New species for EU aquaculture

Deliverable Report

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Deliverable Title	Determine changes in the essential fatty acid requirement (DHA) as a function of developmental stage and ambient salinity in grey mullet		
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Objective: Determine changes in the essential fatty acid requirement (DHA) as a function of developmental stage and ambient salinity in grey mullet

Table of Contents

1. Introduction

It is widely accepted that the long chain polyunsaturated fatty acid (LCPUFA) docosahexaenoic acid (DHA; 22:6n-3) promotes growth more effectively than the other LCPUFAs; eicosapentaenoic acid (EPA; 20:5n-3) and arachidonic acid (ARA; 20:4n-6) in marine fish larvae (Watanabe, 1993, Koven et al., 1992, Wu et al., 2002). The importance of DHA to weight gain lies in its contribution to membrane fluidity and function mainly in the neural membranes of the eyes and brain as well as its involvement in immune function and gene expression (Izquierdo and Koven, 2011; Arts et al., 2009). Optimum DHA levels in larval feeds to

promote growth and survival range from 0.5 % for *Acanthochromys poliacanthus* (Southgate and Kavanagh, 1999) to 2.5 % DW diet for Atlantic halibut *Hippoglossus hippoglossus* (Hamre and Harboe, 2008). The essential fatty acid requirements of larval grey mullet (*Mugil cephalus*) have not been clearly defined although analysis of eggs and carcass content has been reported (Tamaru et al. 1992). In addition, there is evidence for Δ6 desaturase activity (Argyropoulou et al. 1992) in this fish. This is the rate limiting enzyme involved in the biosynthesis of DHA from smaller chain precursors such as α-linolenic acid (18:3n-3), which could modulate the dietary requirement for this essential LCPUFA. However, marine fish larvae are strict carnivores and its natural marine environment would be rich in n-3 LCPUFAs making it somewhat redundant to produce DHA from 18:3n-3 at a physiologically significant rate (Sargent et al., 2002).

In contrast, freshwater fish are capable of producing DHA from 18:3n-3 (Buzzi et al., 1996; Bell et al., 2001) and so must express all the desaturase and elongase activities necessary for this biosynthetic pathway (Izquierdo and Koven, 2011). Interestingly, grey mullet transit from strict carnivores as larvae in a DHA rich marine environment to omnivorous feeding juveniles that move to the lower salinity waters of relatively n-3 LCPUFA poor estuaries and rivers. The question remains how the salinity change and reduction of DHA in the diet of developing mullet affect their dietary requirement for this essential fatty acid and their ability to desaturate and elongate shorter chain n-3 polyunsaturated fatty acids.

Consequently, the aims of this study were (1) to determine the effect of salinity and DHA on grey mullet juvenile growth. (2) To characterize sodium potassium ATPase pump activity in gill epithelium. (3) To quantify expression of genes related to DHA synthesis. (4) To establish the relationship between the expression of these genes and environmental salinity.

2. Materials and methods

Grey mullet eggs were stocked (100 eggs/l) in eighteen 1.5 m^3 tanks where UV treated, filtered (10 µm), ambient sea water (40 ‰) at 25^{o}C entered the bottom of the tanks and exited near the top through a 500 µm filter. The temperature and salinity were computer controlled (Gavish, Israel). The hatching rate (%) of stocked grey mullet eggs and survival of the pre-larva at the end of the day of hatching (0 dph) were calculated by placing a fertilized egg, at the gastrula stage, in each of 12 wells (5 ml) in each of three plastic well plates. The plates were covered and placed in a temperature controlled incubator until hatching where the emerging larvae and surviving newly hatched larvae at the end of 0 dph were counted.

The salinity was incrementally reduced to 25 ‰ and the larvae reared according to the IOLR protocol until 35 dph. At this age, the tanks were divided into two groups where the salinity in each group of 9 tanks was gradually adjusted to 15 or 40 ‰. In each salinity group low, medium and high *Artemia* DHA treatment levels (1.7, 6.6 and 12.2 mg DHA/g DW) were tested on 35-59 dph fish in replicates of three tanks per treatment. On 59 dph, samples of fish were dried and weighed, gills removed for Na^+/K^+ATPase determination and RNA extracted for gene expression of selected genes. The fish, in each salinity group, were then grown from 60 to 89 dph and continued to be fed pellets that tested low, medium and high dietary DHA levels (7.1, 9.8, 13.5 mg DHA/g DW). At 89 dph, samples of fish were dried and weighed, gills removed for Na^+/K^+ATPase determination and RNA extracted from the liver for gene expression of selected genes.

2.1 Dry weight determination

Approximately 130 larvae per tank, were sacrificed with an excess of MS-222 and then washed with distilled water. The fish samples were then dried at 70 ºC for 24 h followed by their weighing on an analytical balance (A&D HD-120, Japan).

2.2 Survival

Survival values were expressed as a percentage of the surviving fish at sampling over the number of the fertilized eggs initially stocked (adjusted for hatching rate and survival after 24 h) in tanks and taking into account the number of sampled fish for analytical purposes.

2.3 RNA extraction, cDNA synthesis and real-time quantitative RT-PCR

Total RNA from fish livers were extracted using Bio-Tri reagent (Bio-Lab LTD., Jerusalem, Israel) according to the manufacturer's protocol. The extracted RNA (5 µg) was treated with 2 units of RNAse free-DNAse I (Ambion®, Life Technologies, Israel) to remove contaminating genomic DNA (30 min at 37 °C), and following DNAse inactivation (5 min at 75 °C). For sequencing of Δ6 desaturase, elongase, PPAR and SREBP1, specific primers were designed according sequences displaying high conservation among Perciformes PCRs were carried out in a final volume of 50 µl using Verso™ 1-step RT-PCR ReddyMix Kit (Thermo Scientific, UK) according to the manufacturer's protocol. The cycling parameters were: 50 °C for 15 min, 95 °C for 2 min, followed by 35 cycles at 95 °C for 20 s, 55 °C for 30 s, 72 °C for 1 min and 72 °C for 5 min. Then, the PCR products were sent for sequencing at the DNA biological services, HyLab, Israel.

1 µg of total RNA were treated as above and cDNA transcribed (qScript™ kit, Quanta Biosciences, USA) according to manufacturer's protocol. Quantitative real time polymerase chain reaction (qPCR) was performed in triplicate in 10 µl reaction volume (Taqman Perfecta® FastMix II kit, Quanta Biosciences, USA) according to manufacturer's protocol. Amplification was carried out in a fast real time PCR system (Applied Biosystems) at the following thermal cycling conditions: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 65 °C for 15 s and 72 °C for 10 s. The specificity was assessed by melting curve run following the PCR. To normalize the levels of selected genes, qPCR for rRNA 18S was also performed with the sample cDNAs. A negative control with sterile water as a template was included in order to check for possible reagent contamination. The results were analyzed by Fast 7500 real-Time PCR system software (Applied Biosystems). Δ6 desaturase, elongase, PPAR and SREBP1 expression levels were calculated by relative expression: $RQ= 2^{-\Delta\Delta ct}$ according Livak and Schmittgen (2001).

2.4 Fatty acid analysis

In fatty acid analysis, the following procedure was carried out. The total lipid in freeze dried tissue samples (20 mg) was chloroform: methanol (2:1) extracted (Folch et al., 1957). The lipid-containing chloroform upper phase was removed and evaporated to dryness under a stream of nitrogen and total lipid weighed (GH-120 analytical balance, A&D, San Jose, CA,USA). This was followed by the addition of the internal standard 17:0 (heptadecanoic acid) to each of the samples. The samples were then transmethylated to their fatty acid methyl esters (FAME) by adding 1 ml/mg lipid of a 14% solution of boron tri- fluoride methanol (BF3) and sonicated for 1 h at 50 °C. To the samples were added 0.5 ml double distilled water (DDW) and 1 ml/mg lipid of analytical grade hexane (99%), which was vortexed and then centrifuged (2300 rpm) for 2 min. The top hexane layer containing FAME was transferred to a vial and stored at −20 °C until analysis in a Varian 450–220 GC/MS/MS (Agilent technologies, California, USA). Injected FAME samples (1 µl) were separated on a Varian WCOT fused silica column (50M×0.32 mm) at a flow rate of 1.5 ml/ min and identified by known purified standards and quantified using a response factor to the internal standard (17:0; Sigma, St. Louis, MO, USA). The 30 min oven temperature program began at 70 °C for 4 min following injection and then increased to 300 °C at 10 °C min−1 for 3 min.

2.5 Na^+/K^+-ATPase activity

Gill filaments were placed in ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and stored at −80 °C. Branchial Na^+/K^+-ATPase activity was determined as described by McCormick (1993) Briefly, ouabain-sensitive Na^+/K^+-ATPase activity was measured by coupling the production of ADP to NADH using lactic dehydrogenase and pyruvate kinase in the presence and absence of 0.5 mmol/l ouabain. Ten µl of samples were loaded (Duplicates) in 96-well microplates at 25 °C and read at a wavelength of 340 nm for 10 min on a BioTek Synergy 2 spectrophotometer (BioTek, Winooski, VT).

Protein concentration of the homogenate was determined using a BCA protein assay (Thermo Fisher Scientific, Rockford, IL).

2.6 Ethics statement

All animal experimental procedures were conducted in compliance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

3. Results

In **Fig. 1a** 59 dph fish demonstrated that whole body DHA correlated with prey DHA level in a dose dependent manner independent of salinity. On the other hand in older 89 dph fish (**Fig. 1b**), whole body DHA was dependent on rearing salinity. Fish reared at 15 ‰ showed no correlation between whole body and prey DHA level while fish grown in 40 ‰ seawater continued to show tissue DHA levels that corresponded with levels of this LCPUFA in the diet. These results are consistent with those in **Fig. 2**, which demonstrated that there was no dietary DHA dose dependent effect on growth in 89 dph fish grown in the lower salinity while there was a dietary DHA dose dependent effect on growth in fish grown in 40 ‰. In **Fig. 3** there was no significant ($P>0.05$) dietary DHA dose dependent effect on Na^+/K^+ ATPase activity in the gills of fish reared at 15 ‰ while there was a significant ($P<0.05$) dietary DHA level effect on the activity of this enzyme in the gills of fish reared at 40 ‰. There was a marked ($P<0.05$) salinity effect on Δ6 desaturase gene expression, which was highest in fish reared at 15 ‰ fed the lowest DHA (**Fig. 4**). Conversely, there was no dietary DHA effect on the expression of this gene independent of salinity. On the other hand, in **Fig. 5** there was a significant ($P<0.05$) inverse effect of dietary DHA on the relative gene expression of elongase in the low salinity fish. In fact, the highest ($P<0.05$) expression of this gene in both salinity treatments was in fish fed the low dietary DHA treatment reared in 15 ‰. Fish reared in 40 ‰ seawater demonstrated no clear effect of dietary DHA on elongase gene expression. **Fig. 6** showed a significant ($P<0.05$) inverse relationship between dietary DHA level and PPAR gene expression in fish reared at both salinities. In contrast, there was an inverse dietary DHA level effect on SREBP1 gene expression only in fish reared at 15 ‰ while cohorts grown in the higher salinity of 40 ‰ demonstrated no pattern of SREBP1 expression with dietary DHA (**Fig. 7**).

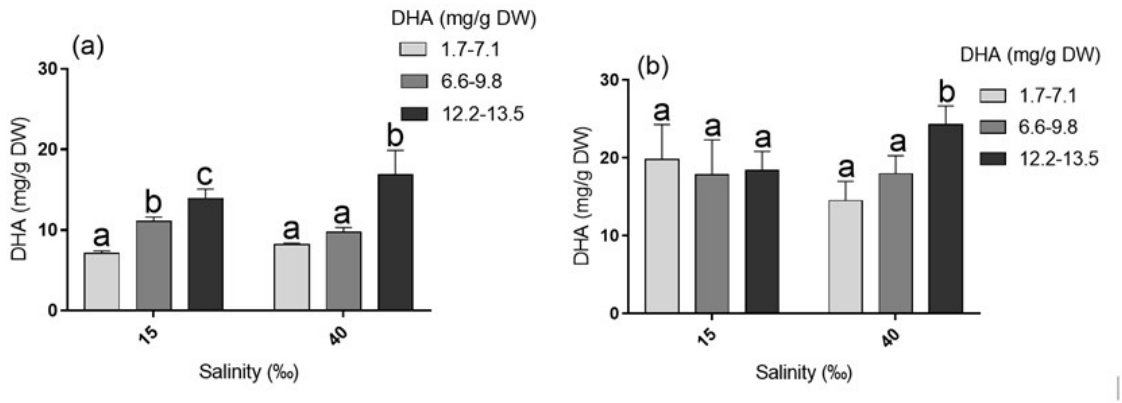

Figure 1. Effect of (a) *Artemia* DHA (1.7, 6.6 and 12.2 mg DHA/g DW) on 58 dph fish DHA levels (mg DHA/g DW) reared at 15 and 40 ‰ and (b) pelleted diet DHA (7.1, 9.8, 13.5 mg DHA/g DW) on 89 dph fish DHA levels (mg DHA/g DW) reared at 15 and 40 ‰. DHA values within a salinity group having different letters were significantly ($P<0.05$) different.

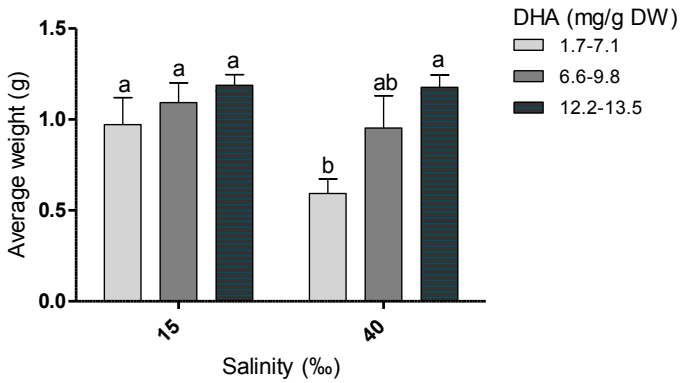

Figure 2. The effect of *Artemia* and pelleted diet different DHA levels on average weight in 89 dph fish reared at 15 and 40 ‰. Average weight values having different letters within a salinity group were significantly (P<0.05) different.

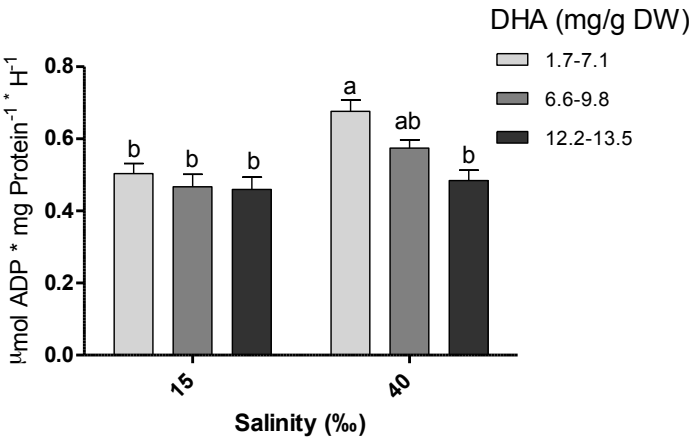

Figure 3. The effect of *Artemia* and pelleted diet different DHA levels on Na+/K+ ATPase activity in 89 dph fish reared at 15 and 40 ‰. Na+/K+ ATPase activity values having different letters were significantly (P<0.05) different.

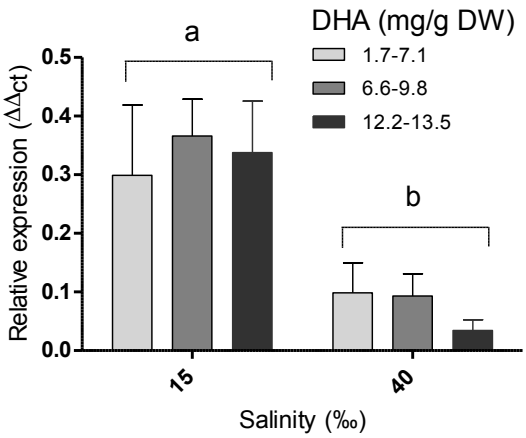

Figure 4. The combined effect of dietary treatments in each of the salinity treatments on the relative gene expression of Δ6 desaturase. Combined values of each of the salinity treatments having different letters were significantly (P<0.05) different.

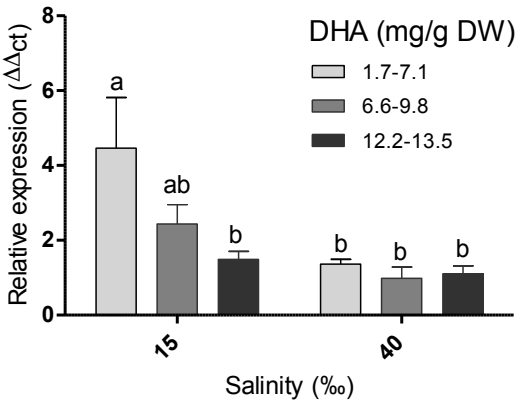

Figure 5. The effect of *Artemia* and pelleted diet different DHA levels on relative gene expression of elongase in 89 dph fish reared at 15 and 40 ‰. Average weight values having different letters within a salinity group were significantly (P<0.05) different.

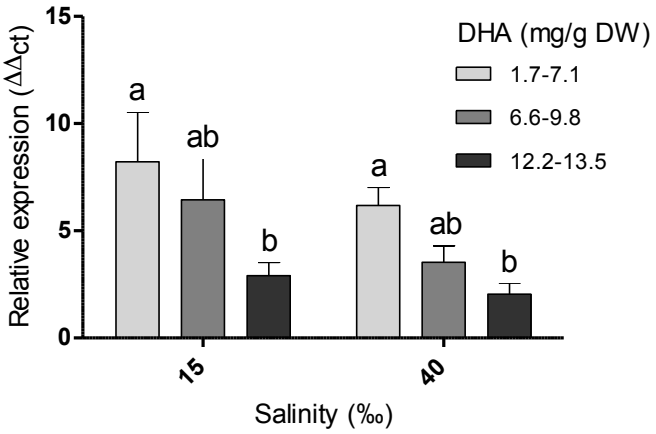

Figure 6. The effect of *Artemia* and pelleted diet different DHA levels on relative gene expression of PPAR in 89 dph fish reared at 15 and 40 ‰. PPAR expression values having different letters within a salinity group were significantly (P<0.05) different.

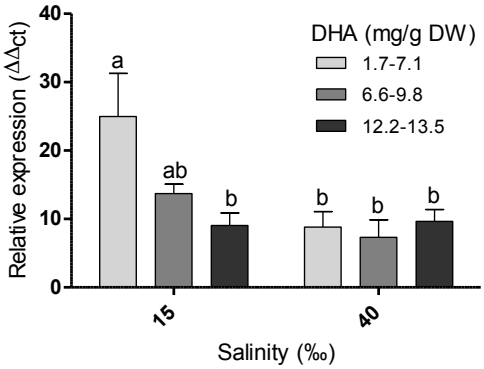

Figure 7. The effect of *Artemia* and pelleted diet different DHA levels on relative gene expression of SREBP1 in 89 dph fish reared at 15 and 40 ‰. SREBP1 expression values having different letters within a salinity group were significantly (P<0.05) different.

4. Discussion

There was a dietary DHA dose dependent effect on fish whole body DHA in 59 dph fish reared in low and high salinity. However, this changed in older 89 dph fish, where there was no dietary DHA dose dependent effect on tissue DHA and growth in fish reared at 15 ‰ while DHA dietary level did significantly ($P<0.05$) modulate fish body DHA and weight gain in cohorts grown at the higher salinity of 40 ‰. This broadly hints that older (89 dph) mullet grown in low salinity are adopting the fresh water model of LCPUFA biosynthesis and have the capability to produce DHA from shorter carbon chain precursors. (Izquierdo and Koven, 2011). DHA would be particularly required in fish grown in high salinity as they must invest 20-68% of their total energy expenditure in osmoregulation (Boef and Payan, 2001) which is regulated by Na^+/K^+ ATPase. It has been reported (Turner et al., 2003) that DHA modulates Na^+/K^+ ATPase activity, which would likely be through its contribution to membrane fluidity (Izquierdo and Koven, 2011) leading to reduced activity of this enzyme and energy demand. On the other hand, fish reared at 15 ‰ will be less challenged with the metabolic requirements of osmoregulation and will be able to mobilize more energy for tissue deposition and growth. This suggests that the DHA requirement would be less in fish exposed to 15 ‰ and/or that these individuals also have the ability to satisfy their requirement for this LCPUFA through biosynthesis. This is suggested by the fact that both biosynthetic enzymes; Δ6 desaturase and elongase were markedly ($P<0.05$) activated by the low salinity treatment. This follows as mullet individuals in nature would be moving to the lower salinity waters of river mouths and estuaries, which are characterized by an environment less rich in LCPUFA and more abundant in smaller chain PUFA precursors. Low salinity upregulated the gene expression of the rate limiting enzyme of LCPUFA biosynthesis; Δ6 desaturase but was independent of DHA dietary level. On the other hand, both low salinity and DHA level upregulated the gene expression of elongase.

There are two transcription factors, SREBP1 (sterol regulatory element binding protein) and PPAR (peroxisome proliferator activated receptors), that are involved in the regulation of fatty acid biosynthesis in mammals (Nakamura and Nara 2003). The enzyme Δ6 desaturase is the rate limiting step in the desaturation and elongation of PUFA to LCPUFA and its gene expression in this study was up-regulated only by the lower salinity. Although both SREBP1 and PPAR expression were highest in 15 ‰ water, PPAR expression was inversely regulated by dietary DHA at both salinities while SREBP1 was inversely regulated by DHA only in the low salinity. DHA likely suppressed the critical proteolytic activation stage of both PPAR and SREBP1 (Nakamura et al., 2004), hence the markedly ($P<0.05$) lower Δ6 desaturase and elongase expression when dietary DHA is high.

Although speculative, salinity appears to be the main trigger in grey juvenile grey mullet and to a lesser degree dietary DHA of the gene expression of the transcription factor SREBP1, which in turn stimulates the rate limiting biosynthesis enzyme Δ6 desaturase. Similarly, the euryhaline rabbitfish (*S. canaliculatus*) showed a 1.56-fold higher Δ6 desaturase gene expression in liver at salinities of 10 ‰ compared to 32 ‰ (Li et al., 2008). However, this appears to be species specific as recent studies suggested the involvement of PPARs in the modulation of Δ6 desaturase gene transcription in fish (Kennedy et al., 2006; Vagner et al., 2011). Moreover, higher Δ6 desaturate mRNA levels were measured in Atlantic salmon (Zheng et al., 2005a,b) and gilthead sea bream (Seiliez et al. 2003; Izquierdo et al., 2008) when fed diets where the fish oil component had been significantly replaced by vegetable oils severely reducing the dietary DHA level. In the present study, dietary DHA in grey mullet appears to be the more dominant factor stimulating SREBP1 and PPAR in an inverse dose dependent manner, which in turn stimulates the biosynthesis of elongase.

Broadly speaking, these results suggest that dietary levels of DHA can be decreased when feeding older juvenile mullet grown in captivity, provided that the salinity is reduced to levels found in estuarine waters. This would translate as a significant savings for farmers as the purchase of feed for the grow-out of fish to market weight can represent 60% of production costs and DHA is costly as a feed additive.

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Deviation from DOW

This deliverable is being submitted much later than planned in the project due to difficulties finding suitable technical staff and students to carry out the experiments.


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