Deliverable Report

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Deliverable Title	Protocol for optimal early fatty acid enrichment to reduce stress sensitivity in pikeperch			
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WP Title:	Nutrition – pikeperch			
Task No:	10.2	**Task Lead beneficiary:**		P21. DTU Aqua
Task Title:	Effects of pikeperch early fatty acid nutrition on long-term stress sensitivity			
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Objective: Protocol for optimal early fatty acid enrichment to reduce stress sensitivity in pikeperch: This deliverable will present a protocol to reduce stress sensitivity in pike perch by the balance in fatty acids contained in enrichment products for pike perch larvae. The deliverable will present the methodology employed and the main results that allowed to develop this protocol including the effects of essential fatty acids and phospholipids on: a) on pikeperch physiology, b) welfare indicators including stressors responses, compromised behaviors and post stress oxygen consumption, c) neural development, d) fish tissue lipid / fatty acid catabolism / re-synthesis, fatty acid composition and eicosanoid activity, d) the tissue capability to synthesize LC-PUFAs, e) in situ hybridization and f) skeleton morphogenesis.

Description: Two main experiments were conducted; - a study to determine the capability of pike perch larvae to metabolize PUFAs under different salinity regimes, and a study on the short and long term consequence of diets low in LC-PUFAs on stress sensitivity. In order to perform the latter experiment additional pilot studies were included. As Deliverable 10.1 also involves performance studies and physiological experiments on larval dietary requirement for phospholipids and LC-PUFAs; deliverable 10.1 and 10.2 should be considered as a whole.

One manuscript has been submitted to a peer reviewed journal entitled:

1) Influence of salinity and linoleic or α-linolenic-based diets on ontogenetic development and metabolism of unsaturated fatty acids in pike perch larvae (*Sander lucioperca*)

Introduction

In modern intensive aquaculture, the robustness and stress resilience are of crucial importance in terms of welfare, health, growth, quality of the end product and thus overall production costs. A major bottleneck for expansion of pikeperch culture today is its high sensitivity to stressors.

Pikeperch larvae resemble most marine carnivorous fish larvae having a high dietary requirement for phospholipids and long chain polyunsaturated fatty acids (LC-PUFAs) (Lund et al., 2017, Deliverable 10.1), which may evoke from the evolutionary marine origin of the species as also indicated by the very high docosahexaenoic acid (DHA; 22:6n-3) content in eggs and larvae and its rapid consumption during early development (Lund & Steenfeldt, 2011). During early larval development, diets low in LC-PUFAs, especially DHA, may provoke increased mortality; shock syndromes (Lund & Steenfeldt, 2011). Information is lacking about the link between the influence of dietary lipid composition early in ontogeny and the robustness of produced fish.

Pikeperch is a piscivorous percid fish originating from the Caspian-Black Sea region, but has spread throughout most of mainland Europe (Craig, 2000). Though regarded as a stenohaline fish, pikeperch populations in brackish water for instance in the Baltic Sea have an anadromous life-history strategy similar to those of salmonids; as they use the coastal environment in the Baltic Sea as forage habitat but migrate to streams and brooks for reproduction. Despite farming of this species takes place in freshwater, studies have shown a high level of osmotic tolerance and degree of hypo-osmoregulatory capacity in a saline environment. This is not common for most other stenohaline freshwater teleosts (Brown et al., 2001), but may suggest a growth potential for rearing in low saline waters or in water iso-osmotic to plasma, which has not been studied so far. As larvae are relatively undeveloped at hatching and do not have the same osmoregulatory capacity (i.e. gills, urinary bladder, completely functional gut, kidneys) as metamorphosed larvae or juveniles, there is a need for a more comprehensive understanding of the influence of salinity on pikeperch larvae development. Successful production of pikeperch juveniles is constrained by the high incidence of skeletal anomalies and cannibalism (Kestemont et al., 2015) a scenario that may be modified by environmental salinity.

The high demand of LC-PUFAs in pikeperch suggests a low elongation/desaturation capacity in this species in contrast to many other omnivorous or carnivorous freshwater fish species, that have a relatively low LC-PUFA requirement and a certain capability to elongate and desaturate C-18 carbon PUFAs (Linoleic acid (LA;18:2n-6), alpha linolenic acid, (ALA); 18:3n-3) to LC-PUFAs of C-20 or C-22 carbons, i.e. 20:4n-6 (ARA), 20:5n-3 (EPA) and DHA (Tocher, 2010, Xie et al., 2015). In Eurasian perch (*Perca fluviatilis*), dietary partial substitution of fish oil with vegetable oils and an increased dietary proportion of LA:ALAdoes not affect growth performance, but increase fatty acid elongation and desaturation activity in liver (Blanchard et al. 2008). Besides, Eurasian perch fed a semipurified fat free diet over 4 weeks followed by different lipid sources (olive oil (OO), safflower oil (SO), linseed oil (LO), or cod liver oil (FO) as the only lipid source showed significant changes in n-3/n-6 ratio and FA composition; indicating that the competition and inhibition between LA and ALA for further desaturation and elongation were greatly influenced by the type of vegetable fat and the content of n-3 and n-6 FA in the diet (Xu & Kestemont, 2002). A dietary incorporation of LA concomitantly to ALA seemed to be beneficial in order to stimulate liver Δ6 and Δ5 desaturation activity, - rate-limiting enzymes involved in LC-PUFA biosynthesis (Xu & Kestemont, 2002; Blanchard et al., 2008).
Salinity may have variable effects on modulation and expression of Δ6 desaturase activity and fatty acid composition in fishes as previously reviewed (Vagner and Santigosa, 2011) and, therefore, affects quantitative PUFA requirements of a same fish species reared at different salinities (Izquierdo and Koven, 2010). In Atlantic salmon (*Salmo salar)*, the LC-PUFA synthesis pathway is affected by environmental cues during transfer to seawater that modulate the expression of fatty acyl desaturase genes in freshwater and seawater phases (Zheng et al., 2005), The rabbitfish *Siganus canaliculatus,* an euryhaline herbivorous, has a higher ability to convert LA and ALA to LC PUFAs when reared in lower salinities (Li et al., 2008; Xie et al., 2015). In the freshwater Mexican silverside (*Chirostoma estor*) LC- PUFA biosynthesis from C18 precursors is detected at low salinities (5-15 ppt) , whereas this pathway is not active in fish reared in freshwater (Fonseca-Madrigal et al., 2012). Studies on the effect of salinity on fatty acids requirements and biosynthesis pathways are scarce (Izquierdo and Koven, 2010). For instance, larvae of the euryhaline common galaxias (*Galaxias maculatus*) reared at different salinities (0-15 ppt) denoted a higher DHA requirement at higher salinities (Dantagnan et al., 2010) and a higher PUFA (18:3n-3) requirement at 0 ppt (Dantagnan et al., 2013).

Results have shown that for marine fishes that rely on marine diets naturally rich in LC-PUFAs the enzymatic activity required for the conversion of C18 FA into LC-PUFAs may be low or down regulated (Sargent et al., 2002).

Experiment 1
Influence of salinity and 18:2n-6 or 18:3n-3 based diets on ontogenetic development and metabolism of unsaturated fatty acids in pike perch larvae (*Sander lucioperca*).

Objectives:
We hypothesized here that pikeperch, which is a freshwater fish and consequently has less availability to dietary LC-PUFAs, may have a higher enzymatic activity and bioconversion efficiency than marine fishes, if exposed to a range of low salinities and LC -PUFA precursors. We further suggest, that salinity and dietary FA composition (n-3 /n-6) may have significant effects on pikeperch larval physiology and ontogenetic development involving enzymatic and hormonal activity and changes in the incidence of skeletal deformities.

Materials and methods

Experimental design

Experimental set up and feeding
Fertilized eggs of pikeperch close to hatching were obtained from Aquapri Innovation, Egtved, Denmark and transferred to DTU Aqua at the North Sea Research Centre, Denmark. Eggs were incubated at 17.5° C in McDonald jar incubators at a flow rate high enough to keep eggs in suspension. Eggs hatched during the following two days and larvae numbers were roughly counted and distributed to 18 conical transparent 46 L tanks in a temperature-controlled flow through system, at 16.5°C with use of temperature regulated seawater/ freshwater from two separate temperature controlled 10 m^3 reservoirs. Salinity in each tank was adjusted by peristaltic pumps (Longer Pumps BT600-2J) which pumped an accurate amount of seawater to the freshwater tank inlet pipe for a thorough mixing.
Each tank was initially stocked with approximately 43 larvae/L; corresponding to approximately 2000 larvae/tank and reared until 30 days post hatch (DPH) at constant 24 h dimmed light conditions (i.e. 30-40 Lux at water surface) provided by LED light bulbs installed over each tank. From 4- 9 DPH larvae were fed a newly hatched unenriched *Artemia* nauplii (strain MC460, *Artemia* Systems INVE, kept at 5°C after hatching) for 2 x 8 h by automatic dispensers each holding a suspension of *Artemia* in seawater programmed to feed every 20 min ensuring that live *Artemia* nauplii were present ad libitum in the tanks. From 10 DPH and onwards larvae were similarly fed by EG *Artemia* nauplii*;* (*Artemia* Systems INVE) enriched subsequent to hatching (+ 12 h, i.e. nauplii stage II) for 24 h with one of two lipid emulsions based on either sunflower oil or linseed oil and supplemented by olive oil (for obtaining similar levels of oleic acid, 18:1n-9 (OA)). The 2 emulsions were formulated to differ in composition of primarily LA and ALA FA (**Table 1**). In both experimental emulsions a vitamin premix was added (40 g kg^{-1}) and soy lecithin was included (70 g kg^{-1}) as an emulsifier(**Table 1**). Organic sunflower oil and linseed oil were commercially available products; the olive oil was highly refined and obtained from Croda Chemicals Europe, Snaith, UK. Soy lecithin and vitamin premix were purchased from BioMar, Tech Centre, Brande, Denmark.

Table 1 Dietary composition of the 2 experimental emulsions and a commercial DHA Selco control.

Diet Ingredients (%)	*DHA Selco	SO	LO
Linseed oil[a]		0	68
Sunflower oil[b]		85	0
Olive oil[c]		4	21
Soy lecithin[d]		7	7
Vitamin Premix[e]		4	4

[abcde] see text ;

*DHA Selco (commercial control emulsion ; 60% fish oil; 33% moisture, 1 % protein) Organic linseed oil[a] and sunflower oil[b] were commercially available products; olive oil[c] was highly refined and obtained from Croda Chemicals Europe, Snaith, UK. Soy lecithin[d] and vitamin premix[e] were purchased from BioMar, Tech Centre, Brande, Denmark.

The experiment was carried out in a triplicate set-up with 3 tanks per feed type at 3 salinities; i.e. 0 ppt; 5 ppt; 10 ppt. equalling a total of 3 x 3 x 2 (18) tanks. An additional group of larvae reared at 0 ppt. salinity on EG *Artemia* enriched by a commercial DHA Selco; (*Artemia* Systems INVE) was used as an experimental control (the salinities chosen were based on pilot studies on newly hatched pike perch, which revealed that salinities > 15 ppt caused reduced growth and severe mortality (- Special course, Master student))
Oxygen saturation and temperature were monitored daily by a hand-held Oxyguard meter from Oxyguard, Birkerød, Denmark. During the experiment oxygen saturation was 88-94% (i.e. 8.8 ±1.3 mg L^{-1}) and temperature (17.5±0.6 °C).
The total flow rate of either freshwater or fresh - and seawater was kept at approximately 11-12 L h^{-1} $tank^{-1}$ and checked/adjusted twice daily and salinity in tanks was measured by a refractometer. On a daily basis the bottom of each tank was vacuum-cleaned for feed remaining; dead *Artemia* and dead larvae and collected in a bucket. The number of dead larvae per tank day^{-1} was then counted and registered.
Confinement mortality
At the end of the trial, all remaining larvae were gently and slowly siphoned from the bottom outlet from each rearing tank by emptying the tanks through a pvc pipe into a submerged net. Larvae were then without air exposure transferred to a 20 x 20 cm wide plastic tray with a 2 cm water level (same salinity as in the rearing tank) and a light exposure of 500 Lux by a LED lamp. Larvae motility was observed for 5 min and subsequent mortality defined as larvae without movement by touch of a set of tweezers was then registered as dead**.** Total number of larvae was counted.

In vivo incubation with individual labelled ^{14}C fatty acids
Larval capability to incorporate into total lipids and metabolize unsaturated FA under the different dietary and salinity regimes was studied by in vivo radio tracing of ^{14}C fatty acids (Fonseca et al., 2012 and Reis et al., 2014). Non fed larvae (12 h feed deprived) at an age of 20 DPH were sampled from the 6 different rearing conditions (i.e. SO and LO groups; 0, 5, 10 ppt salinity) in addition to larvae from the DHA Selco control group at 0 ppt salinity. Larvae were incubated by triplicate in flat-bottom tissue culture plates (10 larvae per plate) provided with gentle stirring for 5 h, with 0.2 µCi (0.3 µM) of ^{14}C labelled FA including LA, ALA, ARA, EPA and DHA. Larvae were incubated in the same water; temperature and salinity as provided in the different experimental larval rearing conditions. The substrates were directly added to the incubation water. For each dietary and salinity treatment a triplicate group of larvae were also incubated without ^{14}C FA for further analysis of their lipid profiles. In order to determine the radioactivity incorporated into whole larvae, after total lipid extraction (Folch et al., 1957) an aliquot of 0.1 mg of corresponding total lipid (TL) extracts from each sample were transferred to a scintillation vial. Radioactivity was quantified in a RackBeta 1214 liquid scintillation β-counter (LKB, Wallac, USA). Results in dpm were transformed into pmol mg $protein^{-1}$ h^{-1} taking into account the specific activity of each substrate and the sample TL and protein contents (Lowry et al., 1951).

To define the esterification pattern of [1− 14 C] FA, a sample of 0.1 mg of TL was also applied to HPTLC plates. Lipid classes were separated by single-dimensional double-development HPTLC as previously described by Tocher and Harvie (1988). After identification of the bands by a brief exposure to iodine vapor atmosphere, the developed HPTLC plates were placed in closed exposure cassettes (Exposure Cassete-K, BioRad, Madrid, Spain) in contact with a radioactive-sensitive phosphorus screen (Image Screen-K, BioRad, Madrid, Spain) for one week. The screens were then scanned with an image acquisition system (Molecular Imager FX, BioRad) and the bands quantified in percentage by image analysis software (Quantity One, BioRad) (see Reis et al., 2014/ Aquaculture 28–33).
Transformation of radiolabeled fatty acids substrates in terms of desaturation/elongation processes, was assessed by subjecting a sample of around 2 mg of TL to acid-catalysed transmethylation to prepare FAME as detailed by Christie, 2003. FAME were separated by TLC using plates impregnated with a solution of 2 g silver nitrate in 20 mL acetonitrile followed by activation at 110 °C for 30 min. The plates will be fully developed in toluene/acetonitrile (95:5, v/v), which resolves the FAME into discrete bands based on both degree of unsaturation and chain length (Wilson and Sargent, 1992). Developed TLC plates were placed in BioRad closed exposure cassettes in contact with Image Screen-K for two weeks. The screens were then scanned with Molecular Imager FX and bands quantified by Quantity One software. The identification of labelled bands was confirmed by radiolabeled standards run on the same plate (Rodríguez et al., 2002).

Growth performance, survival and analytical samplings
Representative samples of *Artemia* were sampled at 12 and 24 DPH for d.w. and FA composition. At 15, 25 and 30 DPH, 25-35 larvae per tank were sampled for growth (mg wet weight ind.$^{-1}$) and a random subsample of 10 larvae per replicate used for FA composition. At 30 DPH additionally 10 larvae per tank were sampled for measurement of eicosanoid PGE_2 and PGE_3 activity. For digestive enzymatic activity 20 larvae per tank were sampled at 30 DPH. Survival was estimated based on the approximate number of larvae inserted at 1 DPH and total number of larvae collected after the confinement study at 30 DPH. Percent survival did not include larvae sampled during the experiment.
All above larval samples were sedated with benzocaine and immediately frozen at -80°C. Additional 20 larvae per tank were sampled for skeleton morphogenesis and mineralization by staining at 30 DPH. These larvae were sedated; fixed and stored in 10 % phosphate buffered formaldehyde until analysis. For relevant genes involved in the skeletal system and organ development 10 larvae per replicate were sampled sedated and stored in RNA later (kept overnight in RNA later at 4°C and then frozen (-80°C) until analysis.

Fatty acid analysis
FA analysis of *Artemia* and larvae was done according to previously described (Lund et al., 2014). Briefly, lipids were extracted by a chloroform/methanol mixture, (2:1 (v/v); Folch et al., 1957) and addition of 40 µl (1 mg/ml) of an internal 23:0 FAME standard from Sigma Aldrich. Larval samples were homogenised by a tissue tearor probe diameter 4.5 mm, Biospec Products, Inc; Bartlesville, USA, while *Artemia* samples were sonicated in an ultrasonic cleaner (Branson 2510) for 30 min in ice water. All samples were allowed standing for 24 h in – 20°C followed by centrifugation. The supernatant was subsequently transferred to clean GC vials and allowed drying out in a Pierce, reacti-therm heating module at 60°C, under a continuous flow of nitrogen. Trans esterification of the lipids were done by addition of 1 mL of acetyl chloride in methanol (40:50:10, HPLC quality) at 95 °C. The fatty acid methyl esters were analyzed by gas chromatography–mass spectrometry (GC–MS). Peaks on a given chromatogram were identified by comparison with the retention time of a commercial mix of a known FAME standard, SUPELCO 18919 (4:0–24:0), from SIGMA. Peaks were quantified by means of the target response factor of the fatty acids and 23:0 as internal standard. Fatty acid concentrations were calculated (MSD Chemstation Data Analysis, G1710FA) based on the quantified peaks of the standard series and the samples as well of dry weight of prey and larvae and expressed as ng sample^{-1}. A total of 34 fatty acids were analyzed, but only the most relevant shown (Table 2).

Digestive enzymes
Experimental larvae samples (Control Selco larvae not analysed) were thawed and the heads and tails were removed to isolate the digestive segment on a glass maintained on ice (0 °C); the stomach region was then

separated from the intestinal segments. Pooled samples from each tank were homogenized in 10 volumes (v/w) cold distilled water. Assays of cytosolic peptidase; leucine alanine peptidase (leu-ala) were performed following the method of Nicholson and Kim (1975) using leucine-alanine (Sigma-Aldrich, St. Louis, MO, USA) as substrate. Alkaline phosphatase (AP) and aminopeptidase N (AN), two enzymes of the brush border membrane, were assayed according to Bessey et al. (1946) and Maroux et al. (1973) using p-nitrophenyl phosphate (Sigma-Aldrich) and L-leucine p-nitroanalide (Sigma-Aldrich) as substrates, respectively.
Pepsin was assayed by the method of Worthington (1982) modified by Cuvier-Péres and Kestemont (2002). Trypsin and amylase activities were assayed according to Holm et al. (1988) and Metais and Bieth (1968) respectively, such as described by Gisbert et al. (2009). Bile salt-activated lipase activity was assayed following the method of Iijima et al. (1998) modified by Gisbert et al. (2009) using pnitrophenyl myristate as substrate. Protein was determined using the Bradford (1976) procedure. Enzyme activities are expressed as specific activities (U or mU mg protein^{-1}).

Quantitative LCMS/MS analysis PGE2 / PGE3
Samples were analysed by Cayman Chemical Company, Ann Arbor Michigan, USA. Fish larvae were thawed on wet ice prior to transferring to preweighed 2 mL Precellys soft tissue homogenizing tubes each containing 1.4 mm ceramic beads. Net wet weight of the samples was then calculated. Homogenization buffer (0.1M potassium phosphate, pH 7.0 containing 1mM EDTA and 10uM indomethacin), 1uL per mg wet tissue, was added to each vial. Samples were then homogenized in a Precellys Evolution tissue homogenizer at 5800 rpm for 2 cycles of 15 s each. Samples were subsequently spun for 15 min on an Eppendorf centrifuge. A 200 uL aliquot of supernatant from each sample was pipetted into a 96 deep well plate, spiked with 50 uL of internal standard (1ng mL PGE_2-d_4^{-1}), diluted with 200uL of deionized water and vortexed thoroughly prior to solid phase extraction. Due to the presence of endogenous PGE_2 and PGE_3 calibrators were prepared in the homogenization buffer. External calibrators were prepared at ten concentrations over the range from 9.77pg ml^{-1} to 5000pg mL^{-1} by serial dilutions of a 100ng mL^{-1} stock solution of PGE_2 and PGE_3 with homogenization buffer. 200uL of each calibrator solution was spiked with 50uLof internal standard and diluted with 200uL deionized water prior to extraction. Solid phase extraction was performed on Waters Oasis MAX microelution plates. Plate wells were conditioned with 200uL of acetonitrile followed by 25% acetonitrile in water. A volume of 450uL of sample or calibrator was added to the SPE plate and then the wells were washed with 200uL of 25% acetonitrile in water followed by 200uL of 100% acetonitrile. Samples were eluted into 25uL of a trapping solution (10% glycerol in water) using 25uL of 50:50 acetonitrile/IPA + 5% formic acid. Prior to mass spectrometry, samples (25uL) were chromatographed on an Acquity UPLC equipped with a Waters BEH C8 100mm x 2.1mm, 1.6um column at 25C. The flow rate was set to 400uL/min and mobile phase A was water + 0.1% formic acid and mobile phase B was acetonitrile + 0.1% formic acid. A gradient of 15%-50% mobile phase B over 12 minutes was used followed by a 1 min hold at 50% B. The mass spectrometer was a Waters TQ-Su triple quadrupole mass spectrometer set to run in negative electrospray ionization mode. The transitions for analytes and Internal Standard were tuned by infusion of 10ug mL^{-1} solutions of the pure materials in 1:1 acetonitrile/water at 5uL minute^{-1} into a mobile phase of 50%B at 400uL min^{-1}. The transitions used for quantitation were: PGE_2 351=>315, PGE_3 349=>269, and PGE_2-d_4 355=>319. The relative response ratio of analytes to internal standard was used for quantitation. Extraction recovery was determined to be >90% for both analytes and calibrator linearity was >0.999 for both analytes over the calibration range. Precision of the method was tested by a LLOQ (10pg mL^{-1}, n=6) on two separate days using a mixture of PGE_2 and PGE_3 in buffer. %RSD for PGE_2 was 10.9% on day 1 and 13.3% on day 2 and PGE_3 was 15.1% on day 1 and 13.3% on day 2.

Larval skeleton anomalies and gene expression
Fixed 30 DPH pikeperch larvae were stained with alizarin red to evaluate the skeletal anomalies and vertebral mineralization following methods (Izquierdo et al., 2013) modified from previous studies (Vandewalle et al., 1998). Larvae were immediately photographed and examined for the occurrence of skeletal anomalies following Boglione et al., 2001 skeletal anomalies classification. The different regions of the vertebral column were divided according to Boglione et al. (2001). Vertebrae were numerated from 1 to

24 using Roman numerals in a cranial to caudal direction. The effects of the different salinities and n-3/n-6 dietary contents on axial skeleton mineralisation were evaluated considering the total number of completely mineralised vertebral bodies within a larval size class (standard length).
Total RNA from larvae samples (average weight per sample 60mg) was extracted using the Rneasy Mini Kit (Qiagen). Total body tissue was homogenised using the TissueLyzer-II (Qiagen, Hilden, Germany) with QIAzol lysis reagent (Qiagen). Samples were centrifuged with chloroform for phase separation (12000g, 15min, 4ºC). The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into an RNeasy spin column where total RNA bonded to a membrane and contaminants were washed away by RW1 and RPE buffers (Qiagen). Purified RNA was eluted with 30µl of RNase-free water. The quality and quantity of RNA were analysed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Synthesis of cDNA was conducted using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Primer efficiency was tested with serial dilutions of a cDNA pool (1, 1:5, 1:10, 1:15,1:20 and 1:25). product size of the real-time q PCR amplification was checked by electrophoresis analyses using PB322 cut with HAEIII as a standard. Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using β-actin as the house-keeping gene in a final volume of 20µl per reaction well, and 100ng of total RNA reverse transcribed to complementary cDNA. Each gene sample was analysed once per gene. The PCR conditions were the following: 95ºC for 3min 30sec followed by 40 cycles of 95ºC for 15sec, 61ºC for 30sec, and 72ºC for 30sec; 95ºC for 1min, and a final denaturing step from 61ºC to 95ºC for 10sec. Data obtained were normalised and the Livak method (2–ΔΔCt) used to determine relative mRNA expression levels. Pikeperch specific gene primers were designed after searching the NCBI nucleotide database and using the Oligo 7 Primer Analysis software (Molecular Biology Insights, Cascade, CO, USA).

Statistics
A 2 way ANOVA in Sigma Plot Ver. 13 was used to compare larval wet weight; FA composition, enzymatic activity; degree of deformities; gene expression; stress confinement mortality and eicosanoid activity using diet and salinity as fixed factors. Prior to analysis normality and homogeneity of data were confirmed within treatment groups. A Tukey HSD test was used to testing significance of mean differences ($P<0.05$) between the treatment groups where applicable. If no interaction between factors (diet and salinity) in the outcome of the two-way ANOVA, a further one-way ANOVA and Tukey's HSD test were used to determine any significant differences. A Holm Sidak all pairwise multiple comparison of means test was applied for testing significance of mean differences ($P<0.05$) between the treatment groups where applicable. Each value is the mean ± standard deviation (SD) from the analysis of triplicate replicates except where otherwise stated. Percent data were arcsine transformed prior to analysis.
For the incorporation of radioactivity into total larval lipids, the statistical analyses were performed using the SPSS 21.0 software package (IBM Corp., New York, USA) for Windows. Results are presented as means and standard deviations (SD); significant differences were similarly considered at $P<0.05$ and variance and homogeneity of data confirmed as above. Data of total average incorporation of radioactivity into larval total lipids were submitted to a one-way ANOVA followed by Tukey´s post hoc test. A two-way ANOVA followed by Bonferroni´s multiple comparison test was used for the incorporation of each radio-labelled fatty acid substrate into larval total lipids, using diet and salinity as fixed factors. If no significant interaction between factors, the comparison between dietary groups was done on the basis of the mean response for the three salinities combined.

Results

Survival, growth and confinement test
Some mortality was observed in most tanks after transfer of larvae to the experimental facilities within the first 5 days after hatching, but no significant correlation with diet or salinity ($P>0.32$). High cannibalism of both types 1 and 2 was observed in all tanks starting from 17 DPH until the end of the study. Final survival at 30 DPH (data not shown) was not significantly ($P=0.652$) different among experimental groups and

ranged between 9 and 12 %. Diet or salinity did not cause any significant differences in mean wet weight of larvae at 15, 20, 25 or 30 DPH (**Fig. 1**), the mean weight at 30 DPH ranging from 20-33 mg. Overall, growth from 15-30 DPH calculated as SGR was not significantly different between groups (P=0.592) and varied from 11.6-16.2 % among experimental groups including the control.

Confinement by gently transfer of surviving larvae from each experimental tank into a white tray with limited water volume and exposed to a strong light intensity at 30 DPH caused high larval mortality in both dietary SO and LO groups. Subsequent to transfer, larvae in both groups reacted by erratic behavior and most larvae died within 30 s irrespectively of salinity. In the control group swimming behavior was similarly affected but erratic movements were less and mortality much lower even after 5 min of exposure (**Fig. 2**).

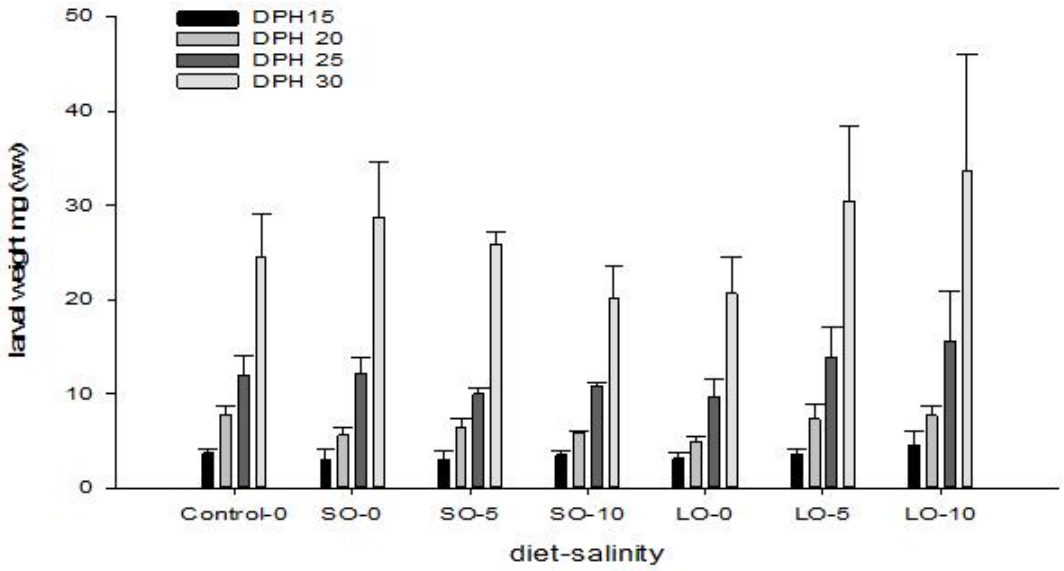

Figure 1. Weight of larvae (mg ww ± std) for each experimental treatment (diet control, SO, LO; salinity 0,5,10 ppt) at 4 sampling points (15 -to 30 days post hatch, DPH).

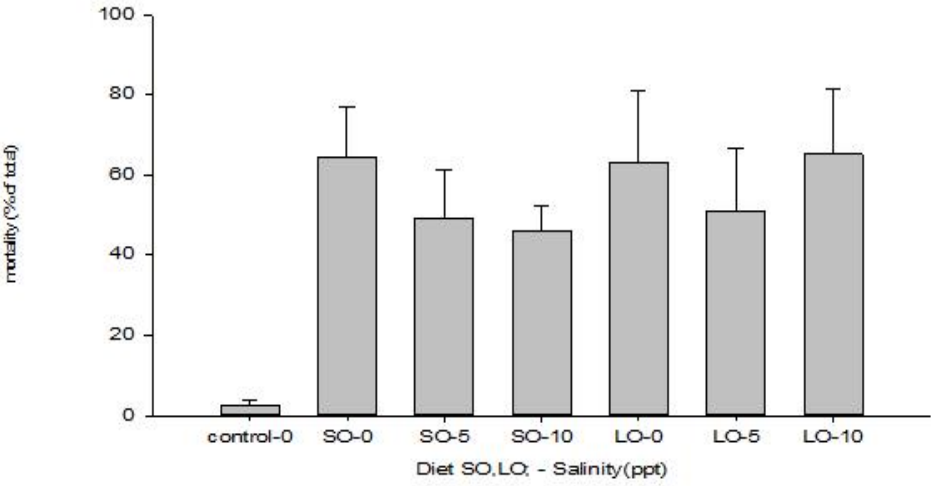

Figure 2. Larval mortality (%) after 5 min confinement and light exposure (500 lux) of all larvae surviving larvae from each treatment at 30 days post hatch.

FA composition of Artemia and larvae during development

FA content of *Artemia* reflected the dietary composition of the emulsions (**Table 2**). LA in SO enriched *Artemia* was about twice the content in LO enriched *Artemia* (P<0.001). Despite that ALA is a major fatty acid in unenriched *Artemia,* ALA content was significantly higher in *Artemia* enriched by use of LO emulsion (P<0.001), constituting 42 % of the FA composition versus 32 % in SO enriched *Artemia* and 25 % in DHA Selco enriched *Artemia*. Consequently, total n-3 content was the highest in LO *Artemia* and n-3:n-6 2.7 times higher in LO *Artemia* than in SO *Artemia.* EPA and DHA content was 5.5 % and 6.5 % (%TFA) in the DHA Selco enriched *Artemia,* respectively, while EPA content in SO and LO *Artemia* was some 1.7% (P<0.001). DHA was not found in *Artemia* from both experimental groups. The above FA differences in *Artemia* influenced significantly on larval tissue FA composition of LA and ALA at both 25 and 30 DPH and affected overall n-6 and n-3 PUFA content. Thus, larval FA composition mirrored the difference in *Artemia* composition.

FA differences became more pronounced along with the growth of the larvae and the duration of the experiment. Five days after introducing the different diets (at DPH 15, **Table 3**) tissue content of LA in SO and LO fed larvae was significantly different (P<0.001) and highest in SO larvae. ALA tissue content was similar between the experimental groups apart from larvae from the LO-10 ppt salinity group. For larvae reared at this salinity ALA content was significantly higher (P<0.001) than larvae in any other groups except for larvae from the LO -5 ppt salinity group. In addition DHA content in larvae of the LO-10 ppt group was significantly lower (P <0.001) than in larvae from the LO-0 ppt and SO-0 ppt groups. At 25 DPH (**Table 4**), we found no significant interacting effect of salinity (P≥0.156). Results on 18:2n-6 content between larvae from the SO and LO groups were similar with results from DPH 15 and significantly different (P>0.001). Analyses of ALA content were not significantly different between groups (P=0.059). DHA content was 6-10 % of TFA, which was only half the larval content at 15 DPH with no significantly differences between experimental dietary groups (P=0.184). In contrast the DHA level in the DHA Selco larval control group was 17% TFA.. At the final sampling point at 30 DPH, (**Table 5**), results revealed no interacting effect of salinity (P≥0.457); similarly as to results on 25 DPH. Tissue LA content was similar to the previous sampling periods and for all SO salinities significantly higher (P<0.001) than in larvae from the 0,5, 10 ppt salinity groups reared on the LO diet.ALA content was significantly higher (P<0.001) in all three salinity groups fed the LO diet than in the salinity groups fed the SO diet. For both dietary experimental groups DHA content was some 4-7% TFA, and differed from the DHA Selco control, for which DHA content was 16 % of TFA.

Table 2 Fatty acid content (% TFA) of *Artemia* enriched with DHA Selco control; sunflower oil (SO) emulsion or linseed oil (LO) emulsion ± Sd, n=2.

Diet	DHA Selco	SFO	LO
TFA (µg g dm^{-1})	239±122	400±173	247±107
FA			
16:0	10.8±0.5	9.7±0.2	9.5±0.4
18:0	5.3±0.2^{a}	6.0±0.2^{b}	6.1±0.1^{ab}
Total SFA	18.9±1.5	17.7±0.7	17.5±0.7
16:1 (n-7)	2.4±0.1^{b}	1.2±0.1^{a}	1.3±0.1^{a}
18:1 (n-9)	25.4±0.1	25.3±0.1	25.9±0.5
Total MUFAs	31.7±1.4	27.7±0.3	28.4±0.8
18:2 (n-6) LA	9.5±0.1^{b}	18.3±0.0^{c}	8.1±0.2^{a}
18:3 (n-6)	0.5±0.0	0.5±0.0	0.6±0.1
20:3 (n-6)	0.1±0.2	0.0±0.0	0.0±0.0
20:4 (n-6) ARA	1.2±0.4	0.5±0.0	0.6±0.1
Total (n-6) PUFA	11.6±1.0^{b}	19.5±0.4^{c}	9.3±0.5^{a}
18:3 (n-3) ALA	24.6±1.0^{a}	32.0±0.8^{b}	41.5±0.6^{c}
20:3 (n-3)	1.3±0.1	1.4±0.0	1.6±0.1
20:5 (n-3) EPA	5.5±0.1^{b}	1.7±0.2^{a}	1.6±0.2^{a}
22:6 (n-3) DHA	6.5±0.1^{b}	0.0±0.0^{a}	0.0±0.0^{a}
Total (n-3) PUFA	37.9±1.4^{a}	35.1±1.0^{a}	44.8±0.9^{b}
DHA/EPA	1.2±0.0^{b}	0.0±0.0^{a}	0.0±0.0^{a}
(n-3)/(n-6)	3.3±0.2^{b}	1.8±0.3^{a}	4.8±0.4^{c}

Values in a row followed by a different superscript are significantly different (P<0.05).

Table 3. Fatty acid content of larvae (% TFA) at 15 days post hatch (DPH) with use of sunflower oil (SO) or linseed oil (LO) at 3 different salinities ± sd values, n=3. DHA Selco control, 0 ppt.- not analysed.

Diet	DHA Selco	SFO			LO		
Salinity (ppt)	0	0	5	10	0	5	10
TFA (µg g dm^{-1})	*NA	92.8±12.8	106.0±18.8	98.2±6.2	110.4±29.6	103.5±8.1	114.5±18.1
FA							
16:0		13.5±0.6	13.6±0.6	14.0±0.6	13.8±0.5	13.6±0.2	14.0±0.3
18:0		7.7±0.5	7.7±0.5	8.0±0.3	7.8±0.4	7.9±0.1	8.1±0.3
Total SFA		23.0±1.2	23.1±1.3	23.8±1.1	23.4±1.0	23.2±0.4	23.8±0.9
16:1 (n-7)		1.3±0.3	1.4±0.3	1.3±0.2	1.5±0.2	1.4±0.1	1.3±0.1
18:1 (n-9)		18.2±0.7^{a}	19.0±1.3^{ab}	19.5±0.7^{ab}	19.2±1.4^{ab}	19.4±1.3^{ab}	21.9±0.9^{b}
Total MUFAs		25.9±1.7	26.1±2.3	26.1±1.6	26.8±2.4	26.7±2.1	28.0±1.9
18:2 (n-6) LA		10.4±0.1^{b}	10.5±0.6^{b}	10.7±0.4^{b}	6.0±0.5^{a}	6.2±0.2^{a}	6.6±0.2^{a}
18:3 (n-6)		0.4±0.1	0.4±0.0	0.4±0.0	0.3±0.0	0.4±0.0	0.4±0.0
20:3 (n-6)		0.3±0.0	0.3±0.0	0.4±0.0	0.3±0.0	0.3±0.0	0.3±0.0
20:4 (n-6) ARA		1.3±0.1	1.3±0.1	1.3±0.1	1.3±0.1	1.3±0.1	1.2±0.1
Total (n-6) PUFA		12.8±1.2^{b}	12.9±0.8^{b}	13.2±0.5^{b}	8.2±0.7^{a}	8.5±0.3^{a}	8.9±0.4^{a}
18:3 (n-3) ALA		12.6±0.5^{a}	14.2±0.6^{a}	14.4±1.7^{a}	15.8±2.5^{a}	16.5±2.2^{ab}	20.6±1.2^{b}
20:3 (n-3)		1.1±0.1^{a}	1.1±0.1^{a}	1.2±0.1^{a}	1.1±0.1^{a}	1.2±0.1^{ab}	1.5±0.0^{b}
20:5 (n-3) EPA		3.1±0.2	3.1±0.1	3.1±0.2	3.1±0.1	3.3±0.2	3.0±0.0
22:6 (n-3) DHA		21.7±2.1^{b}	19.5±2.1^{ab}	18.2±2.8^{ab}	21.4±2.9^{b}	20.5±3.1^{ab}	14.3±1.3^{a}
Total (n-3) PUFA		38.5±2.9	37.9±2.9	36.9±4.7	41.5±5.6	41.6±5.7	39.3±2.5
DHA/EPA		6.9±0.9^{b}	6.3±0.4^{ab}	5.9±0.6^{ab}	6.9±0.7^{b}	6.2±0.8^{ab}	4.8±0.4^{a}
(n-3)/(n-6)		3.0±1.5	2.9±1.5	2.8±1.1	5.0±0.5	4.9±0.9	4.4±0.9

Values in a row followed by a different superscript are significantly different (P<0.05). *DHA Selco control, 0 ppt.; NA: not analysed

Table 4 Fatty acid content of larvae (% TFA) at 25 days post hatch (DPH) with use of sunflower oil (SO) or Linseed oil (LO) at 3 different salinities ± Sd values, n=3. DHA Selco control, 0 ppt

Diet	DHA Selco	SFO			LO		
Salinity (ppt)	0	0	5	10	0	5	10
TFA (µg g dm^{-1})	58.6 n=1	64.5±45.7	40.7±13.7	59.9±14.6	64.6±20.8	101.9±43.2	81.2±33.1
FA							
16:0	13.7	14.4±0.1	14.3±0.2	14.0±0.2	13.9±0.4	14.2±1.7	13.9±1.1
18:0	8.2	8.7±0.2	9.1±0.2	8.7±0.2	8.7±0.4	8.5±0.3	8.6±0.7
Total SFA	24.4	25.2±0.6	25.6±0.6	24.9±0.5	24.7±1.0	24.9±2.1	24.7±2.0
16:1 (n-7)	1.3	1.0±0.1	0.9±0.0	1.0±0.1	1.1±0.2	1.2±0.1	1.1±0.4
18:1 (n-9)	19.1	21.9±0.1^{a}	21.6±0.3^{a}	23.1±0.2^{ab}	23.7±0.8^{ab}	25.4±1.0^{b}	24.7±2.9^{ab}
Total MUFAs	25.7	26.7±0.7	26.2±0.5	27.3±0.7	28.5±1.2	29.6±1.8	28.9±4.5
18:2 (n-6) LA	6.2	12.2±0.8^{b}	12.9±0.5^{b}	13.1±0.3^{b}	8.8±0.7^{a}	9.1±0.7^{a}	9.1±0.3^{a}
18:3 (n-6)	0.3	0.5±0.0	0.6±0.0	0.6±0.0	0.5±0.0	0.5±0.1	0.5±0.0
20:3 (n-6)	0.4	0.5±0.1^{ab}	0.7±0.0^{b}	0.6±0.1^{ab}	0.5±0.0^{a}	0.5±0.0^{a}	0.6±0.1^{ab}
20:4 (n-6) ARA	2.4	1.5±0.1	1.6±0.0	1.4±0.1	1.4±0.2	1.2±0.1	1.4±0.4
Total (n-6) PUFA	9.6	15.2±1.0^{b}	16.3±0.6^{b}	16.2±0.5^{b}	11.6±1.0^{a}	11.7±0.9^{a}	11.9±0.8^{a}
18:3 (n-3) ALA	13.9	17.9±1.7	17.3±0.8	19.4±0.9	20.9±0.6	22.5±2.9	22.6±4.4
20:3 (n-3)	1.2	1.6±0.1	1.8±0.0	1.7±0.1	1.8±0.0	1.9±0.2	1.9±0.1
20:5 (n-3) EPA	8.1	3.3±0.2	3.7±0.1	3.5±0.1	3.4±0.0	3.1±0.2	3.4±0.5
22:6 (n-3) DHA	17.1	10.0±2.3	9.1±0.3	7.1±0.9	9.1±0.5	6.4±2.3	6.5±4.3
Total (n-3) PUFA	40.4	32.8.±4.4	31.9±1.2	31.6±1.9	35.2±1.2	33.9±5.5	34.5±9.3
DHA/EPA	2.1	3.1±0.9	2.5±0.1	2.0±0.2	2.7±0.1	2.1±0.7	1.8±0.9
(n-3)/(n-6)	4.2	2.2±1.0	2.0±0.6	2.0±0.1	3.0±0.3	2.9±1.8	2.9±0.7

Values in a row followed by a different superscript are significantly different (P<0.05) between experimental diets.

Table 5 Fatty acid content of larvae (% TFA) at 30 days post hatch (DPH) with use of sunflower oil (SO) or linseed oil (LO) at 3 different salinities ± sd values, n=3. DHA Selco control, 0 ppt.

Diet	DHA Selco	SFO			LO		
Salinity (ppt)	0	0	5	10	0	5	10
TFA (µg g dm^{-1})	45.1 n=1	51.7 ±20.7	33.6±5.4	49.0±13.0	46.1±12.6	36.3±7.7	35.4±9.0
FA							
16:0	13.6	14.2±0.7	13.7±0.5	14.3±0.2	13.2±0.8	14.2±0.7	14.0±0.3
18:0	7.9	8.1±0.9	7.9±0.5	8.7±0.6	7.9±1.3	8.0±0.5	8.1±1.2
Total SFA	24.2	23.8±1.0	22.6±0.7	23.1±0.5	24.5±1.6	23.5±1.3	23.6±0.7
16:1 (n-7)	1.3	1.1±0.2	1.2±0.1	0.9±0.0	1.2±0.2	1.2±0.2	1.2±0.2
18:1 (n-9)	19.1	23.9±0.9^{ab}	24.7±0.5^{ab}	22.9±0.6^{a}	26.2±1.3^{ab}	26.1±0.5^{ab}	26.4±1.2^{b}
Total MUFAs	25.6	28.0±1.5	28.4±0.7	26.8±1.0	30.1±2.0	30.1±1.0	30.2±1.8
18:2 (n-6) LA	6.4	15.9±0.5^{b}	16.9±0.2^{b}	15.8±0.8^{b}	8.7±0.1^{a}	8.7±0.3^{a}	8.5±0.1^{a}
18:3 (n-6)	0.4	0.6±0.0^{b}	0.7±0.0^{c}	0.6±0.0^{b}	0.5±0.0^{a}	0.5±0.0^{a}	0.5±0.0^{a}
20:3 (n-6)	0.5	0.6±0.0^{ab}	0.7±0.0^{b}	0.7±0.0^{b}	0.5±0.0^{a}	0.5±0.1^{a}	0.5±0.1^{a}
20:4 (n-6) ARA	2.3	1.3±0.1	1.2±0.1	1.6±0.2	1.2±0.3	1.2±0.1	1.2±0.2
Total (n-6) PUFA	9.8	18.9±0.7^{b}	20.0±0.3^{b}	19.2±1.1^{b}	11.3±0.4^{a}	11.3±0.5^{a}	11.1±0.4^{a}
18:3 (n-3) ALA	11.5	17.1±2.0^{a}	18.9±0.5^{a}	17.5±0.9^{a}	25.9±2.2^{b}	24.1±1.3^{b}	25.2±1.1^{b}
20:3 (n-3)	1.1	1.5±0.1^{a}	1.7±0.0^{a}	1.7±0.0^{a}	1.9±0.0^{b}	1.9±0.1^{b}	1.9±0.1^{b}
20:5 (n-3) EPA	8.8	3.0±0.3	3.1±0.2	3.7±0.2	3.0±0.5	3.2±0.3	3.2±0.3
22:6 (n-3) DHA	16.4	7.1±1.8	4.4±0.3	6.0±1.2	4.8±1.0	5.1±0.8	4.3±1.1
Total (n-3) PUFA	39.9	28.7±4.1^{ab}	28.0±1.0^{a}	28.9±2.3^{ab}	35.5±3.7^{b}	34.3±2.5^{ab}	34.6±2.6^{ab}
DHA/EPA	2.1	2.4±0.6^{b}	1.4±0.1^{ab}	1.6±0.3^{ab}	1.6±0.1^{ab}	1.6±0.4^{a}b	1.3±0.2^{a}
(n-3)/(n-6)	4.1	1.5±0.1^{a}	1.4±0.5^{a}	3.2±0.7^{b}	1.4±0.5^{a}	3.0±1.0^{b}	3.1±0.6^{b}

Values in a row followed by a different superscript are significantly different (P<0.05) between experimental diets.

Tissue incorporation, esterification and transformation of labelled ^{14}C fatty acids

Results of the in vivo incubation with ^{14}C18 PUFAs and ^{14}C20-22 PUFA substrates revealed no significantly differences in larval incorporation of radioactivity into total lipids comparing the 2 experimental diets (SO or LO), but a significant effect of rearing salinity (**Table 6**). Thus, larvae reared in freshwater (at 0 ppt.) incorporated more radioactivity from 18:2n-6 in total lipids than larvae reared at 5 ppt salinity, but not different from larvae reared at 10 ppt salinity. Similarly, for 20:4n-6, the highest incorporation was observed at 0 ppt, differing significantly with larvae reared at 10 ppt salinity. For the n-3 LC-PUFAs, 22:6n-3 expressed a higher incorporation at 0 ppt, significantly different from the incorporation at 5 and 10 ppt.

Overall, and independently of salinity and dietary rearing conditions, the average incorporation of radioactivity was significantly higher for EPA than for the other tested FAs,. Similarly, incorporated radioactivity was significantly higher for ARA than for LA, ALA and DHA among which no significantly differences were observed (**Table 7**).

Table 6. Incorporation of radioactivity into total lipid (pmol mg prot^{-1} h^{-1}) of pikeperch larvae reared with sunflower oil (SO) or linseed oil (LO) at 3 different salinities or with DHA Selco control at 0 ppt. and incubated at 20 DPH with [1-14C]FA substrates.

Diet	DHA Selco	SFO			LO					
Salinity (ppt)	0	0	5	10	0	5	10	Diet	Salinity (‰)	
								SFO *vs* LO	0	5 10
Substrate										
18:2n-6 LA	7.36 ± 1.38	9.63 ± 3.71	3.09 ± 0.25	4.05 ± 0.99	9.13 ± 2.37	3.49 ± 2.34	8.72 ± 1.72	NS	b	a ab
18:3n-3 ALA	8.77 ± 1.87	7.28 ± 2.90	4.23 ± 0.15	6.39 ± 2.47	8.25 ± 1.39	4.77 ± 1.65	7.18 ± 3.89	NS	NS	
20:4n-6 ARA	18.68 ± 3.88	21.61 ± 3.19	10.21 ± 1.11	14.73 ± 6.46	22.41 ± 6.77	16.72 ± 3.98	12.15 ± 5.67	NS	b	a a
20:5n-3 EPA	34.61 ± 12.09	52.23 ± 18.86	37.35 ± 11.48	28.80 ± 13.97	33.44 ± 4.93	20.72 ± 3.62	50.31 ± 9.37	NS	NS	
22:6n-3 DHA	5.67 ± 1.20	9.06 ± 2.70	3.49 ± 0.33	4.80 ± 1.66	9.54 ± 3.90	6.20 ± 1.15	5.78 ± 0.64	NS	b	a a

Values are expressed as means ± SD (n=3). NS, non significant; a, b For substrates with a significant effect of salinity and no interaction, values without a common letter are significantly different (P<0.05).

Table 7. Average incorporation of radioactivity into total lipid (pmol mg prot^{-1} h^{-1}) of pikeperch larvae independently of rearing conditions when incubated with [1-14C] FA substrates at 20 DPH.

Substrate	18:2n-6 LA	18:3n-3 ALA	20:4n-6 ARA	20:5n-3 EPA	22:6n-3 DHA
Incorporation	6.67 ± 3.22 ^{a}	6.82 ± 2.52 ^{a}	16.98 ± 5.99 ^{b}	37.58 ± 14.33 ^{c}	6.52 ± 2.72 ^{a}

Values are expressed as means ± SD (n=21). Different superscript letters indicate differences among fatty acids. Comparisons between means were performed by one-way ANOVA followed by Tukey´s post hoc test (P<0.05).

Table 8 – Lipid class composition (%) of 20 DPH pikeperch larvae fed with sunflower oil (SO) and linseed oil (LO) at 3 different salinities (0, 5 and 10 ppt) or a DHA Selco control at 0 ppt.

Diet	DHA Control			SO									LO								
Salinity (ppt)				0			5			10			0			5			10		
UK	0.68	±	0.10	2.43	±	0.26	2.06	±	0.11	1.87	±	0.37	1.90	±	0.27	2.16	±	0.63	1.89	±	1.01
SE	12.46	±	1.39	7.79	±	0.45	14.34	±	4.34	13.37	±	4.07	10.29	±	1.34	12.90	±	5.13	12.54	±	3.86
TAG	24.55	±	1.42	20.57	±	4.25	20.01	±	2.70	20.64	±	2.72	21.83	±	1.34	21.39	±	4.46	23.33	±	3.16
FFA	3.54	±	0.51	4.64	±	0.14	3.96	±	0.44	3.71	±	0.63	3.65	±	0.06	4.10	±	0.28	4.23	±	0.48
CHO	14.23	±	0.63	15.85	±	1.38	14.21	±	1.02	14.53	±	0.80	15.00	±	0.81	14.44	±	0.47	14.18	±	0.41
DGLI	2.02	±	0.13	1.93	±	0.14	1.82	±	0.14	2.06	±	0.08	1.84	±	0.15	1.96	±	0.04	1.84	±	0.18
MAG + PIG	3.76	±	0.90	5.03	±	0.03	4.37	±	1.00	4.10	±	0.15	4.48	±	0.10	3.71	±	0.44	2.85	±	0.92
PE	11.27	±	0.20	12.41	±	0.63	11.41	±	1.10	11.64	±	0.55	11.66	±	0.28	12.02	±	0.39	11.30	±	0.54
PG	1.92	±	0.21	2.08	±	0.22	2.00	±	0.22	2.11	±	0.14	2.18	±	0.05	2.04	±	0.05	2.19	±	0.23
PI	3.44	±	0.20	3.78	±	0.09	3.31	±	0.27	3.45	±	0.50	3.57	±	0.15	3.35	±	0.16	3.35	±	0.70
PS	3.75	±	0.42	4.24	±	0.55	4.07	±	0.36	4.09	±	0.47	4.18	±	0.14	3.93	±	0.52	4.23	±	0.36
PC	17.71	±	0.93	18.66	±	0.75	17.88	±	1.03	17.90	±	0.55	19.10	±	0.69	17.50	±	0.72	17.55	±	0.34
SPH	0.68	±	0.27	0.58	±	0.12	0.56	±	0.23	0.54	±	0.34	0.33	±	0.10	0.49	±	0.17	0.53	±	0.33
TNL	60.80	±	1.93	57.22	±	2.24	59.70	±	2.92	57.94	±	0.92	57.94	±	1.50	59.54	±	2.03	59.97	±	2.02
TPL	39.20	±	1.93	42.78	±	2.24	40.30	±	2.92	42.06	±	0.92	42.06	±	1.50	40.46	±	2.03	40.03	±	2.02

Results represent means ± SD; n = 3. Lipid classes are presented in percentage of total lipid content. UK – unknown; SE – sterol esters; TAG – triacylglycerols; FFA – Free fatty acids; CHO – cholesterol; DGLI – diglycerols; MAG – monoacylglycerols; PIG – pigments; PE – phosphatidylethanolamine; PG – phosphatidylglycerol; PI – phosphatidylinositol; PS – phosphatidylserine; PC – phosphatidylcholine; SPH – sphingomyelin; TNL – total neutral lipids; TPL – total polar lipids.

For a better understanding of the esterification pattern of the radioactive PUFAs into lipid classes, the profile of the lipid classes of the larvae, which came from the same tanks and had been incubated without the addition of any radioactive substrate, is shown in **Table 8.** As can be seen; the general composition, in these larvae had no notable variations of composition related to the dietary regime or environmental salinity. On the other hand, it is noteworthy that in all of them there is an approximate distribution of 60% of neutral lipids (TNL) and 40% of polar lipids (TPL), with triglycerides (TAG) being the most abundant class among neutral lipids and phosphatidylcholine and phosphatidylethanolamine, the most remarkable among polar lipids.

Table 9. Incorporation of radioactivity into lipid classes (%) of pikeperch larvae reared with sunflower oil (SO) or linseed oil (LO) at 3 different salinities or with DHA Selco control at 0 ppt, and incubated at 20 DPH with [1-14C]FA substrates.

[1-14C]18:2n-6

	DHA Control		SO						LO					
			0‰		5‰		10‰		0‰		5‰		10‰	
TAG	15.1	± 3.6	5.7	± 1.8	9.4	± 4.3	9.5	± 1.8	5.3	± 0.3	6.7	± 3.4	11.4	± 1.1
FFA	14.7	± 6.4	15.8	± 2.5	19.5	± 6.4	17.2	± 3.0	15.2	± 1.1	18.2	± 3.7	16.4	± 2.2
PAG	12.1	± 2.2	6.2	± 0.7	6.3	± 3.0	4.0	± 1.4	5.1	± 0.8	4.8	± 1.0	4.8	± 1.1
PE	3.7	± 2.4	6.6	± 0.3	5.6	± 2.0	6.1	± 1.5	7.2	± 0.3	7.3	± 0.4	8.0	± 1.9
PI	1.8	± 1.5	4.4	± 0.7	3.7	± 1.1	4.2	± 0.3	5.1	± 0.4	5.3	± 0.3	5.6	± 0.7
PS	1.5	± 1.3	3.0	± 0.8	2.6	± 1.9	2.6	± 1.8	2.7	± 0.1	3.1	± 1.0	2.8	± 0.7
PC	**51.1**	**± 3.3**	**58.5**	**± 2.8**	**53.0**	**± 8.2**	**56.4**	**± 5.7**	**59.3**	**± 1.3**	**54.5**	**± 5.0**	**51.1**	**± 5.8**
TNL	41.9	± 7.5	27.6	± 4.3	32.0	± 14.5	30.7	± 5.7	25.6	± 1.6	29.8	± 4.2	32.5	± 3.0
TPL	58.1	± 7.5	72.4	± 4.3	68.0	± 14.5	69.3	± 5.7	74.4	± 1.6	70.2	± 4.2	67.5	± 3.0

[1-14C]18:3n-3

	DHA Control		SO						LO					
			0‰		5‰		10‰		0‰		5‰		10‰	
TAG	26.8	± 3.8	7.7	± 2.0	8.7	± 3.6	12.3	± 3.5	8.5	± 3.0	11.4	± 1.2	14.1	± 1.3
FFA	14.1	± 2.4	5.1	± 4.7	4.1	± 1.4	6.1	± 3.0	12.7	± 3.0	11.1	± 3.6	10.1	± 3.9
PAG	6.7	± 1.6	10.2	± 4.9	8.8	± 2.9	8.4	± 2.7	6.0	± 2.8	4.9	± 3.7	4.8	± 4.4
PE	5.8	± 1.1	9.5	± 0.8	9.7	± 1.0	8.8	± 1.6	8.8	± 0.2	9.0	± 1.0	8.4	± 1.7
PI	2.0	± 1.9	4.9	± 1.3	5.6	± 1.2	5.2	± 0.1	4.4	± 0.7	4.1	± 0.9	4.2	± 1.0
PS	0.7	± 0.3	3.7	± 1.2	4.8	± 1.6	3.3	± 1.2	1.7	± 0.5	2.2	± 1.1	2.1	± 0.7
PC	**44.0**	**± 5.9**	**58.9**	**± 4.4**	**58.3**	**± 4.5**	**55.9**	**± 2.7**	**57.9**	**± 4.3**	**57.1**	**± 2.0**	**56.2**	**± 2.4**
TNL	47.6	± 5.8	23.0	± 4.7	21.6	± 4.4	26.8	± 4.5	27.3	± 3.8	27.4	± 2.3	29.1	± 4.4
TPL	52.4	± 5.8	77.0	± 4.7	78.4	± 4.4	73.2	± 4.5	72.7	± 3.8	72.6	± 2.3	70.9	± 4.4

[1-14C]20:4n-6

	DHA Control		SO						LO					
			0‰		5‰		10‰		0‰		5‰		10‰	
TAG	1.3	± 1.5	0.3	± 0.3	1.0	± 0.7	0.9	± 1.1	1.0	± 1.3	3.0	± 2.6	1.6	± 1.2
FFA	2.8	± 0.3	2.5	± 1.2	2.7	± 0.2	2.9	± 0.4	1.2	± 0.5	3.7	± 1.4	3.7	± 1.0
PAG	11.8	± 1.0	10.8	± 0.8	11.4	± 0.2	9.0	± 0.2	10.9	± 1.7	9.8	± 1.0	9.4	± 0.8
PE	10.1	± 0.3	12.1	± 0.9	10.6	± 0.6	11.0	± 0.4	11.2	± 0.7	10.4	± 2.0	10.3	± 0.9
PG	0.9	± 0.3	1.2	± 0.2	1.1	± 0.1	0.9	± 0.4	1.2	± 0.1	0.6	± 0.3	0.3	± 0.3
PI	**36.1**	**± 2.3**	**37.9**	**± 1.5**	**40.8**	**± 0.8**	**43.2**	**± 3.5**	**39.6**	**± 2.8**	**40.8**	**± 2.2**	**43.8**	**± 4.3**
PS	3.8	± 0.4	3.9	± 0.5	3.4	± 1.0	3.2	± 1.1	3.9	± 0.4	3.4	± 0.5	3.1	± 1.0
PC	**33.2**	**± 1.3**	**31.3**	**± 0.3**	**29.1**	**± 0.3**	**28.9**	**± 2.9**	**31.0**	**± 1.7**	**28.3**	**± 1.7**	**27.8**	**± 4.3**
TNL	15.9	± 2.3	13.5	± 0.7	15.0	± 0.5	12.8	± 0.6	13.1	± 0.9	16.5	± 2.0	14.6	± 1.8
TPL	84.1	± 2.3	86.5	± 0.7	85.0	± 0.5	87.2	± 0.6	86.9	± 0.9	83.5	± 2.0	85.4	± 1.8

[1-14C]20:5n-3

	DHA Control		SO						LO					
			0‰		5‰		10‰		0‰		5‰		10‰	
TAG	6.1	± 0.8	0.5	± 0.4	0.9	± 0.8	1.3	± 0.1	0.2	± 0.2	0.3	± 0.4	0.1	± 0.1
FFA	5.0	± 0.8	3.7	± 1.0	4.8	± 0.1	5.4	± 1.1	1.9	± 0.1	3.0	± 1.8	5.0	± 2.2
PAG	5.5	± 0.6	10.3	± 0.8	8.3	± 1.0	8.9	± 0.0	10.3	± 2.1	9.2	± 0.6	7.6	± 0.5
PE	**14.2**	**± 0.9**	**16.7**	**± 0.8**	**17.6**	**± 1.3**	**17.4**	**± 0.4**	**16.5**	**± 1.1**	**17.7**	**± 0.3**	**18.3**	**± 0.9**
PG	2.0	± 0.5	1.9	± 0.3	1.4	± 0.2	1.3	± 0.3	1.7	± 0.4	1.3	± 0.0	0.6	± 0.2
PI	10.0	± 0.2	13.5	± 0.3	13.2	± 0.1	13.5	± 0.6	14.1	± 1.7	14.1	± 0.4	16.0	± 1.2
PS	2.7	± 0.9	4.0	± 0.3	3.9	± 1.5	3.2	± 0.2	3.9	± 1.0	3.7	± 1.0	2.5	± 0.4
PC	**54.5**	**± 0.4**	**49.5**	**± 1.1**	**49.9**	**± 1.2**	**48.9**	**± 2.0**	**51.6**	**± 1.6**	**50.6**	**± 2.2**	**50.0**	**± 2.6**
TNL	16.7	± 1.8	14.5	± 0.8	14.0	± 1.6	15.7	± 1.0	12.3	± 2.2	12.5	± 2.6	12.7	± 2.7
TPL	83.3	± 1.8	85.5	± 0.8	86.0	± 1.6	84.3	± 1.0	87.7	± 2.2	87.5	± 2.6	87.4	± 2.8

[1-14C[22:6n-3

	DHA Control		SO						LO					
			0‰		5‰		10‰		0‰		5‰		10‰	
TAG	8.0	± 0.4	3.4	± 0.1	4.7	± 0.7	6.3	± 2.1	1.7	± 0.8	4.0	± 0.9	3.1	± 0.7
FFA	4.8	± 0.2	5.6	± 0.7	4.9	± 1.1	3.5	± 2.0	6.9	± 0.9	4.9	± 1.3	5.5	± 0.3
PAG	4.1	± 1.2	3.2	± 0.5	4.7	± 0.8	4.3	± 2.4	3.6	± 1.6	3.6	± 2.2	2.8	± 0.9
PE	**26.3**	**± 0.8**	**27.7**	**± 1.1**	**27.9**	**± 1.5**	**28.0**	**± 1.8**	**26.4**	**± 1.4**	**27.4**	**± 2.1**	**27.8**	**± 2.0**
PI	10.6	± 1.3	16.3	± 1.0	14.9	± 1.3	16.6	± 1.9	15.8	± 0.6	15.4	± 1.7	17.6	± 1.6
PS	5.0	± 0.7	5.1	± 1.2	5.1	± 1.7	4.8	± 1.5	5.7	± 1.0	5.1	± 0.5	5.0	± 1.8
PC	**41.3**	**± 0.6**	**38.7**	**± 1.3**	**37.7**	**± 1.3**	**36.5**	**± 3.3**	**39.9**	**± 1.4**	**39.5**	**± 1.6**	**38.2**	**± 1.0**
TNL	16.8	± 1.4	12.2	± 0.2	14.0	± 0.7	14.1	± 3.1	12.2	± 1.0	12.5	± 2.6	11.4	± 1.0
TPL	83.2	± 1.4	87.8	± 0.2	86.0	± 0.7	85.9	± 3.1	87.8	± 1.0	87.5	± 2.6	88.6	± 1.0

As stated in **Table 9**, and according to the level of [1-14C]FFA, more than 80% of each substrate was esterified into different lipid classes, with the three 20-22C essential fatty acids being esterified in greater proportion than those of 18 carbons, and with a marked preference of all of them to be incorporated in the different lipid classes of the polar lipids (TPL). It is noteworthy, however, that in the DHA Selco control treatment, the degree of esterification in the triglycerides (TAG) is greater for both LC-PUFA precursors 18:2 and 18: 3, the result being a lower presence of these fatty acids in TLP than in the neutral fraction (TNL). No less interesting is the fact that there is great competition between the 5 PUFAs tested for incorporating in phosphatidylcholine (PC) and the remarkable incorporation of ARA into phosphatidylinositol (PI) and phosphatidylethanolamine (PE). EPA also seems to have a particular affinity for PE.

Table 10. Distribution of radioactivity (% of total radioactivity incorporated) in pikeperch larvae reared with use of sunflower oil (SO) or linseed oil (LO) at 3 different salinities or DHA Selco control at 0 ppt and incubated at 20 DPH with [1-14C]FA substrates.

Substrate 1-14C	Products	Recovery						
		DHA selco	SO			LO		
		0 ppt	0ppt	5ppt	10ppt	0ppt	5ppt	10ppt
18:2n-6								
	18:2n-6	84.1 ± 4.7	79.9 ± 6.3	74.7 ± 8.7	75.9 ± 9.0	74.1 ± 17.9	71.9 ± 18.1	67.1 ± 19.1
	20:2n-6	4.4 ± 1.3	5.3 ± 0.8	5.3 ± 0.9	4.6 ± 1.3	8.7 ± 4.1	7.5 ± 4.8	8.3 ± 4.7
	18:3n-6	5.0 ± 1.3	4.9 ± 2.2	5.3 ± 3.2	5.7 ± 3.1	5.7 ± 3.6	7.4 ± 4.8	8.5 ± 4.9
	20:3n-6	3.8 ± 0.7	4.2 ± 1.8	4.6 ± 1.8	4.9 ± 1.9	5.3 ± 5.5	4.3 ± 5.7	6.4 ± 5.6
	de novo	2.8 ± 2.4	5.7 ± 2.0	10.2 ± 3.9	9.0 ± 2.8	6.1 ± 5.0	8.9 ± 4.1	9.7 ± 4.3
18:3n-3								
	18:3n-3	87.0 ± 6.4	88.8 ± 3.4	80.5 ± 14.8	77.7 ± 8.8	83.0 ± 4.1	79.6 ± 12.2	74.7 ± 4.8
	20:3n-3	7.1 ± 1.7	7.2 ± 2.0	7.4 ± 4.9	9.2 ± 4.0	9.7 ± 2.9	8.2 ± 1.9	9.6 ± 2.3
	18:4n-3	2.0 ± 1.6	0.8 ± 0.2	2.7 ± 2.7	3.9 ± 1.5	1.6 ± 1.1	2.8 ± 2.6	4.2 ± 0.6
	20:4n-3	1.0 ± 0.5	0.6 ± 0.5	1.9 ± 1.8	3.5 ± 1.4	1.7 ± 1.0	2.0 ± 1.1	3.9 ± 0.9
	de novo	2.9 ± 2.7	2.7 ± 1.0	7.5 ± 5.7	5.7 ± 2.3	4.0 ± 0.5	7.4 ± 7.5	7.5 ± 4.3
20:4n-6								
	20:4n-6	95.0 ± 1.9	93.7 ± 1.1	93.0 ± 2.7	91.0 ± 3.4	94.3 ± 1.2	90.9 ± 3.7	90.5 ± 2.2
	22:4n-6	4.1 ± 1.8	3.5 ± 0.6	3.5 ± 0.8	4.7 ± 1.5	3.3 ± 0.8	4.1 ± 1.8	5.3 ± 1.4
	24:4n-6	1.0 ± 0.5	2.8 ± 0.9	3.5 ± 2.2	4.2 ± 2.1	2.4 ± 0.6	4.9 ± 3.5	4.2 ± 1.7
20:5n-3								
	20:5n-3	90.9 ± 2.4	92.2 ± 2.7	92.1 ± 3.1	87.6 ± 5.3	92.4 ± 2.7	92.9 ± 1.0	91.4 ± 1.5
	22:5n-3	3.8 ± 1.1	3.4 ± 0.9	3.9 ± 1.9	5.0 ± 1.7	2.5 ± 0.7	2.7 ± 0.3	3.7 ± 0.9
	24:5n-3	1.8 ± 0.3	1.4 ± 0.5	1.3 ± 0.2	2.0 ± 0.7	1.6 ± 0.6	1.4 ± 0.5	1.6 ± 0.4
	Uk	2.4 ± 1.1	2.1 ± 1.3	1.7 ± 0.7	3.6 ± 2.1	2.5 ± 1.0	1.9 ± 0.5	2.1 ± 0.4
	de novo	1.1 ± 0.4	0.9 ± 0.4	0.9 ± 0.5	1.9 ± 1.1	1.0 ± 0.6	1.3 ± 0.5	1.1 ± 0.4
22:6n-3								
	22:6n-3	94.7 ± 2.6	93.0 ± 0.7	96.1 ± 1.8	90.7 ± 4.4	93.0 ± 6.8	82.6 ± 21.5	88.8 ± 8.4
	24:6n-3	5.3 ± 2.6	7.0 ± 0.7	3.9 ± 1.8	9.3 ± 4.4	7.0 ± 6.8	17.4 ± 21.5	11.2 ± 8.4

Table 10 Summarizes elongation and/or esterification products detected from each radioactive substrate in pikeperch total lipids after 5 h of incubation. Although most of the radioactivity was obtained in the form of the unmodified substrate itself (ranging from 67.1 and 96.1%), it is possible to highlight some results and trends. As expected, the least modified substrates are LC-PUFA, particularly DHA, which is a practically terminal product in the LC-PUFA synthesis route. In all the conditions tested, both the transformation of DHA and that of the EPA and ARA, only elongation products are identified. Additionally, two unidentified bands are identified from EPA, either because of its lack of definition or because it was not included the corresponding standard. The first, and poorly defined, would correspond to a product of desaturation, while the second is clearly a new short-chain product generated by incorporation of the labeled carbon released from the substrate by beta-oxidation to a short-chain unlabeled substrate.
In the case of the two fatty acids precursors of the LC-PUFA (18:2n-6 and 18:3n-3), a degree of transformation ugfp to 32.9% (18:2; LO at 10ppt) was reached, presenting both substrates, products of elongation and desaturation, and whose formation seems to be favored by dietary deficiency and increasing salinity. In view of the results, the activity of elongase enzymes of 18, 20 and 2 carbon atoms does not seem to be limiting at all. However, only products that match the activity of a Δ6 are detected.

Eicosanoids
Results of prostaglandin tissue levels PGE_2 and PGE_3 revealed no effects of dietary treatment, but a significant effect of salinity (**Table 11**). PGE_2 and PGE_3 content was significantly higher for larvae reared at 0 ppt (including the control group) than larvae reared at 5 or 10 ppt. PGE_2 and PGE_3 levels were similar for larvae at 5 or 10 ppt (P=0.594; P= 0.393, respectively).

Table 11 Eicosanoid content of pikeperch larvae (pg g ww^{-1}± sd values) reared for 30 days with *Artemia* enriched with sunflower oil (SO) or linseed oil (LO) at 3 different salinities (ppt), n=3. DHA Selco control, 0 ppt.

Diet	Control DHA Selco	SO			LO			Two way Anova		
Salinity	0 (n=2)	0	5 (n=2)	10	0	5 (n=2)	10	Diet	Salinity (‰)	Diet *salinity
PGE_2	5510±298	5912±1587	3313±45	3661±411	5754±476	4917±141	3810±1247	NS	P≤0.032	NS
PGE_3	1018±48	1212±322	634±84	678±120	1128±244	997.2±114	618±229	NS	P≤0.028	NS

Digestive enzymatic activity

At 30 DPH pepsin values were lower in larvae for treatment LO-10 ppt salinity, and no pepsin activity was detected for larvae fed the experimental groups SO-5 ppt , LO-5 ppt and SO-10 ppt (**Table 12**). Peptidase (N) activity was the highest for treatments SO-10 ppt and LO-10 ppt (P<0.01), as so was trypsin activity (P<0.05) in larvae fed the SO diet (treatments: SO-0 ppt, SO-5 ppt and SO-10 ppt), without any effect of salinity on trypsin levels. Data also revealed a significantly higher (P<0.05) activity of alkaline phosphatase (AP) in larvae for treatment SO-10 ppt salinity, while no significant effect was found on lipase levels. Regardless of diets, amylase activity tended to increase in larvae exposed to10 ppt. salinity, although without statistical differences. Lipase activity did not differ between dietary treatment and salinity level.

Table 12 Specific enzymatic activity (mU mg protein^{-1}; U mg protein^{-1}) in larvae with use of sunflower oil (SO) or Linseed oil (LO) at 3 different salinities ± Sd values, n=3.

Diet	SFO			LO					
Salinity	0	5	10	0	5	10	Interaction	Diet	Salinity
							Diet *vs* Salinity	SFO *vs* LO	(‰)
Pepsin[1]	6.4±2.4	Nd	Nd	6.6±0.5	Nd	4.3±1.1			
Trypsin[1]	15.3±3.3	12.4±3.3	14.9±4.7	11.2±3.2	12.5±1.3	7.9±1.9	NS	0.03	NS
Aminopeptidase N[1]	9.3±0.6	10.7±1.6	14.6±2.4	8.1±1.2	10.2±2.1	10.4±1.5	NS	0.028	0.007
Alkaline phosphatase AP[1]	34.2±2.3^{b}	36.3±2.8^{b}	45.7±3.6^{a}	40.8±4.8^{ab}	37.7±5.3^{b}	38.4±5.7^{ab}	0.044	NS	NS
Lipase[1]	10.2±2.1	8.2±2.1	9.2±1.6	7.7±1.5	8.1±0.6	8.5±1.5	NS	NS	NS
Leu-ala[2]	722.0±226.0	765.3±256.5	647.0±62.4	879.5±343.6	670.0±263.4	583.5±156.6	NS	NS	NS
Amylase[1]	56.2±12.4	83.3±22.6	125.3±55.9	61.4±6.2	40.0±2.9	127.0±52.5	NS	NS	NS

[1] mU mg protein^{-1}; [2] U mg protein^{-1}. Values in a row followed by a different superscript are significantly different P<0.05 between exp. diets. Nd: not detected. DHA Selco control not analysed

Skeleton anomalies and gene expression

There was a high incidence of cranial anomalies, particularly in maxillary bones (58-83% population), and a low incidence of vertebral anomalies (**Table 13**). The lowest (P<0.05) incidences of severe and maxillary bone anomalies were found in fish reared at 0 ppt, regardless of the dietary fatty acid profile, whereas an increase in salinity lead to a higher incidence of this type of anomalies (**Table 13**, **Fig. 3**). Particularly, pikeperch fed SO diet and reared at 5 or 10 ppt and those fed LO diet and reared at 5 ppt significantly showed the highest incidence of maxillary bone anomalies (**Fig. 3**). Also results from two-way ANOVA showed the effect of salinity on the results, while there was no significant effect from the diets (**Table 13**). No differences were found in mineralization degree in the different size classes (data not showed).
Pikeperch fed SO diet and reared at 10 ppt and those fed LO diet and reared at 5 ppt showed doubled expression in *sox9* and, particularly, *twist2* than fish reared under the other conditions (**Table 14**, Figure 5). Furthermore, results from the two-way ANOVA regarding expression of *twist2* showed interaction between salinity and the diets (**Table 14, Fig. 4.**) However, large standard deviations in expression of the different genes studied did not allow to find significant differences among fish reared with different treatments. No significant differences or specific tendencies were found in *alp* or *Mef2c* expression.

Table 13. Incidence of different bone anomalies found in pikeperch larvae reared for 30 days with *Artemia* enriched with sunflower oil (SO) or linseed oil (LO) at 3 different salinities ± Sd values, n=3. DHA Selco control, 0 ppt.

Diet	SO			LO			Two-way ANOVA		
Salinity	0	5	10	0	5	10	Diet	Salinity (‰)	Diet*Salinity
Anomaly Type (%)									
Severe	76.7 ± 12.6^{a}	98.3 ± 2.9^{ab}	96.7 ± 5.8^{b}	81.5 ± 10.7^{ab}	86.7 ± 5.8^{b}	85.0 ± 15.0^{ab}	NS	NS	NS
Maxilary	58.3 ± 16.1^{ab}	83.3 ± 7.6^{b}	77.1 ± 4.9^{b}	81.7 ± 17.0^{a}	81.7 ± 7.6^{b}	58.3 ± 25.2^{ab}	NS	0.011	NS
Dentary	0.0 ± 0.0	3.3 ± 2.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 2.9	NS	NS	NS
Other cephalic anomalies	28.3 ± 22.5	53.3 ± 7.6	35.8 ± 19.0	51.7 ± 6.8	51.7 ± 10.4	28.3 ± 5.8	NS	0.036	NS
Opercular plate	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	NS	NS	NS
Branchiostegal rays	10.0 ± 13.2	26.7 ± 37.9	13.3 ± 12.6	28.3 ± 8.7	28.3 ± 31.8	10.0 ± 5.0	NS	NS	NS
Cephalic v. scoliosis	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 2.9	NS	NS	NS
Pre-haemal v. kyphosis	1.7 ± 2.9	1.7 ± 2.9	6.4 ± 7.2	1.7 ± 2.9	1.7 ± 2.9	0.0 ± 0.0	NS	NS	NS
Pre-haemal v. lordosis	33.3 ± 7.6	15.0 ± 8.7	31.0 ± 6.6	23.3 ± 22.5	23.3 ± 27.5	30.0 ± 10.0	NS	NS	NS
Pre-haemal v. morphology anomalies	30.0 ± 26.0	18.3 ± 15.3	14.3 ± 24.7	1.7 ± 31.1	1.7 ± 2.9	21.7 ± 15.3	NS	NS	NS
Pre-haemal v. scoliosis	0.0 ± 0.0	8.3 ± 7.6	1.6 ± 2.7	1.7 ± 0.0	1.7 ± 2.9	0.0 ± 0.0	NS	NS	NS
Haemal v. kyphosis	0.0 ± 0.0	0.0 ± 0.0	3.3 ± 2.9	0.0 ± 5.8	0.0 ± 0.0	0.0 ± 0.0	NS	NS	NS
Haemal v. lordosis	1.7 ± 2.9	23.3 ± 27.5	9.9 ± 13.3	16.7 ± 0.0	16.7 ± 20.8	8.3 ± 7.6	NS	NS	NS
Haemal v. morphology anomalies	10.0 ± 13.2	1.7 ± 2.9	6.4 ± 7.2	1.7 ± 8.7	1.7 ± 2.9	6.7 ± 7.6	NS	NS	NS
Haemal v. scoliosis	0.0 ± 0.0	1.7 ± 2.9	1.6 ± 2.7	0.0 ± 0.0	0.0 ± 0.0	11.7 ± 10.4	NS	NS	NS
Caudal v. kyphosis	0.0 ± 0.0	3.3 ± 5.8	1.7 ± 2.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	NS	NS	NS
Caudal v. scoliosis	0.0 ± 0.0	8.3 ± 14.4	4.9 ± 5.0	8.3 ± 0.0	8.3 ± 10.4	31.7 ± 17.6	NS	0.032	NS

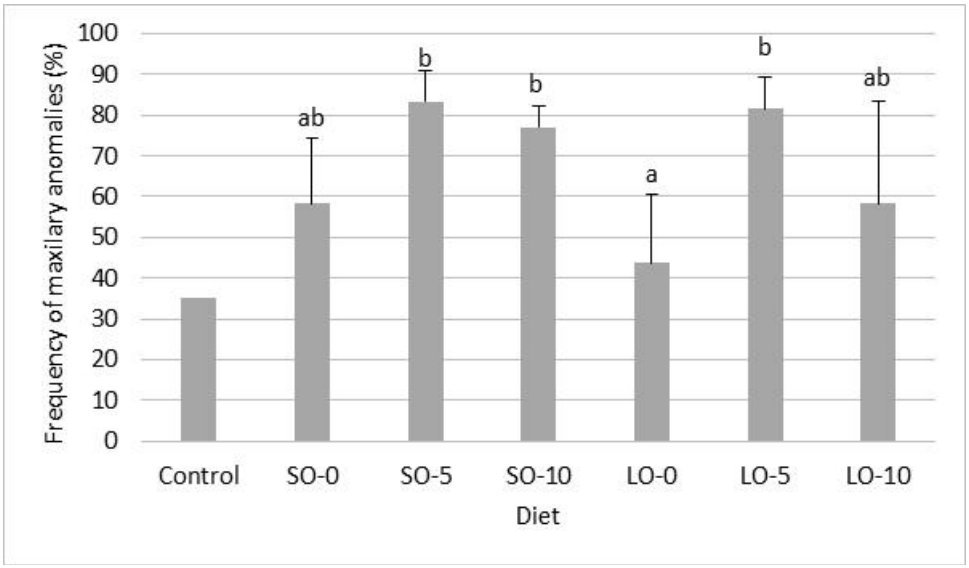

Figure 3. Incidence of total maxillary anomalies in 30 DPH pikeperch larvae fed SO or LO diets at three different salinities, 0, 5 or 10 ppt. Different letters denote significant differences ($p<0.05$)

Table 14 Gene expression found in pikeperch larvae reared for 30 days with *Artemia* enriched with sunflower oil (SO) or linseed oil (LO) at 3 different salinities ± Sd values, n=3. DHA Selco control, 0 ppt.

SO			LO			Two-way ANOVA		
0	5	10	0	5	10	Diet	Salinity (‰)	Diet*Salinity
0.196 ± 0.078	0.228 ± 0.035	0.363 ± 0.279	0.268 ± 0.040	0.246 ± 0.047	0.141 ± 0.080	NS	NS	NS
0.239 ± 0.112	0.259 ± 0.147	0.553 ± 0.332	0.254 ± 0.108	0.521 ± 0.169	0.156 ± 0.077	NS	NS	0.023
0.340 ± 0.325	0.347 ± 0.315	0.330 ± 0.200	0.304 ± 0.075	0.226 ± 0.093	0.213 ± 0.058	NS	NS	NS
0.274 ± 0.210	0.219 ± 0.102	0.468 ± 0.471	0.293 ± 0.078	0.340 ± 0.150	0.192 ± 0.059	NS	NS	NS

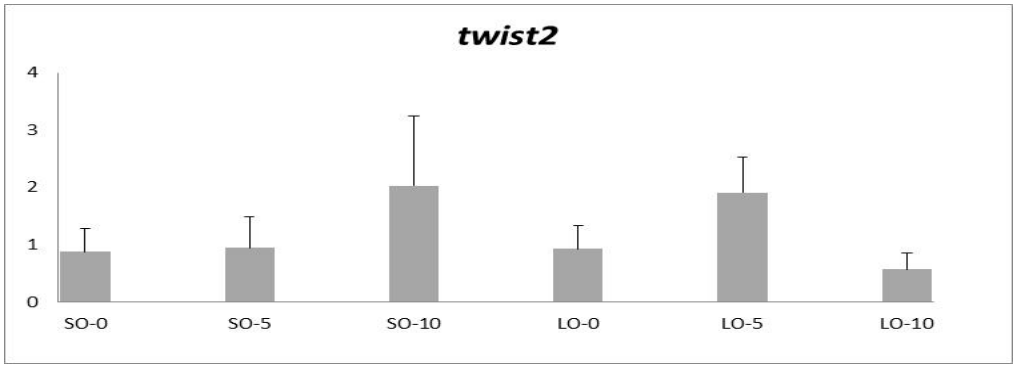

Figure 4. Expression of *twist2* determined by real-time PCR in whole body of 30 DPH pikeperch larvae fed SO or LO diets at three different salinities, 0, 5 or 10 ppt.

Discussion

We exposed pikeperch larvae to different salinities and diets low in LC-PUFA by enrichment of *Artemia* with emulsions rich in VO. The main FA constituents of vegetable sunflower oil and linseed oil; 18:2n-6 and18:3n-3 respectively, significantly affected larval tissue content and gave rise to overall differences in n-6 and n-3 PUFAs content. In fish larvae, only very few studies have examined FA metabolism combining the influence of ambient salinities and diets consistently different in 18:2 n-6 to 18:3n-3 by in vivo [1-C-14] FA incubation. By using direct incubation with 0.2µCi (0.3 µM) of [1−14C] PUFAs we demonstrated for the first time in pikeperch larvae that, regardless of the environmental salinity or dietary regime, pikeperch possess a marked capacity to incorporate and esterify FA into lipids. For LA and ARA we observed, that more radioactivity was incorporated into total lipids under freshwater conditions (0 ppt) as compared to salinity gradients of 5 and 10 ppt. These results seem to agree well with the analysed larval fatty acid profiles of larvae fed the LO diet at 10 ppt salinity (LO-10) at 15 DPH. Thus, this group had relatively higher tissue level of 18-3n-3 and lower levels of DHA, indicating that a combination of diets rich in n-3 PUFA and 10 ppt salinity did not substantially modify LC-PUFA synthesis capabilities. At least not in a degree for which it is able to compensate LC-PUFA dietary deficiencies. In this case, salinity may have more influence on pike perch larval lipid metabolism during early ontogenetic development (at 15 DPH) than at 25 – 30 DPH, as LA, ALA, EPA and DHA tissue content correlated nicely with dietary levels at these sampling points, irrespectively of salinity gradients. The dietary influence of SO - and LO enriched *Artemia* and consequently

FA metabolism and FA tissue composition may further have been affected by cannibalism at 25-30 DPH as compared with 15 DPH, as we visually and analytically observed cannibalism starting around 16 DPH.
EPA was by far the most incorporated PUFA among the tested substrates followed by ARA, while LA, ALA and DHA were less incorporated. This could indicate a physiological need for EPA or a higher assimilation or esterification affinity into tissue for C20 PUFA, but need more studies, as DHA would normally be regarded as the most limiting essential FA in pikeperch larvae. In any case it is evident that there could be a strong competition for incorporation of PUFA into pikeperch and that intended DHA/EPA/ARA ratios in enrichment protocols may greatly finally differ in larval tissues.

As stated before there was a marked preference of all PUFA assayed to be incorporated in the different lipid classes of the polar lipids and in the DHA Selco control treatment, when n-3 LC-PUFA are dietary abundant the degree of esterification 18:2 and 18:3 in the triglycerides decreasing somehow their competition with LC-PUFA for esterification into PL. No less interesting is the fact that there is a great competition between the 5 PUFAs tested for incorporating in (PC) and the remarkable incorporation of ARA into (PI) and (PE) or the EPA affinity for PE. Taking into account the importance of LC-PUFA, and particularly DHA, in larvae phospholipids, all these findings should be taken into account during dietary formulation, particularly when *Artemia* is known to naturally provide high quantities of the competitor ALA.

Δ6 desaturase is the first rate-limiting enzyme in LC-PUFA biosynthesis and salinity may have variable effects on modulation and expression of Δ6 desaturase enzymatic activity and therefore fatty acid composition in fishes. The bioconversion of LAto ARA and from ALA to EPA and DHA involves desaturations at the Δ6 and Δ5 positions in the carbon backbone as well as an intermediate 2-carbon elongation step (Vagner and Santigosa, 2011). Replacing FOs by VOs diets rich in 18:2 n-6 and 18:3n-3 consistently resulted in increased Δ6 activity in several carnivorous anadromous /freshwater fishes as reviewed by Vagner and Santigosa (2011). Hence, our results on the in vivo [1-C-14] FA incubation studies similarly found that Δ6 enzyme desaturase activity was most likely consistent with a significant production of 18:4n-3 from 18:3n-3, or 18:3n-6 from 18:2n-6. Regardless of PUFA substrate most of n-3 elongation/desaturation products were consistent with ELOVLs (fatty acyl elongases) genes, directly producing 20:3n-3 from 18:3n-3 and respectively 22:5n-3 and 24:5n-3 from EPA, however no further or any desaturation activity over EPA was evident, precluding the expression of Δ5 or even Δ4 desaturases in the larvae. In support of this we observed no significant differences in larval tissue content of ARA, EPA and DHA throughout the successive sampling points along the ontogeny of pikeperch at DPH 15, 25 and 30 despite feeding either the LO diet rich in 18:3n-3 or the SO diet rich in 18:2n-6 confirming very limited (i.e 15 DPH) - effects of dietary LC-PUFA deficiency or the increasing salinity on the subsequent steps in LC-PUFA biosynthesis. In the freshwater Mexican silverside (*Chirostoma estor*) LC- PUFA biosynthesis from C18 precursors is detected at low salinities (5-15 ppt) , whereas this pathway is not active in fish reared in freshwater (Fonseca-Madrigal et al., 2012).This trend seems to be also evident for the pikeperch where up to 32.% of 1-C14 18:2n-6 substrate was achieve for LO and 10ppt reared larvae, even though the tissue fatty acid composition showed the although the fatty acid profile of the larval tissue showed the insufficiency of this enzymatic activity.
Growth of fish larvae until 30 DPH (mg ww ind^{-1}) was consistent to results obtained in previous experiments performed with use of live feed (*Artemia*) throughout the rearing period (Lund et al. 2014), but relatively less than in trials with use of microdiets introduced from 15 DPH and reared at 20-21° C (Hamza et al., 2008; Lund et al., 2017, unpublished results). The larval control group reared at 0 ppt salinity on DHA Selco enriched *Artemia* was similar in size as to larvae fed the LO and SO enriched larvae, suggesting that LC PUFA supplementation, salinity levels up to 10 ppt or VO based experimental emulsions (n-3/n-6 ratio) had limited effects on growth. Prior to the present experiment we tested the tolerance of pike perch larvae to several salinity gradients and in none of these 10 ppt seemed to affect growth and survival of early pike perch larvae while >15 ppt had a significant negative influence (unpublished results). In comparison salinities of 10 ppt caused a 50 % reduction in growth rate in Eurasian perch juveniles of app. 1.5- 2 g initial b.w. (Overton et al., 2008) while, in a study with Eurasian perch larvae, survival was significantly affected by salinity as only 2 individuals out of 344 larvae survived at 9.6 ppt (Bein and Ribi, 1994). This indicates a

much higher salinity tolerance in pikeperch, despite that both species naturally inhabit same areas with various strengths of brackish water.

We suggest that a main factor to a relatively slow growth in this experiment was a combination of lower rearing temperature (17.5°C vs 20°-21° C) and the use of only *Artemia* as feed. Pikeperch larvae are able to digest and utilize microdiets from about 10-15 DPH and as a large quantity of amino acid is necessary for sustaining efficient protein synthesis in rapid growing fish larvae (Rønnestad et al. 1999). It is likely that *Artemia* did not provide optimal protein content and amino acid composition throughout the growth period (Helland et al., 2003). In the referred microdiet studies on pikeperch larvae by Hamza (Hamza et al., 2008, 2012) a positive effect on elevated growth and development was additionally attributed to dietary levels of phospholipids independently of LC-PUFA levels. This effect has been reported in other species (Bell et al., 2003; Gisbert et al., 2005; Cahu et al., 2009; Olsen et al., 2014) suggesting, that PUFAs provided as triacylglycerides in live enriched prey (*Artemia*) may have a growth limiting effect, This may further explain the similarity in larval growth between the experimental emulsions without LC-PUFAs and the commercial DHA Selco emulsion. Though no effects on growth, larvae fed DHA Selco enriched *Artemia* had much elevated DHA tissue content as compared with larvae reared on *Artemia* enriched by sunflower oil or linseed oil based emulsions. This likely explains a higher stress resistance as evidenced by less erratic swimming behavior and lower mortality (Fig. 2), when challenged to a combination of confinement and light intensity stressors. These results confirm previous findings on pikeperch larvae and observations of high stress sensitivity and mortality, when fed diets low or deficient in DHA and when exposed to salinity challenge tests at 20 ppt (Lund & Steenfeldt, 2011; Lund et al. 2014). Similarly, red drum (*Sciaenops ocellatus*) larvae reared on high DHA to EPA ratio of 3.8 and challenged to a salinity vitality test exhibited significant stress resistance as compared to larvae on lower DHA to EPA ratios (Brinkmeyer and Holt, 1998) and similar dietary effects of DHA with larvae of other marine fishes have been reported (Izquierdo et al., 1989; Watanabe et al., 1989; Takeuchi et al., 1991). Here, we did not define the exact nutritional requirement of LC-PUFAs or DHA to EPA ratio, but points to the essentially and critical role of dietary DHA supplementation in pikeperch larval nutrition. Results fit nicely with the ^{14}C FA in vivo metabolism and the analytical FA results suggesting no FA products or any desaturation activity over EPA.

ARA and EPA are precursors of eicosanoids namely the 2- series prostanoids and 3- series prostanoids respectively, and the abundance of the two FAs in tissues determines the eicosanoid potency (Bransden et al., 2005). We found elevated prostaglandin levels of both PGE_2 and PGE_3 in larvae reared at 0 ppt salinity compared with larvae reared at 5 or 10 ppt salinity with no interaction effects and displaying similar tissue levels of ARA and EPA. Eicosanoids are known to be responsible for a range of physiological functions in fishes, such as modulating immune and neural function and homeostasis of osmoregulation, and controlling the stress response (Sargent et al., 1999; Koven et al., 2001, 2003; Tocher, 2003). In mammals systemic osmotic imbalances such as chronic salt loading and hydration stimulate renal prostaglandin production that facilitates salt excretion *via* complex pathways (Choe et al., 2006). Cyclooxygenase (COX) activity is the enzyme responsible for the initial rate-limiting conversion of arachidonic acid to prostaglandin G2. In killifish (*Fundulus heteroclitus*) an orthologue of mammalian COX2 has been found in chloride cells, where it may function in systemic and cellular osmoregulation (Choe et al., 2006). However, it is to be examined if a similar functional response of salinity and osmoregulation exist in pike perch juveniles and the possible interactions between salinity, COX activity, levels of PGE prostaglandins, and stress response.

Larvae enzymatic samples only allowed enough material for analyses at 30 DPH, so determining a digestive enzymatic pattern during pikeperch early ontogeny was not possible. The provision of SO – or LO enriched *Artemia* or changes in environmental salinity caused some significant differences in enzymatic larval expression, but interpretation with precautions as larvae age represented a transitional period in which growth and ontogeny can cause substantial changes. A low enzymatic pepsin activity observed and a higher trypsin activity for all larval groups relate to the relative slow growth, causing a likely delay in process of morphogenesis and indicating limited activity of a stomach. The relative high level of alkaline phosphatase enzymatic activity in all larval groups denotes the maturation of enterocytes (Zambonino Infante and Cahu, 2007). Larvae at the same age exposed to extruded PL enriched microdiets and consequently higher protein content expressed a much higher pepsin activity, but lower alkaline phosphatase activity, but also a much faster growth rate until 30 DPH. This suggests that in these larvae the stomach had already differentiated and

an earlier enterocyte maturation leading to a better nutrient absorption and incorporation (Hamza et al., 2008, Lund et al., 2017). In the present experiment overall lipase activity was similar in larval groups and apparently not affected by the relative difference in enrichment with triglycerides in the form of 18:2n-6 or 18:3n-3 as main FA constituents.

In the present experiment there was a high incidence of skeletal anomalies mostly affecting endochondral bones. The low incidence of anomalies related to vertebral bodies, an intra membranous type skeletal element, regardless salinity or dietary treatment, agree well with the similar expression of *alp*. *Alp* is a molecular marker of late mineralization of intra-membranous bones whose expression is markedly affected by EPA and DHA (Saleh et al., 2012; Izquierdo et al., 2013). Therefore, the lack of significant differences in the LC-PUFA contents in 30 DPH pikeperch fed SO or LO *Artemia* agree well with the lack of differences in *alp* expression and the low incidence of vertebral anomalies. Moreover, the control fish fed DHA enriched *Artemia* showed higher DHA body contents and the complete lack of vertebral anomalies. *Mef2c* expression was neither affected by salinity or dietary fatty acid profiles and was not related to the skeletal anomalies found in pikeperch. These results are in agreement with results in other freshwater or marine species where *mef2c* expression was not affected by changes in dietary protein or lipid sources (Lilland et al., 2015; Benedito Palos et al., 2016). On the contrary, *mef2c* may be up-regulated by dietary P:L ratio leading to hypertrophy of myotubes that could be the origin of vertebral anomalies (Overturf et al., 2016).

In addition to the high occurrence of endochondral bones anomalies, an increase in salinity lead to a higher incidence of this type of skeletal anomalies. Changes in environmental salinity may cause an osmoregulatory stress in fish (Deane et al., 2009) and even eurihaline teleosts require adaptive modifications in ion and osmoregulatory processes that may imply changes in blood pH, plasma acid–base or ventilatory rates (Claiborne et al., 1994). This physiological reactions to compensate osmoregulatory stress may increase the risk of peroxidation and the subsequent proliferation of toxic oxidised compounds that induce apoptosis of mammalian bone cells and fish larvae (Izquierdo et al., 2013). Oxidative stress has been associated with particular anomalies in the cranium (Izquierdo et al., 2013), such as maxilar or dentary bones anomalies, skeletal elements that develop from a cartilaginous precursor. In the present study, an increase in salinity leads to a higher incidence of bone anomalies, particularly in maxilar and dentary bones, that related well with the up-regulation of *sox9* and *twist2*. In one hand, chondrocyte ossification is regulated by *twist* genes that are expressed in mesenchymal cells in medaka and affect skull development in mice (Renn et al., 2006). Thus, overexpression of *Twist2* inhibits osteogenesis maintaining cells in a pre-osteoblast phenotype during osteoblast development (Renn and Winkler, 2009). Insufficient *Runx2* activity caused by overexpression of *Twist2* give rise to impaired skull formation (Kronenberg, 2004). On the other hand, *sox9* expression, in cooperation with s*ox5* and s*ox6*, is necessary for early chondrogenic differentiation and in humans mutation of the *sox9b* gene leads to severe cranial defects (Yan et al., 2005; Amano et al., 2009; Choy et al., 2010). In the present study, up-regulation of *Twist2* was related to the increased incidence of skull anomalies in pikeperch reared at higher salinities, possibly due to the impairment of chondrocyte maturation as occurs in medaka. Up-regulation of *sox9,5* in the same fish would promote chondrogenic differentiation to compensate the fail in chondrocyte maturation caused by *Twist2* up-regulation.

Conclusions

The obvious incorporation of all the ^{14}C substrates, and their esterification and transformation patterns points to the suitability of the methodology used to study *in vivo* lipid metabolism of pikeperch larvae as also shown in newly hatched cephalopods (Reis et al., 2014 ; 2016). The present results demonstrate that EPA and ARA are easier incorporated into pikeperch larvae than DHA and that the naturally abundant Artemia´s 18:3n-3 can also compete with DHA for incorporation into total lipids and more specifically into the larval polar lipids. The results also confirm that pikeperch larvae, despite exposed to different salinities and to dietary LC-PUFA deficiencies, have very limited endogenous capability to use PUFA precursors to biosynthesise essential LC-PUFA, displaying some capacity for Δ6 but not forΔ5 activities. Therefore, EFA especially DHA must be supplied in diets of pikeperch larvae to guarantee low stress sensitivity and normal development. Rearing salinity conditions up to 10 ppt had no effects on growth or survival of larvae, but

affected endocrine hormonal prostaglandin production. Results indicate that lipid and FA metabolism could be modified in response to salinity changes by involving a variety of regulatory endocrine processes in the developing fish larvae such as cortisol levels, hormone production and interactions and signal transductions, but further studies are required to .clarify such regulatory processes. A detailed evaluation of the different types of skeleton anomalies as well as the expression of related molecular markers was conducted for the first time in the larval phases of this species. The results pointed out the high occurrence of anomalies in endochondral bones and the increased incidence under LC-PUFA deficiencies and specially at higher salinities and when fed on a sunflower based diet

Experiment 2
Dietary LC-PUFA deficiency early in ontogeny induces behavioral changes in pike perch (*Sander lucioperca*) larvae and fry

The aim of the experiment was to examine if dietary fatty acid composition in larval feed of pike perch affected behavioral responses to challenges in the larval and fry stages, and if they affect learning and the endocrine stress response in the fry stage. This was carried out by studying behavioral responses to visually simulated predator attacks and fast escape responses to mechano-sensory stimuli during the larval stage. During the fry stage the fast escape response test was repeated, spatial learning ability was studied by a maze test and effects on the endocrine stress response were quantified by post stress plasma cortisol levels.

Materials and methods

Formulation of emulsions

Four dietary emulsions were made by the substitution of extra refined virgin olive oil (Seatons 790.1 mg oleic acid/g) with either DHA oil (Incromega DHA500TG, DHA content N500 mg DHA/g; ≤100 mg EPA/g) or a fish oil rich in phospholipids from TripleNine, Esbjerg Denmark (PL: 44.3% weight (i.e. phosphatidyl choline, PC: 16.1%; lysophosphatidylcholine, LPC: 5.4%; phosphatidylethanolamines, PE: 4.5%; APE: 6.3%; spingomyelin, SPH 3.5%, others 8.5%). The main FA in the oil constituted 16:0: 188 mg g−1 oil; 18:1: 109 mg g−1 oil; DHA: 193 mg g−1 oil; EPA: 135 mg g−1 oil. The sum of polyunsaturated FA was 400 mg/g oil. Three emulsions contained either A: 0 g, B: 50 g or C: 500 g kg−1 DHA oil and one emulsion D: 500 g kg−1 phospholipid rich fish oil (i.e. 440 g phospholipids kg−1) (**Table 1**). In all emulsions soy lecithin was included (70 g kg−1) as emulsifier and E-vitamin mix was added (40 g kg−1) as antioxidant (**Table 1**). Olive oil and DHA oil were obtained from Croda Chemicals Europe, Snaith, UK. Fish oil, soy lecithin and E vitamin mix were obtained from BioMar, Brande, Denmark.

Table 1
Analysed TFA Artemia content (mg g^{-1} d.w.) and FA composition (% of TFA) enriched by 4 emulsions. Formulation of emulsions (% inclusion) is shown below.

	A: OO[a]	B: OO[b] +5 DHA	C: OO[c] +50 DHA	D: OO[d] +50 PL
TFA	97.1 ± 37.6	122.1 ± 6.3	128.3 ± 75.5	79.7 ± 20.4
FA				
16:0	11.1 ± 0.0	10.1 ± 0.6	10.6 ± 0.9	11.1 ± 0.0
18:0	6.6 ± 1.2	6.0 ± 0.2	6.2 ± 0.0	6.1 ± 0.0
Total SFA	21.5 ± 2.6	19.4 ± 1.2	21.4 ± 2.1	22.5 ± 1.2
16:1 (n-7)	1.0 ± 0.3	1.1 ± 0.0	1.2 ± 0.0	1.4 ± 0.2
18:1 (n-9)	36.5 ± 0.7[c]	36.6 ± 0.4[c]	25.6 ± 0.9[a]	29.9 ± 0.8[b]
Total MUFAs	40.6 ± 1.8	43.1 ± 1.8	34.2 ± 3.5	39.1 ± 2.2
18:2 (n-6)	5.1 ± 0.2[b]	4.8 ± 0.2[b]	4.2 ± 0.2[a]	4.4 ± 0.4[ab]
18:3 (n-6)	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.1
20:3 (n-6)	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1
20:4 (n-6) ARA	0.3 ± 0.1[a]	0.4 ± 0.1[ab]	0.7 ± 0.0[b]	0.6 ± 0.2[ab]
Total (n-6) PUFA	5.9 ± 0.5	5.8 ± 0.6	5.5 ± 0.5	5.6 ± 0.7
18:3 (n-3)	28.9 ± 2.4	27.8 ± 1.1	27.5 ± 1.5	22.9 ± 0.5
20:3 (n-3)	1.3 ± 0.4	1.1 ± 0.0	1.4 ± 0.2	1.1 ± 0.0
20:5 (n-3) EPA	0.5 ± 0.4[a]	1.4 ± 0.1[b]	3.3 ± 0.2[c]	4.4 ± 0.2[d]
22:6 (n-3) DHA	0.1 ± 0.1[a]	0.6 ± 0.1[a]	5.5 ± 0.2[c]	3.1 ± 0.2[b]
Total (n-3) PUFA	30.9 ± 3.3	30.9 ± 1.3	37.8 ± 2.0	31.6 ± 0.9
DHA/EPA	0.2 ± 0.4[a]	0.4 ± 0.1[a]	1.7 ± 0.0[b]	0.7 ± 0.1[a]
ARA/DHA	3.2 ± 7.0	0.7 ± 0.2	0.1 ± 0.0	0.2 ± 0.0
ARA/EPA	0.5 ± 0.5	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
(n-3)/(n-6)	5.2 ± 0.5[a]	5.4 ± 0.2[ab]	6.9 ± 0.2[b]	5.6 ± 0.3[ab]

Values in a row followed by a different superscript are significantly different $P < 0.05$.
All emulsions included 7% soya lecithin and 4% E vitamin mix.
[a] OO: 89% OO; (OO (olive oil, Seatons refined, ≥79.1% oleic acid).
[b] OO + 5 DHA: 84% OO. 5% Incromega DHA500TG, DHA content ≥51% of total fatty acids.
[c] OO + 50 DHA: 39% OO. 50% Incromega DHA500TG, DHA content ≥51% of total fatty acids.
[d] OO + 50PL: 39% OO. 50% fish oil with phospholipid (PL) content ≥44% total lipids.

Larval and juvenile rearing and feeding

Larvae were obtained from a commercial farm AquaPri Innovation,Egtved, Denmark at 2 DPH. Approximately 1600 larvae were distributed into each of 12 tanks at a density of approximately 36 larvae per litre. The larval rearing tanks had a volume of 46 L, and received a water flow of 8–10 L h−1 from a 10m3 temperature controlled reservoir. Each tank had separate inlet taps with adjustable flowmeters, 500 μm drainage filters and aeration. Larvae were kept under constant dim light provided by light bulbs above the tanks. Temperature and oxygen saturation were monitored daily using a portable DO meter (OxyGuard Handy, OxyGuard, Birkerød, Denmark). The temperature was maintained at 16.6±0.7 ° C during the first 28 days of experimentation. Oxygen content was kept around 7.1–7.5 mg/L in all tanks. Larvae for each treatment were reared in triplicate tanks. Newly hatched un-enriched *Artemia* (MC 450 type, N260.000 nauplii/g, INVE Artemia Systems, Belgium) were used as starter feed from dph 3 until 6 dph for all larval groups. From 7–27 dph, randomly chosen triplicate larval groups were fed EG type *Artemia* (INVE-Artemia Systems) enriched by one of 4 emulsions (0.6 g emulsion L−l for 24 h). *Artemia* were enriched according to normal enrichment procedures at a temperature of 21–22 °C, providing vigorous aeration by airstones (by a mix of air and pure oxygen to ensure oxygen levels N 4 mg/L) at a density of 500–1000 *Artemia* /ml. *Artemia* were harvested in the morning and administered continuously for 2 periods of 6 hours (each morning and afternoon) by automatic dispensers each holding a suspension of *Artemia* in seawater. Buckets

containing the remaining *Artemia* of each enrichment type were kept aerated by airstones in a refrigerator between feedings at 5 °C. The tank bottom of each larval tank was gently vacuumed on a daily basis to remove uneaten *Artemia*, debris and to examine for mortality of larvae, which were counted. From DPH 29–40 all larval groups were fed *Artemi*a enriched by emulsion D (phospholipid rich fish oil) and gradually weaned to an extruded experimental feed composed of fish meal (50%); soy protein concentrate (12.5%); wheat (17.2%); fish oil (10%); rape seed oil (10%); vitamin/mineral (0.3%). Protein and lipid content was 43.6% and 28.1% respectively. The feed was initially crushed to match the size of the growing fish fry and was fed to the fry during the remaining of the study until DPH 140 by 12 h band feeders. Fry were kept in their initial tanks during the entire study and tanks regularly cleaned. Temperature was kept at 19.3–20.4 ° C and oxygen above 5.1 mg/L.

Behavioral studies on larvae and fry;

Avoidance tests

The protocol used for examining avoidance behavior in pike perch larvae was similar to the bouncing ball assay described by Colwill and Creton (2011) and Pelkowski et al. (2011), with slight modifications. The imaging system consisted of a PC running a Microsoft Power Point presentation on a 19 inch LCD monitor placed in horizontal position. The presentation displayed the outline of 4 petri dishes and a 30 mm black bouncing ball animation traversing the upper third of each dish at a velocity of 50 mm sec−1. A camera (HD-4110, Hewlett Packard) with a resolution of 1920 × 1080 pixels was positioned approximately 60cm above the petri dishes, to record the observations from the experiment using Debut Video Capture Software Professional (v. 1.64, NCH Software) at a rate of 5 fps onto a local PC. The entire setup was fitted within a cabinet, which was closed during experimentation. Larvae for avoidance experiments were sampled at random from different dietary treatments at 33 and 34 dph. Fish larvae were isolated individually in 50 mL beakers overnight at room temperature (20 °C). The following day, 4 fish larvae at a time were transferred to individual test arenas (petri dishes with an internal diameter of 115mm) placed on the monitor. Care was taken to avoid air exposure of fish during transfer. The final water volume in each petri dish (test arena) was 70 ml, proving a water level of ~7 mm. Following transfer, fish were allowed to acclimatize for 30 min. Each experimental round was initiated by recording a 5min period without an animated predator stimulus to determine baseline behavior, followed by 25min recordings of behavioral responses to visual predator simulation. All avoidance experiments were completed on 2 consecutive days. Baseline behavior was analysed during a 30 s period following the first minute in the experimental round and the response to the predator stimulus was analysed during a 30 s period following 20 min predator simulation. Adobe Photoshop (Adobe Systems Software) was used to export one frame from every second (every fifth frame), yielding 2 × 30 frames for further analysis for each fish. Video frames were analysed using Image J (v. 1.46r,Wayne Rasband, NIH, USA). The centre x,y coordinates for each petri dish and the length of each fish was established from the first suitable image. For all other images, the x,y coordinates for the snout and centre of mass were recorded (centre of mass was defined as the posterior border of the abdominal cavity which was clearly visible). All coordinates were transferred to a Microsoft Excel, and were used to determine orientation, time spent at the edge of the petri dish (defined as the outermost 10% of the radius), the upper or lower half of the petri dish, swimming speed (body lengths per second, bl s^{-1}) and time spent immobile (defined as moving less than 0.1 bl s^{-1}).

Fast escape response

The fast escape performance studies on larvae (DPH 35–38) and juveniles (DPH 121–124) were conducted in a white semi-translucent polyethylene circular tank with a diameter of 38 cm and a water depth of 5 cm, using a slight modification of previously described methods (Marras et al., 2011). Fish were transferred to the tank without air exposure and allowed to acclimatize to the tank for a period of 1 hour. The experimental setup was covered in black opaque plastic to prevent visual disturbance of the fish. Video recordings were made at

a rate of 250 fps using a Casio high-speed camera (EX-FH100) mounted 80 cm above the water surface. The setup was illuminated from below using a 28Wfluorescent light. The escape response was triggered by mechanical stimulation by releasing an iron rod (ø 10mm, l 15mm) manually from a height of 90 cm above the water surface. To avoid visually stimulating the test subject, the iron rod fell inside a vertical PVC pipe suspended approximately 1 cm above the water surface. Fast escape was determined for single fish and only once per fish larvae, while two repetitions were performed for juveniles with 30 minutes of recovery between tests. There was no water replacement or water current during experiments. Fish were subsequently anaesthetized and measured for standard and total length to the nearest half mm, blotted dry and weighed to the nearest mg or g. Escape responses were analysed using Tracker (v. 4.72, www.cabrillo.edu/~dbrown/tracker). Time 0 was set as the nearest 4 ms interval at which the stimulus broke the water surface. The centre of mass was plotted every 4 ms from stimulus and 20 frames forward. These x,y coordinates were used to calculate escape latency (defined as the time elapsed between stimulus breaking the water surface and the first detectable escape motion of the fish), peak velocity during the escape response (bl s−1), distance covered during the first 80 ms of the escape response, and peak acceleration (m s−2).

Maze spatial learning test. Long-term effects on learning ability and stress responsiveness were investigated at DPH 121–140 by a maze test and cortisol response to confinement. The test was performed in a maze consisting of a 40 × 40 cm square with access to a 10 × 15 cm compartment at each corner. The maze was white and light was provided by two fluorescent tubes (20W,warm white) placed 1.3m above the water surface, in the maze. One of the corner compartments was fitted with an exit, leading fish out of the maze to a darker area with cover. The day before the maze test and in between training sessions during the maze test, fish were kept individually isolated in 20 litre aquaria provided aeration and water exchange. During the training sessions, fish was transported from the isolation aquaria in a 2 litre beaker and gently inserted in the mid-section of the maze. The behavior of the fish was video recorded (HD-4110, Hewlett Packard). Fish showed two stereotypical behavioral patterns in the maze. First, after being inserted in the maze fish did not move, displaying “freezing behavior”. After this, fish showed “seeking behavior”, exploring the maze and corner compartments until locating the exit. Time spent in freezing and seeking behavior was recorded. Time spent freezing and time to leave maze was recorded was 30 min if the fish did not move for a period of 30 min after being inserted in the maze. Fish were exposed to six training sessions during a period of three days (two to three daily training sessions, with a minimum of 3 h in between). Since the behavior and fatty acid profiles were similar within the groups given feed containing low levels of DHA (diet A and B) and high levels of DHA (Diet C and D) at the larval stage, the behavioral data for these two groups was pooled. The group fed low levels of DHA consisted of four fish given diet A and five fish given diet B. The group fed with high levels of DHA consisted of two fish given diet C and eight fish given diet D. Following the maze test, fish were exposed to standardized confinement stress test. Fish were kept in submerged transparent chambers (10 × 5 × 3 cm) for 30 min, whereupon they were anesthetized with an overdose of tricaine methanesulphonate (MS-222, 50mg/L) and frozen (−80 °C) for later whole body cortisol analyses.

Cortisol levels

Whole fish cortisol analyses were carried out on all experimentally used fry. Data presented as pooled values of dietary groups (A and B, n = 11) and (C and D, n = 12). To analyze for whole body cortisol, fish fry were thawed and about 1 g of tissue collected behind the anal fin was dissected out and weighed. The tissue was then homogenized in PBS (1 ml PBS g tissue−1), thereafter cortisol from the homogenate was extracted with ethyl acetate (the relation between homogenate and ethyl acetate was 1 to 5). After vortexing the homogenate / ethyl acetate was centrifuged at 1500 g for ten minutes.1 ml of the supernatant was evaporated using a vacuum centrifuge and the remaining residue was re-suspended in an extraction buffer (ELISA kit extraction buffer). Cortisol content in re-suspended samples was quantified using the ELISA kit standard method (Neogen, Product #402710).

Statistics

Larval dry weight (d.w.); mortality; tissue FA composition; and escape-response tests were compared by one way ANOVA and all pairwise Holm Sidak comparison. Percent data were arcsine transformed prior to analysis. Avoidance behavior and maze tests were carried out by two way repeated measurements ANOVA and all pairwise Holm Sidak comparison. Normality of data was tested by Shapiro Wilks test. All statistics were performed using Sigma Plot (v. 12.5) and P b 0.05 was considered statistically significant.

Growth and mortality

Pike perch larvae had a dry weight of 0.14 ± 0.0 mg at 1 DPH. At 28 DPH larval d.w. was A: 1.81 ± 0.36; B: 1.94 ± 0.36; C: 2.34 ± 0.54; D: 2.41 ± 0.46 mg individual−1 with no significant differences (P = 0.07) between dietary treatments.. Specific Growth Rate, SGR (LnWf−LnWi × 100)/t from 1–27 dph was A: 21.4 ± 0.8; B: 21.6 ± 0.7; C: 22.6 ± 1.1; D: 22.8± 0.9% d−1, and was not significantly different between treatments (P= 0.06). Mortality was relatively low (1.6–4.8% for the 4 treatments, P = 0.73) until the onset of cannibalism.

Avoidance and fast escape response

Baseline (before predator simulation) positioning of larvae in the test arena differed between dietary treatments. (**Fig. 1a–d**). Larvae fed a diet deficient or low in DHA oil (treatment A and B) spent a high proportion of time (~90%) at the edge of the test arena compared to larvae fed a diet with a high DHA or phospholipid inclusion (C and D), which spent b10% of the time at the edge (**Fig. 1a**). When a visual predator simulation was presented in the upper half of the test arena, larvae fed all dietary treatments increased the fraction of time spent at the edge to 50–70%. Simulation of a predator in the upper half of the test arena caused fish larvae to spend a significantly greater amount of time in the lower half (**Fig. 1b**). There was no overall effect of diet, although a preference for diet C to occupy the upper half of the arena was significantly higher than for the other diets. Larvae on dietary treatment A and B with low levels of DHA had significantly higher routine swimming speeds under control conditions, averaging 2 and 1.3 BL s−1 respectively, compared to dietary treatments C and D in which routine swimming speeds were significantly lower (0.1–0.2 BL s−1) (**Fig. 1c**). During predator simulation, diet A and B showed no change in swimming speeds, while diets C and D responded with a 5–8 fold increase in swimming speed. Under control conditions, the fraction of time that fish spent immobile decreased significantly with decreasing DHA oil enrichment, to a minimum of 10% in the groups fed diet A (**Fig. 1d**). In the face of a simulated predator, all dietary treatments decreased the amount of time they were immobile, with no significant differences between dietary treatments. In the series of experiments to a mechano-sensory stimulus and assessment of fast-escape response (**Fig. 2a–d**), larvae reared on diet C and D had higher peak accelerations with a magnitude of 29–33% than larvae fed diets poor in DHA (A). No significant differences were observed in peak velocity or distance covered in 80 ms. There was a tendency towards decreasing escape latencies with increasing DHA content, but this finding was not significant. Escape response experiments performed on juvenile groups 86 days after the larval experiment (**Fig. 3a–d**) showed similar results, with no significant differences observed, except for peak acceleration response in which treatment C was significantly different from treatment A (**Fig. 3d**).

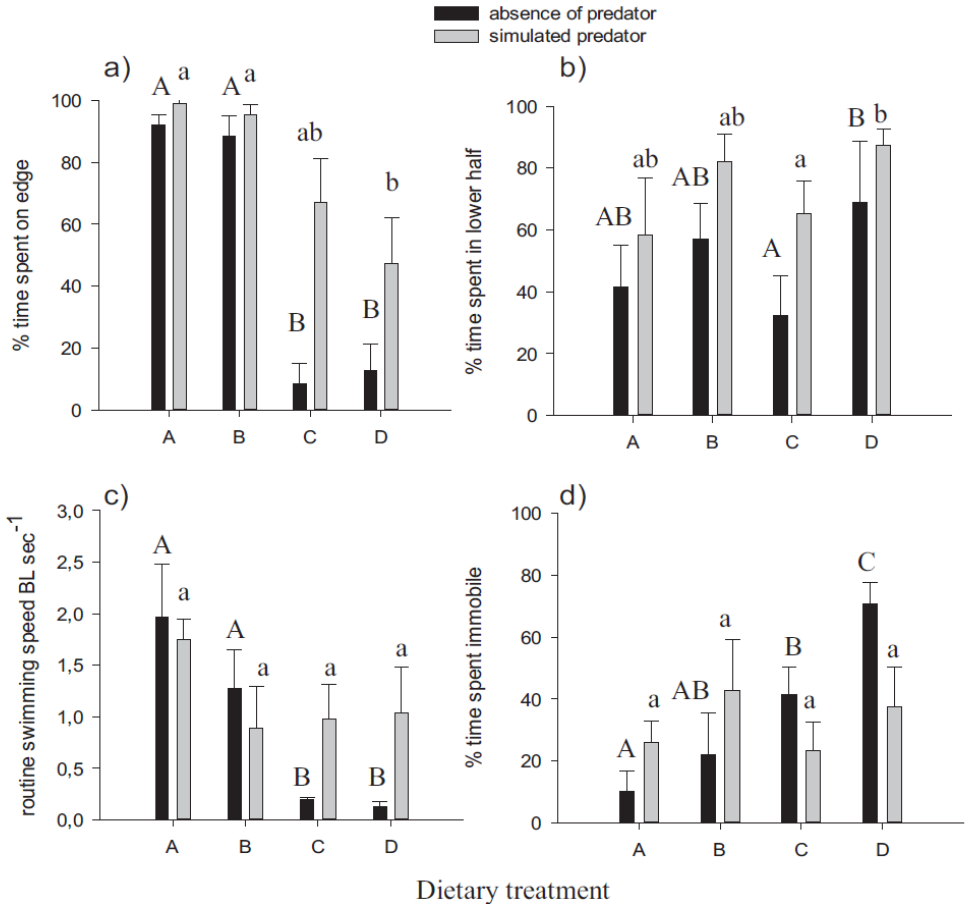

Figure 1. a-d. Avoidance behavior of larvae in the absence and presence of a predator. 1a: Time spent at edge of petri dish; there was a statistically significant interaction between dietary treatment and predator (P = 0.029). 1b: Time spent in lower half of petri dish; There was not a statistically significant interaction between dietary treatment and predator (P = 0.768). 1c: Maximum swimming speed; there was a statistically significant interaction between dietary treatment and predator (P = 0.032); 1d: Time spent holding station: there was a statistically significant interaction between dietary treatment and predator (P = 0.030). Values are presented as the mean ± SEM. Significant differences (P < 0.05) between treatments in presence - or absence of a stressor are shown by different capital or lower case letters respectively. n = 5–8 per treatment. Dietary treatment A: OO;B: OO5DHA; C: OO50DHA; D: OO50PL.

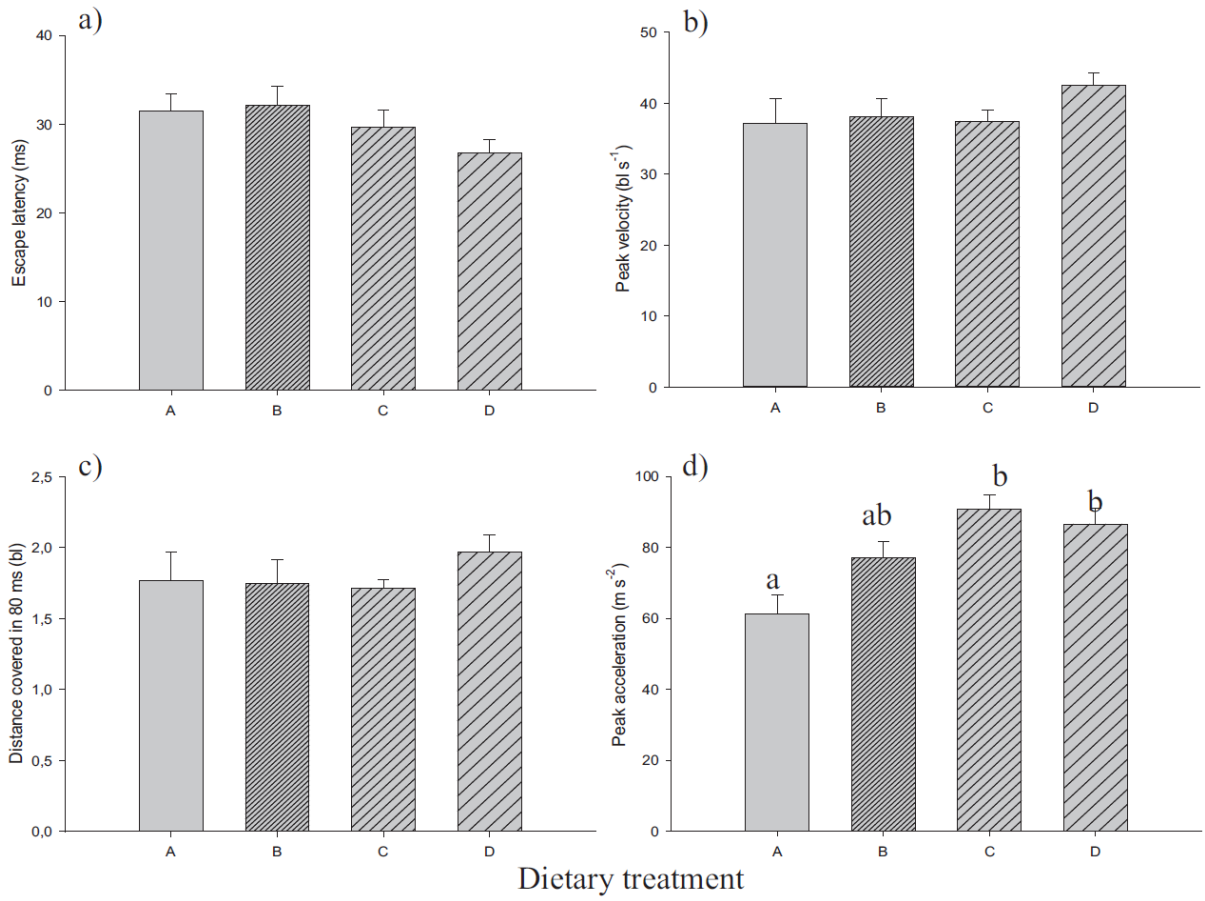

Figure 2. a–d. Larval escape-response to a visual mechano-sensory stimulus. 2a: Escape latency, (P= 0.192); 2b: Peak velocity, (P= 0.399); 2c: Distance covered in 80 ms (P= 0.439); 2d:Peak acceleration (P= 0.001). Values are presented as the mean ± SEM. Significant differences (P =0.192) between treatments are shown by different letters. n= 6–9 per treatment. Dietary treatment A: OO; B: OO5DHA; C: OO50DHA; D: OO50PL.

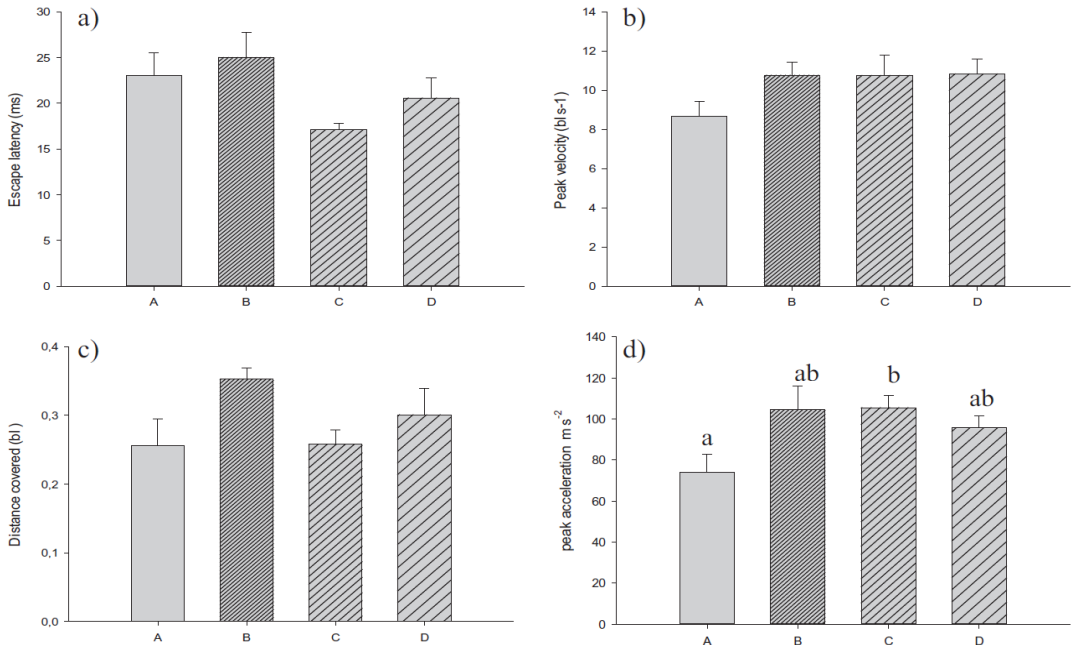

Figure 3. a–d. Fry escape-response to a visual mechano-sensory stimulus. 3a: Escape latency, (P = 0.107); 3b: Peak velocity, (P= 0.206); 3c: Distance covered in 80 ms, (P = 0.093); 3d: Peak acceleration, (P= 0.049). Values are presented as the mean±SEM. Significant differences (P < 0.05) between treatments are shown by different letters. n=6 per treatment. Dietary treatment A: OO; B: OO5DHA; C: OO50DHA; D: OO50PL.

Maze spatial learning test and fry cortisol content

The time fry spent to solve a maze decreased with training (**Fig. 4a–b**), an effect that was related to a decrease in initial freezing time (**Fig. 4b**).Moreover, fry fed diets low or deficient in DHA (A+B) as larvae had longer initial freezing time compared to fry fed diet C or D given diets high in content of DHA and phospholipids, this was independent of training. There was no significant difference (P= 0.25) in tissue cortisol level (mean ± SEM between dietary treatments groups (A and B): 35±3.7 ng g−1 tissue and (C and D): 30±2.5 ng g−1 tissue, respectively. Thus, dietary DHA content or phospholipids did not affect the magnitude of a stress-induced cortisol release in fry.

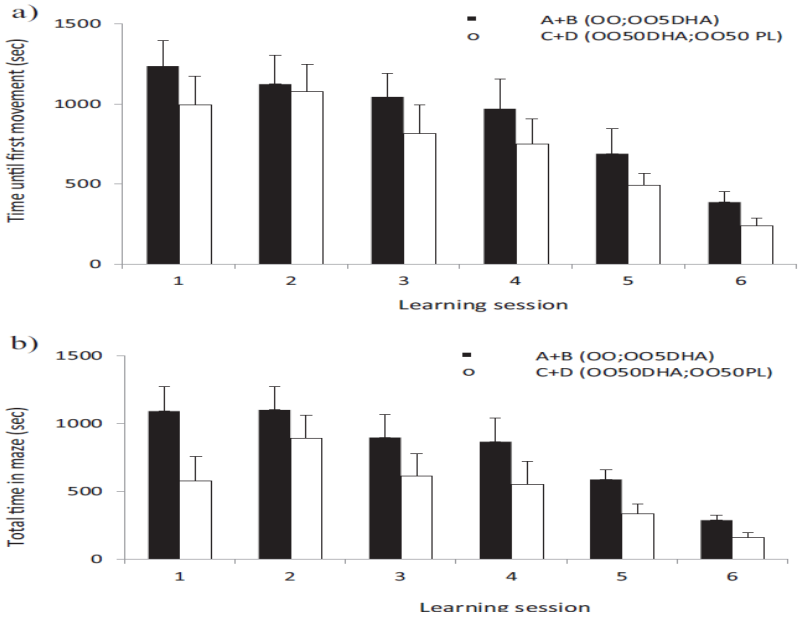

Figure 4. a–b. Learning ability of fry fish to find way out of a maze during 6 repetitious training sessions. 4a: Total time in maze. Two way repeated ANOVA: Learning session: $P < 0.001$; Fatty acid: $P < 0.20$; Fatty acid x learning session: $P= 0.55$. 4b: Time until first movement. Two way repeated ANOVA: Learning session: $P < 0.001$; Fatty acid: $P < 0.05$; Fatty acid x learning session: $P= 0.56$; Values are presented as the mean ± SEM. Dietary treatment A + B: Pooled fish, OO;OO5DHA (n = 7); C + D: Pooled fish, OO50DHA; OO50PL (n = 10).

Discussion

The tolerance to physiological stressors and the display of behavioral abnormalities such as changes in activity levels, risk taking, and escape performance in relation to nutrition and dietary levels of LC PUFAs have only been sparsely investigated in fish. The escape response can be divided into several distinct components; a sensory component in which the fish senses a threat by means of the visual, acoustic or mechanical sensory system (Marras et al., 2011). The sensory output of these systems is transmitted to a pair of reticulospinal neurons, the Mauthner cells, particularly related to escaping behaviour in fish (Benítez-Santana et al., 2012 − which in turn elicit a behavioral response (Eaton and Lee, 2001). The fast start escape response can be analyzed for a variety of variables such as response latency, escape trajectory, velocity, and more (e.g. Domenici et al., 2007). The latency period between stimulus and onset of swimming is dictated by velocity of neural signalling; from the sensory cells to the Mauthner cells to the axial muscle that propels the fish away from the threat (Marras et al., 2011). Impaired functional integrity caused by alterations in the LC-PUFAs may occur at several locations, affecting afferent or efferent neurons or both, and discrimination may be difficult. The present study showed a trend towards lower response times with higher dietary DHA and EPA content in both larvae and in fry. As no effects were observed on the escape latency, it could be hypothesised that effects on peak acceleration rates in larvae from dietary levels of DHA and EPA are related to efferent sensory signalling from Mauthner cells. However, future studies including actual measurements of LC PUFA composition – or activity of Mauthner cells by analysis of the cholinergic neuron density (Marras et al., 2011), are needed to verify this. Larvae fed high DHA levels could achieve higher peak acceleration rates during their escape response. Benítez-Santana et al. (2012) showed that behavioral reactions to visual stimuli were associated with neural and muscular development that occurs early, while responses to sonorous stimuli were associated with the development and mechano-sensory neuromasts of the lateral line system that occurs days to weeks post hatching. Therefore, when assessing the behavioral effects

of dietary n-3 LC PUFA deficiency, and in comparison with other studies, the type of stimulus applied must be considered, as must the species, age (and perhaps more importantly the size) of larvae (Fuiman, 1993). The present study demonstrated a temporal consistency in effects and tendencies for effects of dietary fatty acid composition indicating that a diet with an adequate HUFA profile could not compensate for deficiencies experienced during early ontogeny. The behavioral responses to mechano-sensory stimuli at the larval stage were maintained in fry until 95 days after the dietary treatment period (7–27 DPH) had ceased. This supports and further expands the time frame of the findings by Fuiman and Ojanguran (2011) who observed that feeding larvae a PUFA enriched diet for 3 weeks could not compensate for earlier deficiency. The underlying mechanisms for these long term effects are unknown, but may be caused by differences in brain development and architecture or directly related to tissue DHA deficiency Studies in rats and mice have shown that brain tissue is conservative in terms of DHA content and that complete repletion after being fed a DHA deficient diet takes up to eight weeks (Moriguchi et al., 2001). Further studies are needed to verify, if these long-term effects are directly related to changes in brain developmental pattern and/or the actual LC PUFA composition of the neuronal pathway involved in the fast escape response. The observation that all dietary treatments responded by avoidance to the simulated predator suggests that visual acuity in fish on diets with low DHA or EPA content was not impaired to any significant degree. However, it is important to keep in mind that several studies have shown that larval retina is a primary target for dietary PUFA deficiency (Bell et al., 1995, 1996; Navarro et al., 1997) and an experiment designed to specifically investigate effects on visual capability may have revealed effects of fatty acid composition on visual responses. This has previously been demonstrated in sea bass by Benitez-Santana et al. (2007). A general response, in the present study was that larval groups deficient or low in DHA and EPA displayed higher locomotor activity, including erratic swimming bursts. This is in accordance with other observations in studies of dietary fatty acids composition and performance in pike perch (Lund and Steenfeldt, 2011; Lund et al., 2012 Several studies have shown that cognitive performance and learning ability in mice are impaired when fed n-3 PUFA or n-3 LC-PUFA deficient diets (Carrie et al., 2000; Francès et al., 1995, 1996). Dietary levels of LCPUFAs have been shown to positively affect learning performance in rats (Yonekubo et al., 1994) and mice (Carrie et al., 2000), but the extent with which this would apply to fish has not been examined to date. Although the present study showed that pike perch fry reduced the time required to exit a maze by 75–80% after 6 repeated learning sessions, this effect was independent of dietary treatment. Furthermore, this observation was primarily the result of gradual reductions in initial time lag after transfer to the maze, before fish began to actively explore the maze. This would imply that the effect is more related to habituation than improved spatial learning ability (e.g. fewer mistakes in finding maze exit) as such. These results are slightly in contrast with those reported in rat and mice studies, where DHA deficiency seems to be associated with a slower habituation to new environments (Fedorova and Salem, 2006). In the present study, irrespectively of habituation, the time lag after being transferred to the maze was consistently longer for DHA deficient fish. The longer time lag before first movement for groups low or deficient in DHA and EPA (A + B) was independent of training, and may thus reflect a more anxious behavioral profile of these fish. These results are in line with previous results on mice or rats (Carrie et al., 2000; Enslen et al., 1991). In the study by Carrie et al., 2000 a fish oil diet induced higher exploratory and locomotory activity in mice reflecting both desire to explore an unknown place and the fear of being confronted by novelty) than mice on a palmoil diet. Studies in rats have demonstrated that anxiolytic effects of low LC-PUFA levels can be present after a reconstitution period with diets rich in phospholipids. This is in accordance with the long term effects observed in this study 95 days after the dietary treatment period was terminated and raises questions, about which brain mechanisms that are involved in the long-term anxiolytic effects of DHA deficiency.
The absence of any significant differences in cortisol levels in the fry groups following confinement stress indicates, that the suggested difference in brain function is not directly related to the neuroendocrine stress axis. However, an anxious behavior profile may reflect a general behavioral inhibition in response to stressful events, such as reduced appetite, and thereby may directly affect production parameters.

Conclusions

In this study we present a number of behavioral effects correlated to n-3 LC-PUFA levels in diets for pike perch larvae. Larvae fed low levels of DHA displayed a tendency towards delayed escape responses (latency time increased) and significantly slower peak acceleration rates during escape responses following a

mechano-sensory stimulus. This effect was consistent up to 90 days after the dietary treatment was terminated, demonstrating long-term effects of early nutritional history in fish. A more anxious behavioral profile of the fry low in DHA lends supports to long-term central effects, such as brain developmental pattern, being the cause of these behavioral effects.

Overall conclusions and protocol

Based on the present experiments, it is concluded that pikeperch larvae have a limited ability to elongate and metabolize shorter chain PUFAs to HUFAs (essential FA). Thus, a diet optimized for start feeding pikeperch larvae need to be enriched with essential HUFAs primarily DHA to improve neural development (escape response), stress resistance and to reduce skeletal anomalies. Saline rearing conditions have no obvious positive effect on larval performance or development, but may cause improved FA metabolism and uptake and change eicosanoid activity. Lack of sufficient dietary HUFAs from early larval ontogenetic development have severe effect on long term stress sensitivity and may impair learning abilities and behaviour and must be considered included in early diets to ensure both short - and long term long term fish robustness. Results from deliverable 10.1 show that supplementation of dietary phospholipids in addition to HUFAs improve early growth and development and have several important regulatory physiological functions of liver proteomics and enzymatic activity.

Deviations: This report was scheduled to be delivered in Mo 36, assuming that all analytical work would be finished. Although experiments were finished timely, analytical work from some partners was delayed. Therefore, an extension was requested and was approved by the coordinator.

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