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

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ABSTRACT

Combinations of nutritional requirements and husbandry rearing conditions during early ontogeny are poorly studied in pikeperch (*Sander lucioperca*). The substitution of marine oils with vegetable oils has reduced stress tolerance and caused neurophysiological changes in pike perch larvae, but effects of environmental cues are limited. Saline water influences on a range of physiological functions during early fish larval ontogeny and may affect FA metabolism, − elongation and desaturation - activity when given diets limited in LC PUFAs, but rich in shorter chain n-3 or n-6 PUFAs. Consequently, live *Artemia* differing in 18:2n-6 (LA) and 18:3n-3 (ALA) content by enrichment with sunflower oil (SFO) or linseed oil (LO) were fed to 10 days post hatch (DPH) larvae and reared up to isosmotic salinities (0, 5, 10 ppt) until 30 DPH. Larval tissue FA composition was examined at 15, 25 and 30 DPH. Besides, an *in vivo* assay was performed on 20 DPH larvae with ^{14}C labelled FA including LA; ALA; 20:4n-6 (ARA); 20:5n-3 (EPA) or 22:6n-3 (DHA) to establish FA incorporation and metabolism. At 30 DPH, performance, digestive enzymatic activity, eicosanoid activity, skeletal anomalies and stress sensitivity were further evaluated. Results on larval FA profiles suggest a low desaturation and elongation capability over LA and ALA, with no significant effects of salinity or larval age on modulation of unsaturated fatty acid metabolism. *In vivo* assays revealed that regardless of salinity or diet, pikeperch possess a marked specificity to incorporate ARA and EPA compared to a poorer incorporation of DHA. Larvae exposed to a confinement stress test caused high acute mortality in all experimental groups except for a control group fed with *Artemia* enriched by a commercial DHA Selco emulsion. Growth performance was not significantly affected by salinity or dietary enrichment with SFO or LO, but influenced on larval enzymatic activity of pepsin, aminopeptidase, trypsin and alkaline phosphatase, while lipase activity was not significantly affected. Increased saline conditions significantly decreased hormonal prostaglandin eicosanoid PGE_2, PGE_3 activity with the highest activity at 0 ppt. The prevalence of severe skeletal anomalies was generally high, affecting over 75% of the larval population with negative effects by increase in salinity. The incidence of anomalies was higher on endochondral bones, namely maxillary, ranging from 58 to 83% of the population. These results agree well with those from expression of *sox 9* and *twist2* genes; involved in chondrocyte ossification and differentiation.

1. Introduction

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. Pikeperch aquaculture has gained commercial interest in recent years and farming methods in Europe are in transition from traditional more extensive freshwater pond farming to intensive closed recirculation aquaculture systems

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(RAS) (Dalsgaard et al., 2013; Kestemont et al., 2015). Despite farming of this species takes place in freshwater, studies have shown a relatively 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. Larvae are relatively undeveloped at hatching and do not have the same osmoregulatory capacity (*i.e.* integument, gills, urinary bladder, complete functional gut, kidneys) as metamorphosed larvae or later stage juveniles, but most teleost's larvae are able to osmoregulate due to a high density of ionocytes in the prelarval integument that acts as temporarily osmoregulatory site. Limited knowledge exist about influence of hyperosmotic or isosmotic salinity on early pike perch larval ontogeny or physiology. Environmental changes in salinity have shown to result in modification of physico – chemical conditions of the gut lumen in other fish larvae and may exert a direct action on digestive enzyme performance (Boeuf and Payan, 2001) as well as food transition time (Gheisvandi et al., 2015), that may have implications on growth performance.

Additionally, salinity has shown variable effects on the modulation and expression of Δ6 desaturase activity and fatty acid composition in fishes as previously reviewed (Vagner and Santigosa, 2011) affecting 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 LNA 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 was 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).

The limitations in production of marine oils as due to the increasing demand has caused interest in research into effects of partial or total substitution by oils of plant origin. The early dietary deprivation of EPA, DHA and ARA by use of vegetable oils may give valuable information on dietary programming and implications for physiological pathways in fish larvae as observed in rainbow trout fry (Geurden et al., 2013; Balasubramanian et al., 2016). Diets low in LC-PUFAs, especially DHA, may not affect growth of pike perch, but has provoked increased mortality; shock syndromes; short and long-term stress sensitivity; and deficiency in neural development during early ontogeny, that may affect behavior and learning (Lund and Steenfeldt, 2011; Lund et al., 2014). This suggests a low elongation/desaturation capacity in pikeperch in freshwater 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 C18 carbon PUFAs such as linoleic acid (18:2n-6, LA) and α-linolenic acid (18:3n-3, ALA) to LC-PUFAs of C20 or C22 carbons, *i.e.* ARA, 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/ALA did not affect growth performance, but increased fatty acid elongation and desaturation activity in liver (Blanchard et al., 2008). Besides, Eurasian perch fed a semi-purified fat free diet over 4 weeks followed by different lipid sources (olive oil, safflower oil, linseed oil, or cod liver oil) as the only lipid source showed significant changes in n-3/n-6 ratio and FA composition; indicating that the competition and inhibition between ALA and LA 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 and Kestemont, 2002). A dietary incorporation of ALA concomitantly to LA seemed to be beneficial in order to stimulate liver Δ6 and Δ5 desaturation activity, rate-limiting enzymes involved in LC-PUFA biosynthesis (Xu and Kestemont, 2002; Blanchard et al., 2008). In this sense, and in order to improve the understanding of nutritional physiology of marine fish larvae, studies on metabolic fates of fatty acids through radio tracing methodologies are also currently available (reviewed by Conceição et al., 2007). Among them, *in vivo* incubation of marine larvae with 14C-labelled fatty acid substrates directly added to the rearing water has been successfully used in order to investigate competition and inhibition fates as well as their capability to metabolize dietary FAs (Reis et al., 2014, 2017).

Salinity and dietary ALA/LA ratio may thus influence C20 EPA, DHA and ARA synthesis with possible implications for effects on prostaglandin eicosanoid activity, a group of active hormone-like agents formed from C20 EFA. Their transitory nature fits them to an important role in modulating rapid physiological responses to stimuli while imbalances may impair skeletogenesis during early stages of larval development (Boglino et al., 2014). Successful production of pikeperch juveniles is constrained by the high incidence of skeletal anomalies (Kestemont et al., 2015) and salinity markedly affected the occurrence of skeletal abnormalities in other fishes (Lall and Lewis-McCrea, 2007). Fish possess a wide diversity of cartilages and bones, that are formed during embryonic and larval development (Lall and Lewis-McCrea, 2007), including endochondral and intra-membranous bones (Witten and Huysseune, 2009). A series of molecular markers of bone development have been associated with skeletal anomalies (Saleh et al., 2013a, 2013b). For endochondral bones, *sox9* is a key cartilage transcription factor important for craniofacial bone development, being expressed in zebrafish in the splanchnocranium and pectoral girdle cartilage (Yan et al., 2002). Subsequently, endochondral ossification is regulated by *runx2*, also required for osteoblast differentiation, which is inhibited by Twist proteins (Chen et al., 2014). Alkaline phosphatase gene (*alp*) is a marker of osteoblast differentiation essential for bone matrix mineralization in formation of intra-membranous bones (Hessle et al., 2002). *Alp* is up regulated by dietary fatty acids in larval gilthead sea bream larvae (Saleh et al., 2013a, 2013b). Twist2 plays an important role in bone development by binding to Runx2, and reducing the formation of osteoblasts from its precursors (Kronenberg, 2004). Finally, *mef2c (*myocyte enhancer factor 2), a transcription factor that regulates muscle and cardiovascular development, also controls bone development activating chondrocyte differentiation (Arnold et al., 2007). In addition, up-regulation of *mef2c* causes precocious chondrocyte hypertrophy and dwarfism (Arnold et al., 2007).

The present study thus examined possible effects of dietary n-3/n-6 and salinity by enrichment of *Artemia* with either linseed oil (LO) (high content of ALA) or sunflower oil (SFO) (high content of LA), at low saline rearing conditions (0 ppt; 5 ppt; 10 ppt) in addition to a control group enriched with a commercial DHA Selco emulsion at 0 ppt salinity. Results were assessed by analyses of larval FA composition during development and their *in vivo* capability to incorporate selected EFA (LA, ALA, ARA, EPA and DHA) into total lipids, as well as enzymatic and eicosanoid activities, incidence of skeletal deformities, and stress and performance parameters in pike perch larvae until 30 days post hatching (DPH).

2. Materials and methods

2.1. 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 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 seawater/freshwater from two separate temperature-controlled 10 m^3 reservoirs. Salinity in each tank was adjusted by peristaltic pumps (Longer Pumps BT600-2 J, Hebai, China) 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 near 2000 larvae/tank and reared until 30 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 to 9 DPH, larvae were fed a newly hatched unenriched *Artemia* nauplii (strain MC460, Artemia Systems INVE, Dendermonde, Belgium) kept at 5 °C after hatching for 2 × 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, Dendermonde, Belgium) 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 (SFO) or linseed oil (LO) and supplemented with olive oil (for obtaining similar levels of 18:1n-9, OA). The two emulsions were formulated to differ in composition of primarily LA and ALA FAs (Table 1). In both experimental emulsions a vitamin premix (40 g kg^{-1}) and soy lecithin as an emulsifier (70 g kg^{-1}) were added (BioMar, Tech Centre, Brande, Denmark) (Table 1). Organic sunflower oil and linseed oil were commercially available products; olive oil was highly refined and obtained from Croda Chemicals Europe, Snaith, UK.

The experiment was carried out in a triplicate set-up with 3 tanks per feed type at 3 salinities; *i.e.* 0 ppt; 5–6 ppt; 10–11 ppt equalling a total of 3 × 3 × 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, Dendermonde, Belgium) was used as an experimental control.

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.

The entire experiment and procedures were approved by the ethical committee at DTU Aqua.

2.2. Confinement mortality

At the end of the experiment 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 × 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. After evaluation all fish were killed by an overdose of benzocaine.

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

Diet ingredients (%)	*DHA Selco	SFO	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 Material and Methods section; *DHA Selco: a commercial control emulsion (INVE, Artemia Systems, Dendermonde, Belgium; composition: 60% fish oil, 33% moisture, 1% protein).

2.3. In vivo incubation with labelled ^{14}C fatty acids

Larval capability to incorporate unsaturated FA into total lipids under the different dietary and salinity regimes was studied by *in vivo* radio tracing of ^{14}C fatty acids. More specifically, in the present study the method described by Reis et al. (2014) was adapted to determine the *in vivo* dynamic of incorporation of 5 different radiolabelled FA into pikeperch larvae total lipids, including the C18 FA precursors (18:2n-6; 18:3n-3) particularly abundant in the assayed vegetable oils of the experimental diets, and also their main LC-PUFA physiologically active derivatives (20:4n-6, 20:5n-3 and 22:6n-3) present in the control diet.

Thirty non-fed larvae per tank (12 h feed deprived) at an age of 20 DPH were sampled from the 6 different rearing conditions (*i.e.* SFO 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 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 (PerkinElmer Inc., Waltham, Massachusetts, USA), ARA, EPA and DHA (American Radiolabelled Chemicals Inc., St. Louis, Missouri, USA). Larvae were incubated by triplicate 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 group of 30 larvae incubated without ^{14}C FA were also assessed for fatty acid composition. In order to determine the radioactivity incorporated into whole larvae, 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 sample TL and protein contents (Lowry et al., 1951).

2.4. Growth performance, survival and analytical samplings

Representative samples of *Artemia* were sampled at 12 and 24 DPH for dry weight, and FA composition. At 15, 20, 25 and 30 DPH, 25–35 larvae per tank were sampled and weighed (mg w.w. $ind.^{-1}$) for specific growth rate calculation (SGR) according to the formula:

$$SGR\ (\%day^{-1}) = 100\ x\ (\ln\ w.\ w.\ f - ln\ w.\ w.\ i)\ t^{-1}$$, where ln w. w. f, i = the natural logarithm of the final and initial wet weight, t = time (days)

A random subsample of 10 larvae per replicate was used for FA composition. Additionally, 10 and 20 larvae per tank were sampled at 30 DPH for measurement of prostaglandin PGE_2 and PGE_3, and digestive enzymatic activities, respectively.

All above larval samples were sedated with benzocaine and immediately frozen at −80 °C. Additional 20 larvae per tank were also taken at the end of the experimental period for skeleton morphogenesis and mineralization by staining. These larvae were sedated, fixed and stored in 10% phosphate buffered formaldehyde until analysis. Finally, for relevant genes involved in the skeletal system and organ development 10 larvae per replicate were sedated and stored in RNA later overnight at 4 °C and then frozen at −80 °C until analysis.

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.

2.5. 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 40 μL (1 mg mL^{-1}) of an internal 23:0 FAME standard from Sigma Aldrich was added. Larval samples (10 larvae per replicate) were homogenized 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, Hampton, New Hampshire) 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 was 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 analysed 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 (St. Louis, MO, USA). 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 analysed, but only the most relevant are shown.

2.6. Digestive enzymes

Larval samples (except for Control Selco larvae, *i.e.* 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 and 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, ubiquitously present in larvae) and aminopeptidase N (AN, an enzyme of the brush border membrane), were assayed according to Bessey et al. (1946) and Maroux et al. (1973) using *p*-nitrophenyl phosphate 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 *p*-nitrophenyl myristate as substrate. Protein was determined using the Bradford (1976) procedure. Enzyme activities are expressed as specific activities (U or mU mg $protein^{-1}$).

2.7. Quantitative LCMS/MS analysis PGE2 / PGE3

Larval 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 (0.1 M potassium phosphate, pH 7.0 containing 1 mM EDTA and 10 μM indomethacin), 1 μL 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 μL aliquot of supernatant from each sample was pipetted into a 96 deep well plate, spiked with 50 μL of internal standard (1 ng mL PGE_2-d_4^{-1}), diluted with 200 μL 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 9.77 pg mL^{-1} to 5000 pg mL^{-1} by serial dilutions of a 100 ng mL^{-1} stock solution of PGE_2 and PGE_3 with homogenization buffer. 200 μL of each calibrator solution was spiked with 50 μL of internal standard and diluted with 200 μL deionized water prior to extraction. Solid phase extraction was performed on Waters Oasis MAX microelution plates. Plate wells were conditioned with 200 μL of acetonitrile followed by 25% acetonitrile in water. A volume of 450 μL of sample or calibrator was added to the SPE plate and then the wells were washed with 200 μL of 25% acetonitrile in water followed by 200 μL of 100% acetonitrile. Samples were eluted into 25 μL of a trapping solution (10% glycerol in water) using 25 μL of 50:50 acetonitrile/IPA + 5% formic acid. Prior to mass spectrometry, samples (25 μL) were chromatographed on an Acquity UPLC equipped with a Waters BEH C8 (Milford Massachusetts, USA) / 100 mm × 2.1 mm, 1.6 μm column at 25 °C. The flow rate was set to 400 μL min^{-1} 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 min was used followed by a 1 min hold at 50% B. The mass spectrometer was a Waters TQ-Su triple quadrupole (Milford Massachusetts, USA) set to run in negative electrospray ionization mode. The transitions for analytes and Internal Standard were tuned by infusion of 10 μg mL^{-1} solutions of the pure materials in 1:1 acetonitrile/water at 5 μL min^{-1} into a mobile phase of 50% B at 400 μL 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 (10 pg 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.

2.8. Larval skeleton anomalies and gene expression

Fixed 30 DPH pikeperch larvae (20 per replicate) 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 mineralization were evaluated considering the total number of completely mineralised vertebral bodies within a larval size class (standard length).

Total RNA from larvae samples (10 larvae per replicate, average weight per sample 60 mg) was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total body tissue was homogenized using the TissueLyzer-II (Qiagen) with QIAzol lysis reagent (Qiagen). Samples were centrifuged with chloroform for phase separation (12,000 g, 15 min, 4 °C). 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 30 μL 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, Hercules, CA, USA) according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad).

Table 2
Sequences of primers used for gene expression analysis.

Gene	Nucleotide Sequence
Alp	F: 5′-GCTGTCCGATCCCAGTGTAA-3′
	R: 5′-CCAGTCTCTGTCCACACTGT-3′
Twist2	F: 5′-CCCCTGTGGATAGTCTGGTG-3′
	R: 5′-GACTGAGTCCGTTGCCTCTC-3′
Mef2c	F: 5′-GCGAAAGTTTGGCCTGATGA-3′
	R: 5′-TCAGAGTTGGTCCTGCTCTC-3′
Sox9	F: 5′-TCCCCACAACATGTCACCTA-3′
	R:5′-AGGTGGAGTACAGGCTGGAG-3′

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 *HAE*III as a standard. Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad) using β-actin as the house-keeping gene (*i.e.* others were tried, but β-actin was chosen due to its stability) in a final volume of 20 µL per reaction well, and 100 ng of total RNA reverse transcribed to complementary cDNA. Each gene sample was analysed once per gene. The PCR conditions were the following: 95 °C for 3 min 30 s followed by 40 cycles of 95 °C for 15 s, 61 °C for 30 s, and 72 °C for 30 s; 95 °C for 1 min, and a final denaturing step from 61 °C to 95 °C for 10 s. 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). Detailed information on primer sequences and accession numbers is presented in Table 2.

2.9. Statistics

A two-way ANOVA in Sigma Plot Ver. 13 was used to compare larval wet weight, FA composition, incorporation of radioactivity into total larval lipids, enzymatic activity, degree of deformities, gene expression, stress confinement mortality and eicosanoid activity using diet and salinity as fixed factors. 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. Prior to analysis normality and homogeneity of data were confirmed within treatment groups. A Tukey HSD test was used to determine significance of mean differences ($P < .05$) 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 ($P < .05$) 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.

3. Results

3.1. 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 ($P > .32$) was found. Two types of cannibalism (1, 2); were observed in all tanks starting from 17 DPH until the end of the study; (1) prey partly ingested; (2) whole prey swallowed. Final survival at 30 DPH (data not shown) was not significantly different ($P = .652$) among experimental groups and ranged between 9 and 12%. An apparent trend for a decrement of

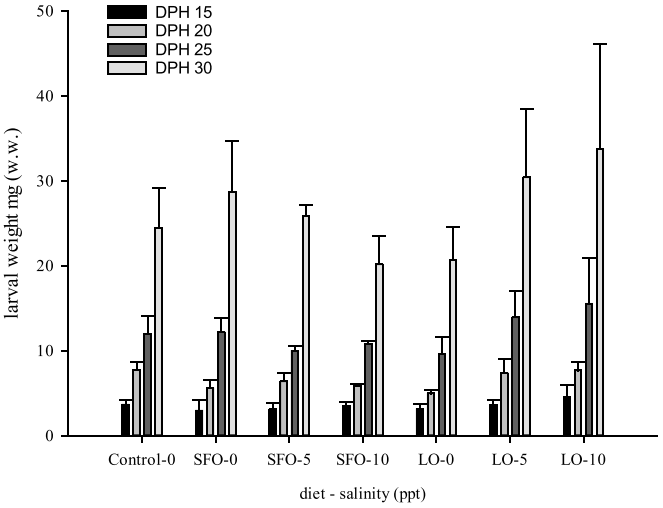

Fig. 1. Weight of larvae (mg w.w. ± SD) for each experimental treatment (diet; Control, SFO, LO; salinity; 0, 5, 10 ppt) at 4 sampling points (15 to 30 days post hatch, DPH).

SFO30 DPH larval growth with increasing salinity and the opposite trend applying for LO diet, however dietary or salinity regime 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 to 33 mg in a wet weight basis. Overall, growth from 15 to 30 DPH calculated as SGR was not significantly different between groups ($P = .592$) 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 experimental dietary SFO 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).

3.2. FA composition of Artemia and larvae during development

FA content of *Artemia* reflected the dietary composition of the emulsions (Table 3). LA in SFO enriched *Artemia* was about twice the

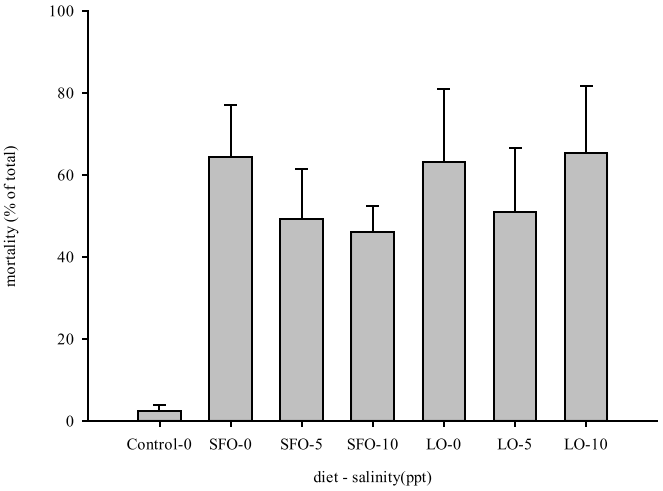

Fig. 2. Larval mortality (%) after 5 min confinement and light exposure (500 lux) for each experimental treatment (diet; Control, SFO, LO; salinity; 0, 5, 10 ppt) at 30 days post hatch.

Table 3
Total fatty acid content (TFA, μg g dm^{-1}) and main fatty acid content (% TFA) of *Artemia* enriched with DHA Selco control; sunflower oil (SFO) emulsion or linseed oil (LO) emulsion (mean ± 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 ± 0.2a	6.0 ± 0.2b	6.1 ± 0.1ab
Total SFA	18.9 ± 1.5	17.7 ± 0.7	17.5 ± 0.7
16:1 (n-7)	2.4 ± 0.1b	1.2 ± 0.1a	1.3 ± 0.1a
18:1 (n-9) OA	25.4 ± 0.1	25.3 ± 0.1	25.9 ± 0.5
Total MUFA	31.7 ± 1.4	27.7 ± 0.3	28.4 ± 0.8
18:2 (n-6) LA	9.5 ± 0.1b	18.3 ± 0.0c	8.1 ± 0.2a
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 ± 1.0b	19.5 ± 0.4c	9.3 ± 0.5a
18:3 (n-3) ALA	24.6 ± 1.0a	32.0 ± 0.8b	41.5 ± 0.6c
20:3 (n-3)	1.3 ± 0.1	1.4 ± 0.0	1.6 ± 0.1
20:5 (n-3) EPA	5.5 ± 0.1b	1.7 ± 0.2a	1.6 ± 0.2a
22:6 (n-3) DHA	6.5 ± 0.1b	0.0 ± 0.0a	0.0 ± 0.0a
Total (n-3) PUFA	37.9 ± 1.4a	35.1 ± 1.0a	44.8 ± 0.9b
DHA/EPA	1.2 ± 0.0b	0.0 ± 0.0a	0.0 ± 0.0a
(n-3)/(n-6)	3.3 ± 0.2b	1.8 ± 0.3a	4.8 ± 0.4c

Values in a row followed by a different superscript are significantly different ($P < .05$).

content in LO enriched *Artemia* ($P < .001$). Despite ALA is a major fatty acid in unenriched *Artemia,* its content was significantly higher in *Artemia* enriched by use of LO emulsion ($P < .001$), constituting 42% of the total fatty acids (% TFA) *versus* 32% in SFO enriched *Artemia* and 25% in DHA Selco enriched *Artemia*. Consequently, total n-3 content was the highest in LO *Artemia* and the n-3/n-6 ratio, 2.7 times higher in LO *Artemia* than in SFO *Artemia.* EPA and DHA content was 5.5% and 6.5% (% TFA) in the DHA Selco enriched *Artemia,* respectively, while EPA content in SFO and LO *Artemia* was some 1.7% ($P < .001$). 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 differences in *Artemia* composition (Table 4).

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 4) tissue content of LA was significantly higher in SFO fed larvae than in LO larvae ($P < .001$). ALA tissue content was similar between the experimental groups apart from larvae from the LO-10 ppt salinity group, where its content was significantly higher ($P < .001$) than larvae in any other groups except for those from the LO-5 ppt salinity. In addition, DHA content in LO-10 ppt larvae was significantly lower ($P < .001$) 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 ($P \geq .156$). Results on

Table 4
Total fatty acid content (TFA, μg g dm^{-1}) and main fatty acid composition (% TFA) of larvae at 15, 25 and 30 days post hatch (DPH) fed *Artemia* enriched with sunflower oil (SFO) or linseed oil (LO) at 3 different salinities (mean ± SD, n = 3). DHA Selco control, 0 ppt.

Diet	DHA Selco	SFO			LO		
Salinity (ppt)	0 ($n = 1$)	0	5	10	0	5	10
		15 DPH					
TFA (μg g dm^{-1})	101.0	92.8 ± 12.8	106.0 ± 18.8	98.2 ± 6.2	110.4 ± 29.6	103.5 ± 8.1	114.5 ± 18.1
Total SFA	23.6	23.0 ± 1.2	23.1 ± 1.3	23.8 ± 1.1	23.4 ± 1.0	23.2 ± 0.4	23.8 ± 0.9
Total MUFAs	26.0	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	8.5	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}
20:4 (n-6) ARA	1.6	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	11.5	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	13.6	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}
20:5 (n-3) EPA	5.4	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	19.1	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	41.5	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	3.5	6.9 ± 0.7^{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)	4.2	3.0 ± 1.5	2.9 ± 1.5	2.8 ± 1.1	5.0 ± 0.5	4.9 ± 0.9	4.4 ± 0.9
		25 DPH					
TFA (μg g dm^{-1})	58.6	64.5 ± 45.7	40.7 ± 13.7	59.9 ± 14.6	64.6 ± 20.8	101.9 ± 43.2	81.2 ± 33.1
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
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}
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
20:5 (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
		30 DPH					
TFA (μg g dm^{-1})	45.1	51.7 ± 20.7	33.6 ± 5.4	49.0 ± 13.0	46.1 ± 12.6	36.3 ± 7.7	35.4 ± 9.0
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
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}
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}
20:5 (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^{ab}	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 ($P < .05$) between experimental diets.

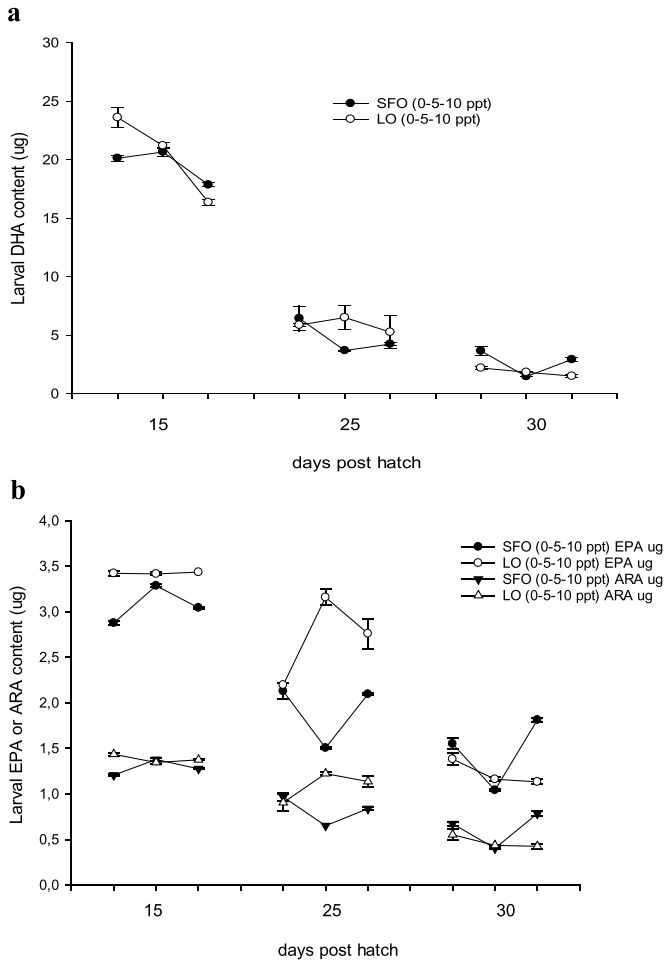

Fig. 3. a-b. Total larval content (μg) of DHA (Fig. 3a), EPA and ARA (Fig. 3b) for each experimental treatment (SFO, LO; salinity; 0, 5, 10 ppt) at 15, 25 and 30 days post hatch.

LA content between larvae from the SFO and LO groups were similar to those from 15 DPH, thus significantly higher in SFO fed larvae than in LO larvae ($P < .001$). On the contrary, ALA content was not significantly different between groups ($P = .059$). DHA was 6–10% of TFA, which was only half the larval content at 15 DPH with no significantly differences between experimental dietary groups ($P = .184$). In contrast, the DHA level in the DHA Selco larval control group was 17% TFA. At the final sampling point at 30 DPH (Table 4), results revealed no interacting effect of salinity ($P \geq .457$); similarly to 25 DPH. Tissue LA content was similar to the previous sampling periods, and for all salinities assayed significantly higher ($P < .001$) in SFO-larvae than in groups reared on the LO diet. On the contrary, ALA content was significantly higher ($P < .001$) in all three salinity groups fed the LO diet than in those fed the SFO diet. DHA tissue content in larvae for both dietary experimental groups was some 4–7% TFA, and differed from the DHA Selco control larvae, for which DHA content was 16% of TFA. The absolute larval fatty acid content (ug) of DHA, EPA and ARA at 15, 25 and 30 DPH is illustrated (Fig. 3) for each experimental treatment. For all three FAs there was a gradual but steep decline over time illustrating the dietary deprivation.

3.3. Tissue incorporation of labelled ^{14}C fatty acids

Results of the *in vivo* incubation with $^{14}C18$ and $^{14}C20–22$ PUFAs substrates revealed no significant differences in larval incorporation of radioactivity into total lipids comparing the 2 experimental diets (SFO or LO), but a significant effect of rearing salinity (Table 5). Thus, larvae reared in freshwater (at 0 ppt) incorporated more ^{14}C 18:2n-6 in total lipids than larvae reared at 5 ppt salinity, but not different from larvae reared at 10 ppt. Similarly, for 20:4n-6 and for the n-3 LC-PUFA 22:6n-3, the highest incorporation was observed at 0 ppt, differing significantly from larvae reared at 5 and10 ppt. The average incorporation pattern of radioactivity into larval tissues independently of salinity and dietary rearing conditions was 20:5n-3 > 20:4n-6 > 18:2n-6 = 18:3n-3 = 22:6n-3 (Table 6).

3.4. Eicosanoids

Prostaglandin tissue levels PGE_2 and PGE_3 revealed no effects of dietary treatment, but a significant effect of salinity (Table 7). PGE_2 and PGE_3 content was significantly higher for larvae reared at 0 ppt (including the control group) than for larvae reared at 5 or 10 ppt. PGE_2 and PGE_3 levels were similar for larvae at 5 or 10 ppt ($P = .594$; $P = .393$, respectively).

3.5. Digestive enzymatic activity

At 30 DPH, 4 of the 7 analysed digestive enzymes displayed a significant difference, while no differences were observed in activity of lipase, Leu-ala or amylase. Pepsin activity was lower in larvae from treatment LO-10 ppt salinity whereas no pepsin activity was detected for larvae from the experimental groups SFO-5 ppt, LO-5 ppt and SFO-10 ppt (Table 8). Independently of dietary treatment, aminopeptidase (N) activity was highest for larvae reared at 10 ppt ($P < .01$), as so was trypsin activity ($P < .05$) in larvae fed the SFO diet, which was not affected by salinity. Data also revealed a significantly higher ($P < .05$) activity of alkaline phosphatase (AP) in larvae for treatment SFO-10 ppt. Regardless of diets, amylase activity tended to increase in larvae exposed to10 ppt salinity, although without statistical differences.

Table 5
Incorporation of radioactivity into total lipid (pmol mg prot^{-1} h^{-1}) of pikeperch larvae at 20 DPH fed *Artemia* enriched with sunflower oil (SFO) or linseed oil (LO) at 3 different salinities (ppt) and incubated with [1-14C]FA substrates. DHA Selco control, 0 ppt (Mean ± SD, n = 3).

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

NS, not significant; a, b. For substrates with a significant effect of salinity and no interaction, values without a common letter are significantly different ($P < .05$).

Table 6
Incorporation of radioactivity into total lipid (pmol mg prot^{-1} h^{-1}) of pikeperch larvae at 20 DPH independently of rearing conditions when incubated with [1-14C] FA substrates (Mean ± SD, n = 21).

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 followed by a different superscript are significantly different ($P < .05$).
Comparisons between means were performed by one-way ANOVA followed by Tukey's *post hoc* test.

3.6. Skeleton anomalies and gene expression

Of the 17 skeletal anomaly typologies observed, only 4 were significantly affected by the different treatments (Table 9). There was a general high incidence of cranial anomalies, particularly in maxillary bones (58–83% population), and a low incidence of vertebral anomalies (Table 9). The lowest ($P < .05$) 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. Particularly, pikeperch fed SFO diet and reared at 5 or 10 ppt and those fed LO diet and reared at 5 ppt showed the significantly highest incidence of maxillary bone anomalies (Table 9). Also the two-way ANOVA showed the effect of salinity on the results, while there was no significant effect from the diets (Table 9). No differences were found in degree of mineralization according to the size of the larvae (data not shown). Despite the lack of significant differences in the expression of *twist2* among the different treatments, pikeperch fed SFO diet and reared at 10 ppt and those fed LO diet and reared at 5 ppt showed approximately doubled expression in *twist2,* than fish reared under the other conditions. Furthermore, results from the two-way ANOVA regarding expression of *twist2* showed interaction between salinity and the diets (Table 10). No significant differences or specific tendencies were found in *alp* or *mef2c* expression.

4. Discussion

Pikeperch larvae were exposed to different salinities and diets low in LC-PUFA by enrichment of *Artemia* with emulsions rich in vegetable oils (VO). The main FA constituents of vegetable sunflower oil (SFO) and linseed oil (LO); 18:2n-6 and 18:3n-3 respectively, significantly affected larval tissue FA 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 LA and ALA along the ontogeny with an *in vivo* ^{14}C PUFA incubation assay. By using direct incubation with 0.2 μCi (0.3 μM) of ^{14}C PUFAs we demonstrated for the first time in pikeperch larvae that, regardless of the environmental salinity or dietary regime, larvae possess a marked capacity to incorporate 20:5n-3 and 20:4n-6 compared to the other PUFAs assayed (22:6n-3, 18:3n-3 and 18:2n-6). In fact, EPA was by far the most incorporated PUFA among the tested substrates followed by ARA, while LA, ALA and DHA were less and similarly incorporated. This could either indicate a physiological need for EPA and ARA or a higher affinity for incorporation and esterification of C20 LC-PUFA into tissue, which need further studies as DHA would normally be regarded as the limiting most essential FA in pikeperch and other fish larvae (Watanabe, 1993; Takeuchi, 1997; Lund et al., 2014). This dynamic of incorporation should be particularly taken into account during enrichment protocols, considering that DHA must compete for incorporation with EPA or even with 18:3n-3, which is naturally abundant in the *Artemia*. Additionally, and 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 fatty acid profiles of larvae at 15 DPH and fed the LO diet at 10 ppt salinity (LO-10). Thus, this group had relatively higher tissue levels of 18:3n-3 and lower levels of DHA, indicating that a combination of diets rich in n-3 C18 PUFA and 10 ppt salinity do not favour LC-PUFA incorporation, neither synthesis as previously described for *Chirostoma estor* (Fonseca- Madrigal et al., 2012). In addition, 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 with dietary levels at these sampling points, irrespective to salinity gradients.

Δ6 desaturase is the first rate limiting enzyme in LC-PUFA biosynthesis and according to bibliography, salinity may have variable effects on modulation and expression of Δ6 desaturase enzymatic activity and therefore fatty acid composition in different fish species. The bioconversion of linoleic acid to ARA and from linolenic acid 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 LA and ALA consistently resulted in increased Δ6 activity in several carnivorous anadromous/freshwater fishes as reviewed by Vagner and Santigosa (2011). In some vertebrates it has been generally accepted that LA and ALA may compete for Δ-6 desaturase substrate necessary for the biosynthesis of n-3 and n-6 LC PUFAs (Emery et al., 2013). Results presented here cannot totally rule out some effect of dietary regime (LA, ALA ratio), or environmental salinity on expression and *in vivo* activity of these enzymes. However, we observed no significant differences in larval tissue content of ARA, EPA and DHA (apart from the aforementioned DHA content for LO-10 at 15 DPH) throughout the successive sampling points along the ontogeny of pikeperch at DPH 15, 25 and 30. This was evident despite feeding either the LO diet rich in ALA or the SFO diet rich in LA which caused a gradual depletion of especially larval tissue DHA but also larval EPA and ARA, suggesting no

Table 7
Eicosanoid content (pg g ww^{-1}) of pikeperch larvae fed for 30 days with *Artemia* enriched with sunflower oil (SFO) or linseed oil (LO) at 3 different salinities (ppt), (Mean ± SD, n = 3). DHA Selco control, 0 ppt.

Diet	DHA Selco	SFO			LO					
Salinity	0 (n = 2)	0	5 (n = 2)	10	0	5 (n = 2)	10	Interaction	Diet	Salinity
								Diet *vs* Salinity	SFO *vs* LO	(‰)
PGE_2	5510 ± 298	5912 ± 1587^{b}	3313 ± 45^{a}	3661 ± 411^{a}	5754 ± 476^{b}	4917 ± 141^{a}	3810 ± 1247^{a}	NS	NS	$P \leq .032$
PGE_3	1018 ± 48	1212 ± 322^{b}	634 ± 84^{a}	678 ± 120^{a}	1128 ± 244^{b}	997 ± 114^{ab}	618 ± 229^{a}	NS	NS	$P \leq .028$

Values in a row followed by a different superscript are significantly different ($P < .05$).

Table 8
Specific enzymatic activity (mU mg protein^{-1}; U mg protein^{-1}) in pikeperch larvae fed for 30 days with *Artemia* enriched with sunflower oil (SFO) or linseed oil (LO) at 3 different salinities (Mean ± SD, $n = 3$).

Diet	SFO			LO					
Salinity	0	5	10	0	5	10	Interactin	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^{a}	36.3 ± 2.8^{a}	45.7 ± 3.6^{b}	40.8 ± 4.8^{ab}	37.7 ± 5.3^{a}	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

Values in a row followed by a different superscript are significantly different $P < .05$ between experimental diets.
Nd: not detected. DHA Selco control not analysed. * No ANOVA performed on pepsin as value below detection in some treatments.
[1] mU mg protein^{-1}.
[2] U mg protein^{-1}.

Table 9
Incidence of different bone anomalies found in pikeperch larvae fed for 30 days with *Artemia* enriched with sunflower oil (SFO) or linseed oil (LO) at 3 different salinities (Mean ± SD, $n = 3$). DHA Selco control, 0 ppt, $n = 1$.

Diet	Control	SFO			LO					
Salinity (‰)	0	0	5	10	0	5	10	Interaction	Diet	Salinity
								Diet *vs* salinity	SFO *vs* LO	(‰)
Anomaly Type (%)										
Severe	90	76.7 ± 12.6^{a}	98.3 ± 2.9^{b}	96.7 ± 5.8^{b}	81.5 ± 10.7^{ab}	86.7 ± 5.8^{b}	85.0 ± 15.0^{ab}	NS	NS	NS
Maxilary	35	58.3 ± 16.1^{ab}	83.3 ± 7.6^{b}	77.1 ± 4.9^{b}	43.7 ± 17.0^{a}	81.7 ± 7.6^{b}	58.3 ± 25.2^{ab}	NS	NS	0.011
Other cephalic anomalies	55	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	NS	0.036
Caudal v. scoliosis	0	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	NS	0.032

Values in a row followed by a different superscript are significantly different ($P < .05$) by means of a one-way ANOVA followed by Tukey's *post hoc* test.

- or limited (*i.e.* 15 DPH) - effects of diet or salinity on the conversion of C18 FA precursors into LC-PUFA.

Size of larvae and growth until 30 DPH was consistent to results obtained in previous experiments performed with the use of live feed (*Artemia*) throughout the rearing period (Lund et al., 2014), but relatively lower (*i.e.* SGR 11–16% day^{-1}) than in trials with microdiets introduced from15 DPH and reared at 20–21 °C (Hamza et al., 2008; Lund et al., 2018, unpublished data, *i.e.* SGR 21–24% day^{-1}). The enzymatic data provided no clear evidence as to the influence of salinity or diet. The observed low enzymatic pepsin activity at 30 DPH and a higher trypsin activity for all larval groups suggest a limited activity of a functional stomach indicating a relatively slow growth by use of *Artemia* feeding and consequently a delay in process of morphogenesis. The larval control group reared at 0 ppt salinity on DHA Selco enriched *Artemia* was similar in size to larvae fed the LO and SFO enriched larvae, suggesting that LC-PUFA supplementation, VO based experimental emulsions (n-3/n-6 ratio) or salinity levels up to 10 ppt has limited effects on growth at these ages.

Prior to the present experiment we tested the tolerance of first feeding pike perch larvae to several isosmotic salinity gradients and in none of them 10 ppt seemed to affect growth and survival, 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 (*P. fluviatilis*) juveniles of 1.5–2 g initial body weight (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 explain a relatively slow growth in this experiment was a combination of lower rearing temperature 17.5 °C *vs* 20–21 °C (Hamza et al., 2008) and the use of *Artemia* as feed. Pikeperch larvae are able to digest and utilize microdiets from about 12–15 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), *Artemia* will most likely 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

Table 10
Gene expression in pikeperch larvae reared for 30 days with *Artemia* enriched with sunflower oil (SFO) or linseed oil (LO) at 3 different salinities (Mean ± SD, $n = 3$). DHA Selco control, 0 ppt.

Diet	Control	SFO			LO					
Salinity	0	0	5	10	0	5	10	Interaction	Diet	Salinity (‰)
Alp	1 ± 0	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
Twist2	1 ± 0	0.239 ± 0.112	0.259 ± 0.147	0.553 ± 0.332	0.254 ± 0.108	0.521 ± 0.169	0.156 ± 0.077	0.023	NS	NS
Mef2c	1 ± 0	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
Sox9	1 ± 0	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

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 (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. In the present experiment, overall lipase activity was similar in larval groups and apparently not affected by the relative difference in an enrichment protocol based on triglycerides in the form of 18:2n-6 or 18:3n-3 as main FA constituents.

Though no effects on growth, larvae fed DHA Selco enriched *Artemia* had much elevated LC- PUFA, especially 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 and 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 fed on lower DHA to EPA ratios (Brinkmeyer and Holt, 1998). Similar dietary effects of DHA with larvae of other marine fishes have been reported (Izquierdo et al., 1989; Takeuchi et al., 1991). Although the aim of the present study was not to define the exact nutritional requirement of LC-PUFAs or DHA to EPA ratio, results point out to the essentially and critical role of dietary DHA supplementation in pikeperch larval nutrition.

ARA and EPA are precursors of eicosanoids namely the 2- 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 (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) 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). What seems to be evident from the present results is that the assayed increasing salinities reduced PGE2 and PGE3 production, which somehow might modify the osmoregulation homeostatic status of the larvae. However, it is to be examined if a similar functional response of salinity and osmoregulation exists in pike perch juveniles and the possible interactions between salinity, COX activity, levels of PGE prostaglandins, and stress response.

We observed a high incidence of skeletal anomalies affecting endochondral bones. In addition, an increase in salinity lead to a higher incidence of this type of skeletal anomalies in SFO larvae while a similar effect was observed for LO fed larvae at 5 ppt. Changes in environmental salinity may cause an osmoregulatory stress in fish (Deane and Woo Norman, 2009). Even euryhaline 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). These 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 maxillary or dentary bones anomalies, skeletal elements that develop from a cartilaginous precursor. In the present study, an increase in salinity lead to a higher incidence of bone anomalies in SFO larvae, particularly in maxillary bones, that related well with the interaction between factors (salinity and diet) in *twist2* expression (Table 10). On the one hand, chondrocyte ossification is regulated by *twist* genes that are expressed in mesenchymal cells in medaka (*Oryzias latipes*) 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). Expression of the gene Runx2 is necessary for osteoblast differentiation (Bialek et al., 2004). Insufficient *runx2* activity caused by overexpression of *twist2* gave rise to impaired skull formation (Kronenberg, 2004). In the present study, up-regulation of *twist2* was related to the increased incidence of skull anomalies in pikeperch reared at higher salinities (in the SFO diet), possibly due to the impairment of chondrocyte maturation as occurs in medaka. Despite not being significantly different, 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. *Sox9* expression in cooperation with *sox5* and *sox6*, is necessary for early chondrogenic differentiation and in humans, mutation of the *sox9b* gene leads to severe cranial defects.

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 (Izquierdo et al., 2013; Saleh et al., 2013a, 2013b). Therefore, the lack of significant differences in the LC-PUFA contents in 30 DPH pikeperch fed SFO or LO *Artemia* agree well with the lack of differences in *alp* expression. Moreover, the control fish fed DHA enriched *Artemia* showed higher DHA and EPA body contents coinciding with 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 those obtained in other freshwater or marine species where *mef2c* expression was not affected by changes in dietary protein or lipid sources (Liland et al., 2015; Benedito-Palos et al., 2016). On the contrary, *mef2c* may be up-regulated by dietary PL ratio leading to hypertrophy of myotubes that could be the origin of vertebral anomalies (Overturf et al., 2016).

5. Conclusions

The obvious incorporation of all the ^{14}C substrates indicates the suitability of the complementary methodology used to study *in vivo* lipid metabolism of pikeperch larvae as also shown in newly hatched cephalopods and other marine organism's larvae (Reis et al., 2014, 2017). The present results showed a highest incorporation of EPA and ARA into pikeperch lipids, probably indicating a physiological need of this species for these FAs during its first stages of life or a higher affinity for incorporation and esterification of C20 PUFA. Nonetheless, future studies regarding esterificaction and elongase/desaturase patterns towards incubated C14 PUFA, should be performed in order to further elucidate FA metabolism by pikeperch larvae. Rearing salinity conditions up to 10 ppt had no effects on growth or survival of larvae and no clear effects on digestive enzymatic activity, but affected endocrine hormonal prostaglandin production. Results indicate that lipid and FA metabolism could be modified in response to salinity changes and may involve 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. Our present research pointed out the high occurrence of anomalies in endochondral bones and the increased incidence at higher salinities. Finally, and although no exact nutritional requirement of LC-PUFA was defined, the results of this study suggest the essentiality and critical role of LC PUFA especially dietary DHA supplementation in pikeperch larval nutrition.

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Conflict of interest

“None”.

Ethics and animal welfare

The study has been conducted in accordance with national and international guidelines according to animal welfare ethics and approved. The ARRIVE guidelines for animal research studies have been followed.

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