 2	3	10	15
NL-C	27.66 ± 1.52^a	24.5 ± 0.7^a	25.5 ± 1.37^a	29.66 ± 4.5^a
LI -C	$26 + 0^a$	21.6 ± 1.52^b	24.5 ± 1.97^a	29.66 ± 3.51^a

- Values in the same row followed by different superctipt letter are signifacantly different (P< 0.05)

Table V. Effect of the light intensity over photoreceptor cells apparition.

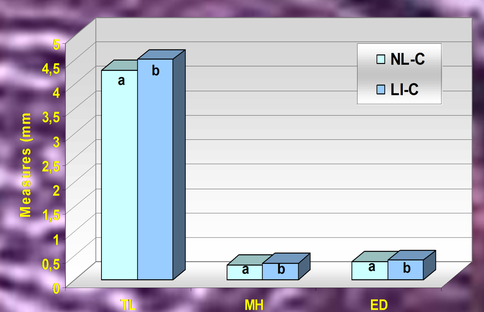

Figure 3: Morphometric measures of 10 day larvae rearing under different light intensity conditions.

Light Quality

Light quality in terms of total spectrum or fluorescent light did not affected larval growth of gilthead seabream fed with B rotifers. However larval under total spectrum light showed bigger eye diameter (figure 4) (Table VI).

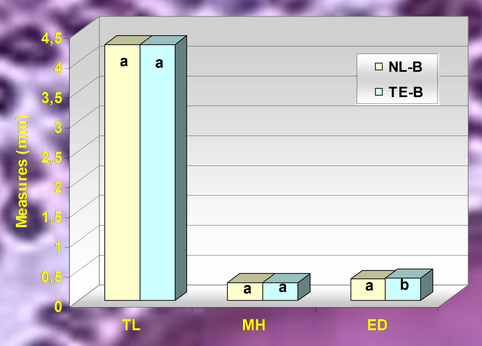

Figure 4: Morphometric measures of 10 day larvae rearing under different light quality conditions.

Treatment	Photoreceptors number / 0.05mm of retina — Larval age (days) 2	3	10	15
NLDHA	27.66 ± 1.52^a	24.5 ± 0.7^a	$25.4 + 1.67^a$	30.0 ± 4.58^a
TEDHA	27 ± 1^a	23.3 ± 0.57^b	25.33 ± 1.63^a	29.0 ± 4.35^a

- Values in the same row followed by different superctipt letter are significantly different (P< 0.05).

Table VI. Light quality effect over photoreceptor cells apparition.

DISCUSSION

Histologically, gilthead seabream eye structure was similar to those described by Kawamura (1984) and (Roo *et al.* 1999) for *Pagrus major* and *Pagrus pagrus,* respectively. *Sparus aurata* larvae eye diameter continued growing with increasing body size. Under common light conditions, low n-3 HUFA contents in A rotifers caused a low growth which was improved when n-3 HUFA contents increased up to 3.43 % in B rotifers. Although further increase in the n-3 HUFA contents (5.16 %) by elevation of EPA levels in C rotifers did not significantly improved growth in terms of TL or miotome height, it caused an increase in eye diameter. Increased levels of dietary EFA have been found to affect not only the whole brain weight (Ushio *et al.*, 1996) but also specific regions. The present study has shown as well a significant correlation between the dietary n-3 HUFA and the eye diameter. Increased eye diameter in larvae fed C rotifers, rich in n-3 HUFA, together with their high density of cone photoreceptors, would imply a total higher number of cones in this larvae and a potentially improved visual accuracy.

Despite the low levels of n-3 HUFA found in A rotifers, eye polar lipids of larvae fed these rotifers showed an especific retention of EPA and, particularly, DHA, denoting the importance of this fatty acid in this tissue. Both photoreceptors in the eye, rods and cones, accumulate and selectively retain DHA in the external segments, rods containing twice as much DHA than cones (Bell and Dick, 1993). Although only cones were present in the larval eyes in the present experiment, rods appearing in gilthead seabream after the start of the exogenous feeding about 18th day after hatch (authors´ own data), specific retention of DHA in the eye at this stage denote the importance of this fatty acid also for cone formation.

Despite n-3 HUFA levels in B rotifers were able to meet the metabolic demand of n-3 HUFA for larval growth, these levels were not high enough to keep the high n-3 HUFA demand for eye tissue formation. Thus, eye polar lipid of these larvae were low in n-3 HUFA, particularly EPA which would be incorporated into phospholipids of other tissues contributing to larval growth, and eye diameter was lower than in larvae fed higher n-3 HUFA levels. Elevation of dietary EPA levels (C rotifers) did not further promote larval length as discussed above, but allowed a higher incorporation of EPA into eye polar lipids. Furthermore, increased EPA levels in C larvae caused an spare effect on DHA levels which increased in eye polar lipids in comparison with larvae fed rotifers B despite both rotifers (C and B) had the same DHA content. Increased especific incorporation of DHA into larval PL (particularly PE and PC) when dietary EPA levels are slightly increased has been also found in gilthead seabream fed microdiets (Izquierdo *et al.*, 2000).

During first larval stages wild sparids larvae live in shallow waters under high intensity light levels. In the culture conditions of the present experiment, low light intensity caused a reduction in the density of photoreceptors at the 3rd day of life. Similarly, Pankhurst (1992) found in adult fish reared under low light intensity reduced densities of cones, ganglion cells and bipolar cells compared to fish under normal light.

Light intensity was also related with an increased growth in larvae fed C rotifers. Optimum light intensity conditions seem to differ among species and have a minimum threshold requirement bellow which the larvae are unable to detect and catch food and die after yolk sac reabsorption (Boeuf *et al.*, 1999). Our lowest light intensity conditions were above the threshold for gilthead seabream according to Ounais (1989) and Chatain (*et al.* 1991). Better growth rates obtained in the present experiment when larvae were fed high n-3 HUFA rotifers and low light conditions could be related with a lower stress conditions in this larvae. Tandler and Mason (1983) showed a positive relationship on growth and survival with the increased in light intensity from 600-1300. These authors fed larvae with rotifers growth under *Nannochloropsis oculata* and possibly with a lower EFA contents, such as DHA, which is essential for rodopsin formation and consequently for the success in prey capture under low light intensity.

There is a general lack of published information about the effect of light quality on fish larvae. Our results shows that fish reared in daylight type conditions, had a bigger eye diameter. The biological advantage of this fact for the larvae is uncertain since no differences were found in larval growth. Stefanson and Hansen (1989) studied Atlantic salmon (*Salmo salar*) reared in five different light qualities including natural light but no differences were founded on fish growth.

Changes in the photosensitivity along larval development associated with rod apparition would suggest the need to apply different light intensity conditions during larval rearing.

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