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Dietary DHA and ARA level and ratio affect the occurrence of skeletal anomalies in pikeperch larvae (*Sander lucioperca*) through a regulation of immunity and stress related gene expression

Najlae El Kertaoui [a,*], Ivar Lund [b], Monica B. Betancor [c], Camille Carpentier [a], Daniel Montero [d], Patrick Kestemont [a]

[a] Research Unit in Environmental and Evolutionary Biology (URBE), Institute of Life, Earth & Environment (ILEE), University of Namur, Rue de Bruxelles, 61 - 5000 Namur, Belgium
[b] Technical University of Denmark, DTU Aqua, Section for Aquaculture, The North Sea Research Centre, P.O. Box 101, DK-9850 Hirtshals, Denmark
[c] Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, United Kingdom
[d] Instituto ECOAQUA, Universidad de Las Palmas de Gran Canaria, Grupo de Investigación en Acuicultura (GIA), Muelle de Taliarte s/n, 35200 Telde, Las Palmas, Canary Islands, Spain

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ABSTRACT

Several causative factors have been proposed for the occurrence of skeletal anomalies in fish larvae, among which we quote nutritional factors, such as LC-PUFAs. This study aimed to investigate the effect of different dietary DHA and ARA level and ratio on pikeperch (*Sander lucioperca*) larval development and performance, digestive capacity, fatty acids composition, skeleton anomalies and molecular markers of oxidative stress status (*sod, gpx*, and *cat*), stress response (*StAR*, *gr*, *pparα, hsl* and *pepck*), fatty acid synthesis (*fadsd6*, *elovl5*), eicosanoids synthesis (*pla2*, *cox2*, *lox5*, *pge2*, and *lta4h*), and bone development (*twist*, *mef2c*, *sox9*, and *alp*). Pikeperch larvae were fed six microdiets containing two different dietary levels of DHA (0.5% and 3.5%) combined with three levels of ARA (1.2%, 0.6%, and 0.3%). Dietary fatty acid changes did not affect growth performance but significantly influenced enzymatic activities. A significant increase in skeletal anomalies with DHA intake increment was recorded. *StAR*, *cox2*, *pla2* and *hsl* expression were significantly depressed in 2.5% DHA larvae. An opposite effect of dietary DHA elevation was recorded in *gpx* expression. Both DHA and ARA had a significant effect on *pparα*, *gr*, and *pge2* expressions. Although no significant interactions were found, *pge2*, *gr*, and *pparα* displayed a differential pattern of expression between the different treatments. A strong association was found for the larval tissue amount of ARA and DHA with eicosanoid metabolism, stress response and skeleton anomaly related genes. These results denoted the effects of dietary LC-PUFAs on immune/stress gene regulation and their potential implication in skeleton development.

1. Introduction

Pikeperch (*Sander lucioperca*) is recognized as one of the main freshwater species with a great potential for the expansion of the EU aquaculture industry mainly because the good flesh quality and the high market value (Alexi et al., 2018). The major bottlenecks for further expansion of pikeperch culture today include low larval survival and high incidence of skeletal anomalies (Kestemont et al., 2015). Pikeperch larvae are very stress sensitive to lack or low levels of n-3 dietary essential long chain polyunsaturated fatty acids (LC-PUFA, n-3) causing

Abbreviations: ANOVA, Analysis of variance; Alp, Alkaline phosphatase; ARA, Arachidonic; Cat, Catalase; Cox2, Cytochrome c oxidase subunit; DHA, Docosahexaenoic acid; Elovl5, Elongation of very long chain fatty acids protein 5; EPA, Eicosapentaenoic acid; Fadsd6, Fatty acid desaturase 2/acyl-coa 6-desaturase 6; GC, Glucocorticoids; Gpx, Glutathione peroxidase; Gr, Glucocorticoid receptor; Hsl, Hormone-sensitive lipase; LC-PUFA, Long chain polyunsaturated fatty acids; Lox5, Arachidonate 5-lipoxygenase; Lta4h, Leukotriene A (4) hydrolase; Mef2c, Myocyte enhancer factor 2C; Pepck, Phosphoenolpyruvate carboxykinase; Pge2, Prostaglandin E synthase 2; Pla, Phospholipases; Pparα, Peroxisome proliferator-activated receptor; Sod, Superoxide dismutase; Sox9, Transcription factor Sox9; StAR, Steroidogenic acute regulatory protein.

* Corresponding author.
E-mail address: najlaeelkertaoui@gmail.com (N. El Kertaoui).

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lower performance, higher mortality; deficiency syndroms and deformities (Lund and Steenfeldt, 2011; Lund et al., 2014). Thus, recent studies suggested requirements similar to those of marine carnivorous fish larvae for both phospholipids and LC-PUFAs (Hamza et al., 2015; Lund et al., 2019). Moreover, at a physiological level, oxidative risk is particularly high in the fast-growing larvae due to the high metabolic rate, oxygen consumption and water content in the larval tissues (Betancor et al., 2012). Fish have an endogenous antioxidant defense system with a wide range of antioxidant mechanisms to maintain an adequate oxidative balance (Filho et al., 1993). Among them, various antioxidant enzymes such as catalase (cat), superoxide dismutase (sod) and glutathione peroxidase (gpx) (Bell et al., 1987).

Glucocorticoids (GCs) are central steroid hormones on endocrine stress response modulation and whole-body homeostasis in vertebrates, well known to affect glucose metabolism, immune system, reproduction as well as bone metabolism regulation (Subramaniam et al., 1992; Sapolsky et al., 2000; Suarez-Bregua et al., 2018). Endogenous GC hormones regulate the expression of target genes through glucocorticoid receptor (*gr*) signaling within bone cells, and affecting skeletal development and metabolism (Suarez-Bregua et al., 2018). Also, *gr* is considered as an indicator of lipid nutrition effect on stress response in fish (Alves Martins et al., 2012). In trout, it has been shown that unsaturated fatty-acids inhibit glucocorticoid receptor-binding of hepatic cytosol (Lee and Struve, 1992). Previous studies report possible regulation by *gr* of the transcription of hormone-sensitive lipase (*hsl*) (Alves Martins et al., 2012; Le et al., 2005; Lampidonis et al., 2008). Furthermore, the gene expression of lipolytic enzymes such as *hsl* were regulated by dietary modifications (Turchini et al., 2003; Ma et al., 2013; Peng et al., 2014). In this respect, Alves Martins et al. (2012) suggested that fatty acids and their derivatives can-indirectly- modulate metabolic pathways related to energetic metabolism (*hsl* and phosphoenolpyruvate carboxykinase *pepck*).

LC-PUFAs are important ligands for nuclear receptors and transcription factors such as peroxisome proliferator-activated receptor (*ppar*) (Lin et al., 1999). Beside the regulation of the expression of genes that participate in fatty acid oxidation, transcription factor *pparα* have been reported to modulate genes involved in cholesterol uptake and transport (Xie et al., 2002) which is central in steroidogenesis. Previous studies have reported the implications of LC-PUFAs and their derivatives in steroidogenesis in sea-bream (*Sparus aurata*) (Ganga et al., 2006, 2011). Interactions between Pparα and steroidogenic acute regulatory protein mitochondrial (StAR) have been addressed in Atlantic salmon (*Salmo salar*) (Pavlikova et al., 2010).

On the other hand, the ratio among dietary fatty acids, such as eicosapentaenoic (EPA), docosahexaenoic (DHA) and arachidonic (ARA) acids constitutes a critical factor for broodstock and larval performance due to competitive interaction among them (Bell and Sargent, 2003; Izquierdo, 2005). Hence, regardless of the need to study the optimum absolute dietary values for LC-PUFAs in this species, optimum dietary ratios must be defined. In fact, LC-PUFAs (specially EPA and ARA) are precursors for highly bioactive eicosanoids. These PUFA-derived mediators (eicosanoids and resolvins), are recognized of high importance in signaling molecules playing roles in biological processes such as inflammation (Kremmyda et al., 2011). Eicosanoids are involved in a great variety of physiological functions and are produced in response to stressful situations. The major precursor of eicosanoids in fish is ARA, while eicosanoids formed from EPA are less biologically active than those formed from ARA (Tocher, 2003). Initially, eicosanoids production is catalyzed by phospholipases (pla), mainly cpla2. The free ARA can undergo several possible enzymatic pathways to create bioactive eicosanoids, among them cyclooxygenase – governed by cytochrome *c* oxidase subunit (coxs) such as cox2 that mediate the production of prostaglandins -including prostaglandin E synthase 2 (pge2); and lipoxygenase pathway which consists of arachidonate 5-lipoxygenase (lox5) enzymes as well as their products such as leukotrienes -including leukotriene A(4) hydrolase (lta4h) (Kremmyda et al., 2011; Hannah and Hafez, 2018). Furthermore, cox2 seems to play a key role in osteogenic differentiation (Kirkham and Cartmell, 2007).

Initially a multifactorial approach was used to investigate the effects of various dietary nutrients (fatty acids, vitamins and minerals). Results of this screening experiment showed a significant interaction between EPA + DHA and ARA in pikeperch larvae, especially on deformity occurrence, suggesting the importance of a balanced n-3 HUFA/n-6 HUFA ratio in this species (El Kertaoui et al., 2019). Based on this result, the present experiment was carried out in the facilities of DTU Aqua (Dannmark). The objective of the present study is to understand how dietary DHA/EPA/ARA ratios affect tissue fatty acid profiles and antioxidant and stress response capacity, as well as the relationship between the deformity occurrence and the stress status in pikeperch. In this sense, the present data evaluated -particularly- larval development and performance, digestive capacity, skeleton deformities and molecular markers of oxidative stress status including: Sod, Gpx, and Cat; stress response including: StAR, Gr, Pparα, Hsl and Pepck; fatty acid synthesis such as fatty acid desaturase 2/acyl-coa 6-desaturase 6 (Fadsd6) and elongation of very long chain fatty acids protein 5 (Elovl5); eicosanoids synthesis such as Pla2, Cox2, Lox5, Pge2 and Lta4h: status and bone development such as twist related protein (Twist), myocyte enhancer factor 2C (Mef2c), transcription factor Sox9 (Sox9) and alkaline phosphatase (Alp).

2. Materials and methods

2.1. Ethical standards

The Animal Welfare Committee of DTU Aqua ensured, that protocols and all fish handling procedures employed in the study complied with Danish and EU legislation (2010/63/EU) on animal experimentation. All experiments were performed at the Technical University of Denmark (DTU Aqua) facilities in Hirtshals, Denmark. Fish larvae were not exposed to any surgery and sampled larvae for analyses were kept to an absolute minimum and euthanized by an overdose of clove oil. The dietary nutrient profiles provided were within the range that could reasonably be expected to be encountered in vivo.

2.2. Larvae and rearing conditions

Newly hatched larvae were obtained from AQUPRI Innovation, Egtved, Denmark and transferred to DTU Aqua at North Sea Research Centre, Denmark, where the experiment was carried out. Larvae were distributed into conical tanks (0,7 m in height and a diameter of 0.3 m), and from 3 dph larvae were fed on unenriched *Artemia* nauplii (AF and EG strains) (INVE, Dendermond, Belgium) until they reached 14 dph, followed by a co-feeding period from 15 to 17 dph using *Artemia* nauplii and a mixture of the experimental diets. The experiment was carried out in a triplicate set-up with 3 tanks per diet. Pikeperch larvae (initial body weight 3.15 ± 1.08 mg) were randomly distributed into 18 experimental conical tanks (50 L) at a density of 1300 larvae per tank in a flow through system with adjustable light and temperature control. Oxygen concentration and temperature were monitored daily by a hand-held Oxyguard meter from Oxyguard, Birkerød, Denmark. During the experiment, oxygen saturation was kept at a mean saturation of 74.8 ± 3.0% for all tanks with no significantly difference between treatments ($P \geq 0.480$), and temperature was kept at 20.6 ± 0.7 °C. Larvae in each tank were fed with one of six experimental diets. Feed was administered by automatic feeders from 8 am to 6 pm. To ensure feed availability, daily feed supply was maintained at app. 15–20% of larval wet biomass per tank during the first week (particles of 200–400 μm/400–700 μm) and 10–15% per tank biomass (particles of 400–700 μm) during the rest of the experimental period approximately every 20–30 min. Daily, bottom of tanks were vacuum cleaned to remove feed waste. Photoperiod was kept at 12 h light: 12 h dark.

2.3. Experimental diets

Two different dietary levels of DHA were formulated: 0.5% (low) and 3.5% (high) combined with three levels of ARA 1.2%, 0.6% and 0.3% (Table 1). Therefore, six isonitrogenous and isolipidic diets were formulated and fabricated by SPAROS S.A. (Portugal) as cold extruded feed pellets of 200–400 μm and 400–700 μm. Experimental diets were formulated using a mix of oils as sources of EPA, DHA and ARA to reach the required fatty acid content and to equalize the lipid content in each diet. Moisture (A.O.A.C. 1995), crude protein (A.O.A.C. 1995) and crude lipid (Folch et al., 1957) contents of diets were analyzed. The proximate composition of the main nutrients is shown in Table 1. Feeds were tested according to DHA, ARA and DHA/ARA ratios respectively. (See Table 2.)

2.4. Samplings, husbandry variables and analyses

Final survival was calculated by individually counting all living larvae in each tank at the end of the experiment, and expressed as the percentage of the initial numbers of larvae. Representative samples of pikeperch larvae were sampled at 27, 32 and 40 dph for wet weight, and digestive enzymatic assays. Specific growth rate (SGR) was calculated according to the formula (SGR = (ln w.w. f − ln w.w. i × 100)/t, 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 at 32 and 40 dph. Additional 50 larvae per tank were also taken at the end of the experimental period for skeleton morphogenesis and mineralization by staining. These larvae were sedated by an overdosis of clove oil, fixed and stored in 10% phosphate buffered formaldehyde until analysis. Finally, for the molecular study 10 larvae per replicate were similarly sedated and stored in RNA later overnight at 4 °C and then frozen at −80 °C until analysis.

Table 1
Formulation and the proximate composition (%) of the experimental diets.

	0.6% DHA			2.5% DHA		
	1.2% ARA	0.6% ARA	0.3% ARA	1.2% ARA	0.6% ARA	0.3% ARA
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Ingredients	5.00	5.00	5.00	5.00	5.00	5.00
MicroNorse	5.00	5.00	5.00	5.00	5.00	5.00
CPSP 90	5.00	5.00	5.00	5.00	5.00	5.00
Squid meal 80 ETOX	5.00	5.00	5.00	5.00	5.00	5.00
Krill meal (Low fat)	50.00	50.00	50.00	50.00	50.00	50.00
Fish gelatin	1.20	1.20	1.20	1.20	1.20	1.20
Wheat gluten	10.00	10.00	10.00	10.00	10.00	10.00
Potato starch gelatinised (Pregeflo)	9.50	9.50	9.50	9.55	9.55	9.55
Algatrium DHA70	0.00	0.00	0.00	2.85	2.85	2.85
VEVODAR	3.20	1.55	0.75	3.20	1.55	0.75
Krill oil	1.50	1.50	1.50	0.00	0.00	0.00
Vit & Min Premix PV01	1.00	1.00	1.00	1.00	1.00	1.00
Soy lecithin - Powder	6.20	6.20	6.20	4.80	4.80	4.80
Antioxidant powder (Paramega)	0.40	0.40	0.40	0.40	0.40	0.40
MAP (Monoammonium phosphate)	2.00	2.00	2.00	2.00	2.00	2.00
Proximate composition(%)						
Crude protein, % feed	54.2	54.2	54.2	54.2	54.2	54.2
Crude fat, % feed	20.2	20.2	20.2	20.2	20.2	20.2
Starch, % feed	9.7	9.7	9.7	9.7	9.7	9.7
Ash, % feed	9.0	9.0	9.0	9.0	9.0	9.0
Total P, % feed	1.67	1.67	1.67	1.62	1.62	1.62
Ca, % feed	1.52	1.52	1.52	1.52	1.52	1.52
Ca/P	0.91	0.91	0.91	0.93	0.93	0.93
LNA (C18:2n-6), % feed	0.53	0.40	0.33	0.50	0.37	0.30
ALA (C18:3n-3), % feed	0.13	0.13	0.13	0.10	0.10	0.10
ARA, % feed	1.20	0.59	0.30	1.19	0.59	0.30
EPA, % feed	1.19	1.19	1.19	1.22	1.22	1.22
DHA, % feed	0.61	0.61	0.61	2.49	2.49	2.49
EPA/ARA	0.99	2.00	3.95	1.02	2.07	4.12
DHA/EPA	0.52	0.52	0.52	2.04	2.05	2.05
Total phospholipids, % feed	7.76	7.76	7.76	6.22	6.22	6.22

Table 2
Main fatty acid content (% TFA) of feeds.

Diet	0.6%DHA			2.5%DHA		
	1.2% ARA	0.6% ARA	0.3% ARA	1.2% ARA	0.6% ARA	0.3% ARA
Σ Saturated	71.35	71.78	71.27	65.32	66.27	66.68
Σ Monoenes	7.41	9.20	10.57	6.33	9.83	9.69
Σ n-3	10.25	10.10	10.40	16.55	15.92	15.81
Σ n-6	11.19	8.35	7.28	11.28	7.47	7.32
Σ n-3 LC-PUFA	9.65	9.50	9.79	16.04	15.40	15.30
Σ n-6 LC-PUFA	4.94	2.49	1.35	5.61	2.16	2.08
18:1 n-9	4.94	7.44	8.83	4.68	8.17	8.03
18:2 n-6	6.00	5.71	5.84	5.40	5.18	5.13
18:3n-6	0.25	0.14	0.09	0.27	0.12	0.12
18:3 n-3	0.6	0.59	0.62	0.51	0.51	0.51
ARA	4.72	2.37	1.27	5.37	2.06	1.97
EPA	7.46	7.37	7.57	8.43	8.16	8.07
DHA	2.10	2.05	2.16	7.51	7.17	7.18
EPA/ARA	1.58	3.11	5.94	1.57	3.97	4.09
DHA/EPA	0.28	0.28	0.28	0.89	0.88	0.89
DHA/ARA	0.44	0.87	0.69	1.40	3.49	3.64
n-3/n-6	0.92	1.21	1.43	1.47	2.13	2.16
n-3 LC-PUFA/n-6 LC-PUFA	1.95	3.81	7.26	2.86	7.12	7.37

2.5. Fatty acid analysis

FA analysis of feeds and larvae was done according to previously described method (Lund et al., 2014). 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 (Denmark A/S) was added. A fixed amount of each feed (2-3 mg) was weighed and for larval samples (10 larvae per tank) were weighed and homogenized by a Tissue Tearor probe diameter 4.5 mm, Biospec Products, Inc.; Bartlesville, USA. 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, reactitherm 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 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-Aldrich (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 $sample^{-1}$.

2.6. RNA extraction and reverse-transcriptase quantitative PCR

Samples were homogenized in 1 ml of TriReagent® (Sigma-Aldrich, Danmark A/S) RNA extraction buffer using a bead tissue disruptor (Bio Spec, Bartlesville, Oklahoma, USA). Total RNA was isolated following manufacturer's instructions and quantity and quality determined by

spectrophotometry using a Nanodrop ND-1000 (Labtech Int., East Sussex, UK), and electrophoresis using 200 ng of total RNA in a 1% agarose gel. cDNA was synthesized using 2 μg of total RNA and random primers in 20 μl reactions and the high capacity reverse transcription kit without RNase inhibitor according to the manufacturer's protocol (Applied Biosystems, Warrington, UK). Gene expression was determined by qPCR of candidate genes: *pparα*, *fadsd6*, *elovl5*, *pepck*, *hsl*, *gr*, *StAR*, *pge2*, *pla2*, *lta4h*, *cox2*, *5-lox*, *gpx*, *sod*, *cat*, *twist*, *mef2c*, *sox9*, *alp,* and intestinal fatty-acid binding protein (*i-fabp*), Elongation factor-1α (*elflα*) and β-actin (*β actin*) were used as reference genes. The cDNA was diluted 20-fold with milliQ water. The efficiency of the primers for each gene was previously evaluated by serial dilutions of cDNA pooled from the samples to guarantee it was >90% for all primer pairs. qPCR was performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate 20 μl reaction volumes containing 10 μl of Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific, Hemel Hempstead, UK), 1 μl of the primer corresponding to the analyzed gene (10 pmol), 3 μl of molecular biology grade water and 5 μl of cDNA (1/20 diluted). In addition, amplifications were carried out with a systematic negative control (NTC, no template control) containing no cDNA. Standard amplification parameters contained an UDG pretreatment at 50 °C for 2 min, an initial denaturation step at 95 °C for 10 min, followed by 35 cycles: 15 s at 95 °C, 30 s at the annealing Tm and 30 s at 72 °C. Primer sequences for genes are given in Table 3. Data obtained were normalized and the Livak method (2–ΔΔCt) used to determine relative mRNA expression levels. Sequence alignment was done and conserved domains obtained were used to design primers with Primer3 (v. 0.4.0) program and subsequent sequencing of PCR products and BLAST of them. Sequences of genes encoding for *pparα*, *fadsd6*, *elovl5*, *pepck*, *hsl*, *gr*, *i-fabp*, *StAR*, *pge2*, *pla2*, *lta4h*, *cox2*, *5-lox*, *gpx*, *sod* and *cat* were obtained by identifying the sequences from Sequence Read Archives (SRA) SRX1328344 and SRX1385650. The set of contiguous sequences were assembled using CAP3 (Huang and Madan, 1999) and identity of the deduced aa sequences confirmed using the BLASTp sequence analysis service of the NCBI. Sequences for *alp*, *twist22*, *mef2c* and *sox9* were available for the species of interest (Lund et al., 2018, 2019). Pikeperch specific gene primers were designed after searching the NCBI nucleotide database and using Primer3. Detailed information on primer sequences is presented in Table 3.

Table 3
Sequences of primers used for gene expression analysis.

Genes	Sens	Primer sequence (5′to 3′)	Efficiency
5-lox	Forward	CAACACCAAGGCCAGAGAAC	0.89
	Reverse	AACTCTTGGTAGCCTCCCAC	
pla2	Forward	TGTGCTGTGGTTTGATCTGC	0.84
	Reverse	CACCTTCATGACCCCTGACT	
elovel5	Forward	CGAAGTATGTATGGCCGCAG	0.83
	Reverse	ATGCCCTGTGGTGGTACTAC	
cat	Forward	TACACTGAGGAGGGCAACTG	0.85
	Reverse	CTCCAGAAGTCCCACACCAT	
cox-2	Forward	GGAACATAACCGGGTGTGTG	0.88
	Reverse	ATGCGGTTCTGGTACTGGAA	
pge2	Forward	CTCGCGCACAATGTAGTCAA	0.84
	Reverse	CTGTGAACGAACGTGGGAAG	
gr	Forward	GTCCTTCAGTCTCGGTTGGA	0.85
	Reverse	TCTTCAGGCCTTCTTTCGGT	
lta4h	Forward	ATCCAGATGTTTGCGTACGG	0.88
	Reverse	GCGTCGTGTCGTACTGATTT	
gpx	Forward	ACACCCAGATGAACGAGCTT	0.93
	Reverse	TCCACTTTCTCCAGGAGCTG	
hsl	Forward	CAGTTCAGTCCAGGCATTCG	0.84
	Reverse	TTCTGCCCCTCTCAACTCTG	
pepck	Forward	CGAACACATGCTGATCCTGG	0.89
	Reverse	CGGGAGCAACACCAAAGAAA	
ppar	Forward	GCCCCAGTCAGAGAAGCTAA	0.87
	Reverse	TTTGCCACAAGTGTCTGCTC	
fadsd6	Forward	GGTCATTTGAAGGGAGCGTC	0.90
	Reverse	TGTTGGTGGTGATAGGGCAT	
sod	Forward	TGTGCTAACCAGGATCCACT	0.87
	Reverse	TCGCTCACATTCTCCCAGTT	
StAR	Forward	CTGGAGACTGTAGCCGCTAA	0.95
	Reverse	TGACGTTAGGGTTCCACTCC	
i-fabp	Forward	ATGTCAAGGAGAGCAGCAGT	0.89
	Reverse	TGCGTCCACACCTTCATAGT	
sox9	Forward	TCCCCACAACATGTCACCTA	0.95
	Reverse	AGGTGGAGTACAGGCTGGAG	
mef2c	Forward	GCGAAAGTTTGGCCTGATGA	0.91
	Reverse	TCAGAGTTGGTCCTGCTCTC	
alp	Forward	GCTGTCCGATCCCAGTGTAA	0.99
	Reverse	CCAGTCTCTGTCCACACTGT	
twist2	Forward	CCCCTGTGGATAGTCTGGTG	0.85
	Reverse	GACTGAGTCCGTTGCCTCTC	
elflα	Forward	TGATGACACCAACAGCCACT	0.81
	Reverse	AAGATTGACCGTCGTTCTGG	
b-actin	Forward	CGACATCCGTAAGGACCTGT	0.93
	Reverse	GCTGGAAGGTGGACAGAGAG	

2.7. Skeleton anomalies

To determine the presence of skeletal anomalies, 50 larvae per tank were fixed and stored in buffered (10% phosphate) formalin at the end of the experiment. Staining procedures with alizarin red and alcian blue were conducted to evaluate skeletal anomalies following a modified method from previous studies (Izquierdo et al., 2013). Classification of skeletal anomalies was conducted according to Boglione et al. (2001). Anomalies were expressed as frequency of total severe anomalies and specific anomalies, such as jaw deformities, scoliosis, lordosis, cleithrum and branchiostegal rays within each dietary group (Fig. 1).

2.8. Digestive enzyme activities

The head and tail of 10 pikeperch larvae were dissected on a glass maintained on ice to isolate the digestive segment, and the stomach region was separated from the intestinal segments. Pooled samples from each tank were homogenized in 10 volumes (*v/w*) cold distilled water. Alkaline phosphatase (AP) and aminopeptidase (N), two enzymes of 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 (2001). Trypsin activity was assayed according to Holm et al. (1988), such as described by Gisbert et al. (2009). Protein was determined using the Bradford (1976) procedure. Enzyme activities are expressed as specific activities (U or mU mg protein^{-1}).

2.9. Statistics

Data are expressed as the mean ± standard error (SEM). Kolmogorov and Smirnov's test was used to assess the normality of data sets ($p < 0.05$) and Bartlett's test was conducted to evaluate variance homogeneity ($p < 0.05$). Two-way ANOVA was used to compare the different endpoints using DHA and ARA dietary levels as fixed factors. The statistical analyses were performed using the JMP 12.1 software (SAS Institute Inc., North Carolina, USA). A Tukey HSD test was used to determine significance of mean differences ($P < 0.05$) between the treatment groups where applicable. If no interaction between factors (DHA and ARA dietary levels) 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 according to the DHA/ARA ratio effect. Data with no normality and/or homogeneity of variances were tested with Kruskall-Wallis tests and post-hoc pair-wise Wilcoxon comparison test. The relationship between the expression of the target genes and larval fatty acid profiles were performed using the R software; first association between paired samples was checked using one of Pearson's product moment correlation coefficient, the correlation matrix was generated using corrplot package and the significance levels (*p*-values) was generated using lattice package. Then, multivariate principal

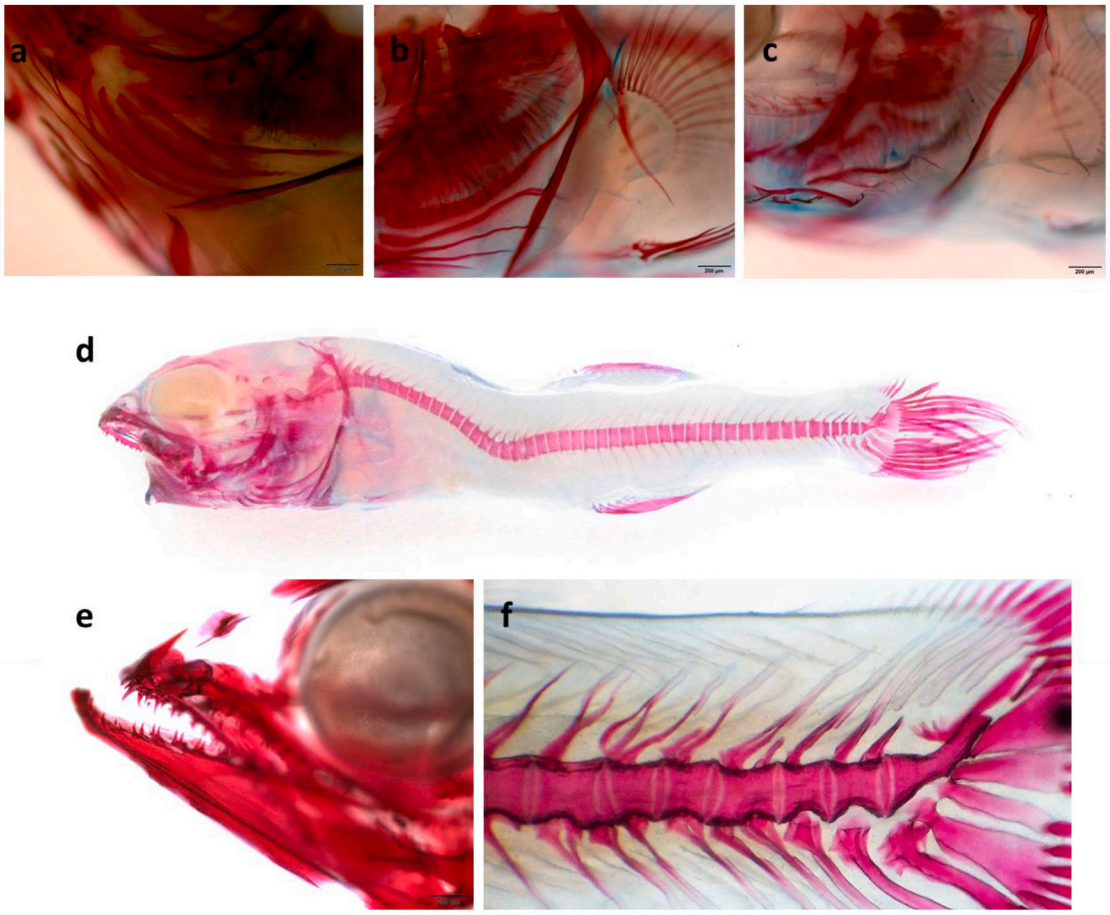


Fig. 1. Examples of some skeletal anomalies observed in 40 dph pikeperch *Sander lucioperca* larvae. (a) Larvae showing normal branchiostegal rays morphology. (b) Larvae showing a slightly deformed branchiostegal rays. (d) Twisted and fused branchiostegal rays. (d) Larvae showing a severe lordosis and cranium anomaly with marked lower jaw reduction. (e) Lower jaw increment. (f) Larvae showing vertebral body compression and fusion with neural spinal anomalies.

component analysis (PCA) combined with co-inertia analysis (CIA) were applied to the cross-platform comparison of gene-expression and fatty acid content datasets. Component scores were further clustered according to RVAideMemoire package. PCA and co-inertia analyses were performed with ADE-4 package. All statistical computations were considered significant when resulting p-values were: < 0.05.

3. Result

3.1. Growth and survival

The growth was similar in the different groups of larvae with no significant differences of individual body wet weight at 27, 32 and 40 dph (Table 4). Meanwhile, at the end of the experiment at dph 40, juveniles fed D4 exhibited a lower growth performance compared to the larvae fed D2. Specific Growth Rate (SGR) from 17 to 40 dph ranged between 12.45 ± 0.67 and 13.32 ± 0.33 d^{-1}, and was not significantly different between treatments.

Overall survival at 40 dph was similar with a tendency for a better survival for D4. The apparent mortality (dead larvae siphoned and counted) and the total mortality (including lost larvae due to type II cannibalism) showed no significant differences between groups.

3.2. Larval fatty acid composition and gene expression

Fatty acid compositions of 32 dph and 40 dph pikeperch larvae are presented in Tables 5 and 6. Higher levels of DHA, 18: 3n-3, total n-3 LC-PUFA and total n-3 larval contents were found in 40 dph larvae fed diets 4, 5 and 6 (p: 0.0005, 0.0375, 0.0153 and 0.0219 respectively) as a consequence of higher dietary DHA levels, while no significant

Table 4
Effects of dietary treatments on specific growth rate, individual weight, apparent mortality rate, cannibalism and survival rate. Data are presented as mean ± SEM (n = 3).

Diet	0.6% DHA			2.5% DHA			Two way ANOVA		
	1.2% ARA	0.6% ARA	0.3% ARA	1.2% ARA	0.6% ARA	0.3% ARA	DHA	ARA	DHA*ARA
SGR (% day^{-1})	13.05 ± 0.35	13.32 ± 0.33	13.02 ± 0.45	12.45 ± 0.67	12.99 ± 0.75	12.82 ± 0.55	ns	ns	ns
Apparent mortality (%)	25.62 ± 1.63	27.10 ± 9.40	31.28 ± 5.19	35.03 ± 8.88	34.31 ± 3.91	31.85 ± 7.86	ns	ns	ns
Survival (%)	22.69 ± 4.46	21.13 ± 3.07	22.46 ± 3.09	26.64 ± 4.10	21.15 ± 5.87	19.54 ± 6.02	ns	ns	ns
Cannibalism (%)	51.69 ± 6.09	51.77 ± 7.84	46.26 ± 5.81	38.30 ± 7.52	44.54 ± 2.58	48.62 ± 2.09	ns	ns	ns
Weight at 27dph (mg)	17.40 ± 0.83	14.41 ± 0.86	16.23 ± 1.60	16.08 ± 1.58	15.38 ± 1.38	16.68 ± 3.14	ns	ns	ns
Weight at 32 dph (mg)	23.10 ± 1.77	21.64 ± 2.21	24.74 ± 3.12	22.42 ± 0.99	23.47 ± 1.14	22.25 ± 4.49	ns	ns	ns
Weight at 40 dph (mg)	63.46 ± 5.09	67.48 ± 5.07	63.13 ± 6.65	55.59 ± 8.23	63.16 ± 10.37	60.41 ± 7.63	ns	ns	ns

Table 5
Main fatty acid content of larvae (% TFA) at 32dph.

Diet	0.6%DHA			2.5%DHA			Two way ANOVA		
	1.2%ARA	0.6%ARA	0.3%ARA	1.2%ARA	0.6%ARA	0.3%ARA	DHA	ARA	DHA*ARA
Σ Saturated	32.08 ± 6.51	34.29 ± 5.33	31.11 ± 1.80	33.67 ± 8.26	33.00 ± 4.40	33.61 ± 4.58	ns	ns	ns
Σ Monoenes	21.10 ± 3.16b	23.98 ± 6.17ab	25.66 ± 1.07a	18.72 ± 3.50b	24.17 ± 3.52ab	25.54 ± 1.60a	ns	*	ns
Σ n-3	15.87 ± 7.28	13.44 ± 4.64	19.48 ± 3.06	17.79 ± 8.99	18.30 ± 6.21	21.77 ± 5.40	ns	ns	ns
Σ n-6	30.60 ± 3.70a	27.93 ± 6.73ab	23.43 ± 0.31b	29.46 ± 2.83a	24.24 ± 1.61ab	18.79 ± 0.79b	ns	**	ns
Σ n-3 LC-PUFA	14.98 ± 7.20	12.62 ± 4.62	18.56 ± 3.10a	17.13 ± 8.92	17.54 ± 6.21	21.10 ± 5.36	ns	ns	ns
Σ n-6 LC-PUFA	15.87 ± 4.26a	13.72 ± 6.47ab	8.27 ± 0.02c	17.76 ± 2.89a	11.73 ± 1.61ab	8.44 ± 0.34c	ns	**	ns
18:1 n-9	16.47 ± 2.83b	19.35 ± 5.66ab	21.18 ± 1.12a	14.13 ± 3.30b	19.38 ± 3.55ab	20.89 ± 1.93a	ns	*	ns
18:2 n-6	14.20 ± 0.77a	13.75 ± 0.48ab	14.83 ± 0.31a	11.16 ± 0.05 cd	12.13 ± 0.62bc	10.13 ± 1.10d	***	ns	**
18:3n-6	0.53 ± 0.11a	0.47 ± 0.12a	0.33 ± 0.01c	0.54 ± 0.01a	0.38 ± 0.03ab	0.23 ± 0.01c	ns	**	ns
18:3 n-3	0.89 ± 0.08a	0.83 ± 0.11ab	0.92 ± 0.04a	0.66 ± 0.06b	0.76 ± 0.03ab	0.68 ± 0.03b	***	ns	ns
ARA	15.40 ± 4.19a	13.28 ± 6.37ab	7.97 ± 0.05c	17.28 ± 2.90a	11.41 ± 1.61ab	8.25 ± 0.36c	ns	**	ns
EPA	9.04 ± 2.94	7.71 ± 1.98	12.29 ± 0.36	7.71 ± 2.37	9.29 ± 1.68	9.94 ± 0.73	ns	*	ns
DHA	5.75 ± 4.25	4.74 ± 3.25	6.17 ± 3.43	9.25 ± 6.57	8.14 ± 4.62	11.07 ± 4.63	ns	ns	ns
EPA/ARA	0.61 ± 0.27b	0.62 ± 0.17b	1.54 ± 0.05a	0.44 ± 0.07b	0.81 ± 0.03b	1.21 ± 0.14a	ns	***	*
DHA/EPA	0.57 ± 0.33	0.61 ± 0.33	0.51 ± 0.29	1.07 ± 0.64	0.83 ± 0.40	1.09 ± 0.40	*	ns	ns
DHA/ARA	0.39 ± 0.34b	0.35 ± 0.14ab	0.77 ± 0.43a	0.50 ± 0.33b	0.68 ± 0.34ab	1.36 ± 0.61a	ns	*	ns
n-3/n-6	0.52 ± 0.25b	0.47 ± 0.05b	0.83 ± 0.14ab	0.59 ± 0.26ab	0.75 ± 0.23ab	1.15 ± 0.25a	*	**	ns
n-3 LC-PUFA/n-6 LC-PUFA	0.99 ± 0.60bc	0.95 ± 0.09c	2.24 ± 0.37ab	0.92 ± 0.39c	1.46 ± 0.37bc	2.52 ± 0.72a	ns	***	ns

Different superscript letters within a row denote significant differences among diets determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$).
* $p < 0.05$.
** $p < 0.01$.
** $p < 0.001$.

Table 6
Main fatty acid content of larvae (% TFA) at 40 dph.

Diet	0.6%DHA			2.5%DHA			Two way ANOVA		
	1.2%ARA	0.6%ARA	0.3%ARA	1.2%ARA	0.6%ARA	0.3%ARA	DHA	ARA	DHA*ARA
Σ Saturated	32.68 ± 0.39	34.94 ± 2.55	36.27 ± 2.92	37.03 ± 5.01	33.03 ± 10.20	33.18 ± 5.75	ns	ns	ns
Σ Monoenes	18.03 ± 1.29c	24.21 ± 1.26b	27.08 ± 0.71a	16.33 ± 0.50c	24.65 ± 2.04b	28.20 ± 1.96a	ns	***	ns
Σ n-3	11.97 ± 1.65	13.09 ± 1.93	13.76 ± 1.29	13.91 ± 1.67	16.25 ± 4.29	17.58 ± 2.27	*	ns	ns
Σ n-6	37.01 ± 0.23a	27.48 ± 1.31bc	22.62 ± 1.66c	32.44 ± 2.94ab	25.85 ± 4.53bc	20.81 ± 2.68c	ns	***	ns
Σ n-3 LC-PUFA	11.18 ± 1.60	12.28 ± 1.87	12.97 ± 1.23	13.29 ± 1.58	15.55 ± 4.16	16.84 ± 2.18	*	ns	ns
Σ n-6 LC-PUFA	22.04 ± 0.24a	12.79 ± 0.67b	7.70 ± 0.67c	21.23 ± 1.07a	13.02 ± 1.60b	7.08 ± 0.33c	ns	***	ns
18:1 n-9	13.96 ± 1.55b	20.00 ± 1.54a	22.69 ± 0.95a	12.75 ± 0.34b	20.58 ± 1.82a	19.69 ± 6.23a	ns	**	ns
18:2 n-6	14.36 ± 0.22	14.30 ± 1.07	14.60 ± 1.26	10.68 ± 1.85	12.47 ± 3.15	13.49 ± 2.73	ns	ns	ns
18:3n-6	0.61 ± 0.06a	0.40 ± 0.05bc	0.33 ± 0.01c	0.53 ± 0.08ab	0.35 ± 0.06 cd	0.25 ± 0.02d	*	***	ns
18:3 n-3	0.79 ± 0.05	0.80 ± 0.10	0.80 ± 0.06	0.62 ± 0.12	0.70 ± 0.13	0.74 ± 0.11	*	ns	ns
ARA	21.52 ± 0.30a	12.41 ± 0.64b	7.39 ± 0.66c	20.79 ± 1.03a	12.78 ± 1.54b	6.90 ± 0.5c	ns	***	ns
EPA	7.79 ± 0.41b	9.27 ± 0.67ab	10.09 ± 0.65a	7.81 ± 0.88b	8.79 ± 0.85ab	9.90 ± 0.86a	ns	***	ns
DHA	3.20 ± 1.45	2.89 ± 1.38	2.78 ± 0.65	5.31 ± 0.63	6.66 ± 3.27	6.87 ± 2.14	***	ns	ns
EPA/ARA	0.36 ± 0.02c	0.75 ± 0.03b	1.37 ± 0.08a	0.38 ± 0.02c	0.69 ± 0.02b	1.44 ± 0.14a	ns	***	ns
DHA/EPA	0.41 ± 0.19	0.31 ± 0.13	0.27 ± 0.05	0.68 ± 0.01	0.74 ± 0.29	0.70 ± 0.23	**	ns	ns
DHA/ARA	0.15 ± 0.07c	0.23 ± 0.10bc	0.37 ± 0.05b	0.25 ± 0.02bc	0.51 ± 0.18b	1.01 ± 0.37a	*	*	**
n-3/n-6	0.32 ± 0.04c	0.48 ± 0.06bc	0.61 ± 0.03b	0.43 ± 0.02c	0.62 ± 0.07b	0.85 ± 0.11a	***	***	ns
n-3 LC-PUFA/n-6 LC-PUFA	0.51 ± 0.08e	0.96 ± 0.09 cd	1.68 ± 0.05b	0.62 ± 0.04de	1.18 ± 0.17c	2.39 ± 0.42a	ns	***	*

Different superscript letters within a row denote significant differences among diets determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$).
* $p < 0.05$.
** $p < 0.01$.
** $p < 0.001$.

differences were detected at 32 dph except for 18: 3n-3 ($p = 0.0001$). The group of larvae fed a higher dietary ARA content showed an increase in their ARA body content and resulted also in a higher n-6 LC-PUFA and total n-6 at 32 dph as well as at 40 dph. In contrast, monounsaturated acid content was significantly higher in larvae fed 0.3% ARA ($p = 0.0442$, 0.0005 respectively at 32 dph and 40 dph) principally due to a higher percentage of oleic acid (18: 1n-9) in these larvae ($p = 0.0334$ and 0.0087 respectively at 32 dph and 40 dph). Similarly, a decrease in dietary ARA resulted also in graded increase in EPA/ARA ratio ($p = 0.0001$, 0.0005 at 32 dph and 40 dph respectively) and EPA larval content ($p = 0.047$, 0.0001 at 32 dph and 40 dph respectively) was negatively correlated with ARA larval content (Fig. 2; $R = 0.9708$ at 40dph). Significant interactions between DHA and ARA were found in DHA/ARA and n-3 LC-PUFA/ n-6 LC-PUFA ratios in 40 dph larvae ($p = 0.00978$ and 0.039 respectively), while the total content of saturated

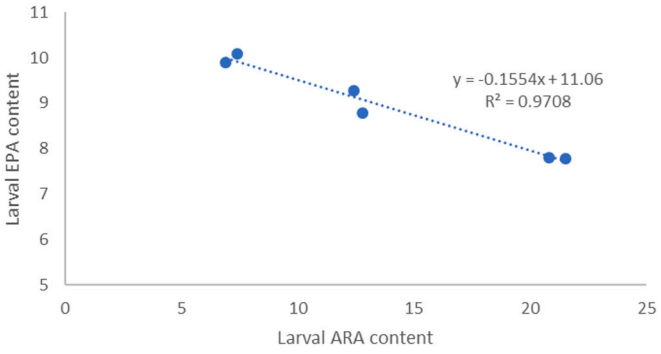


Fig. 2. Correlation between EPA and ARA levels (% total fatty acids) in the whole body of pikeperch larvae fed different experimental diets.

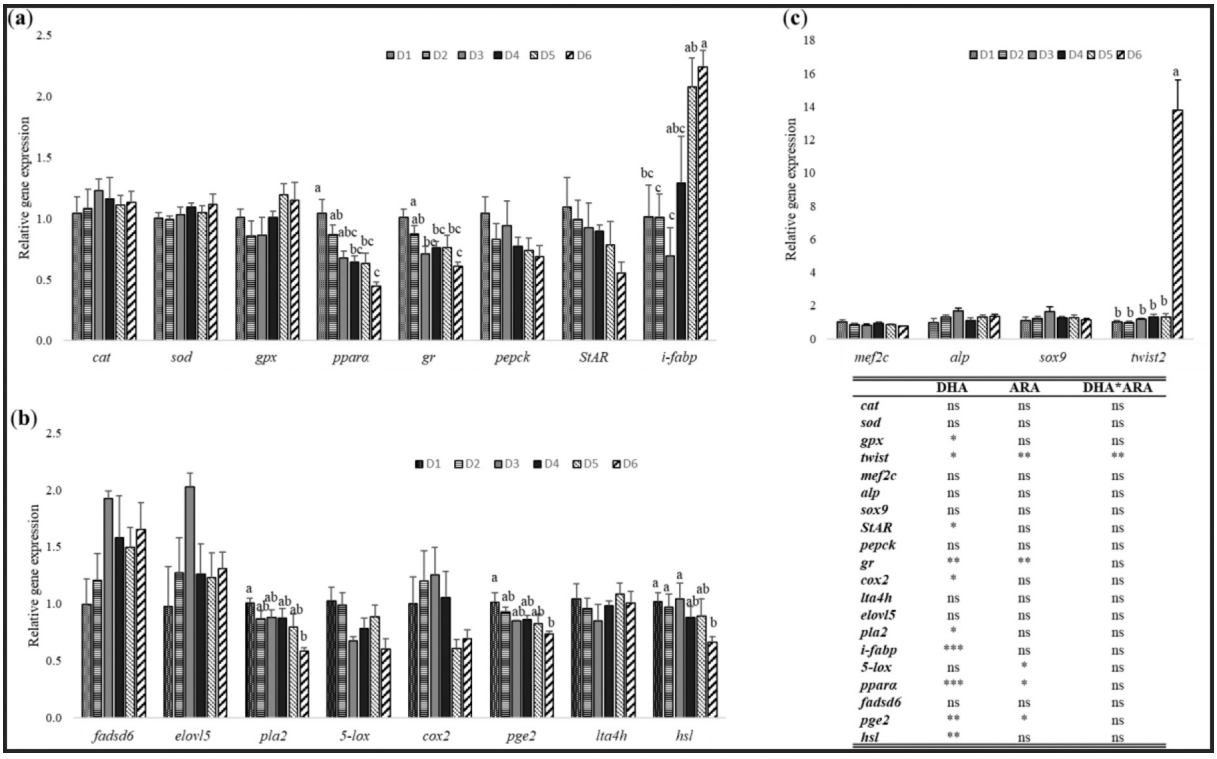
fatty acids (SFA) was similar among larvae fed the different experimental diets.

Among the 20 studied genes, 11 target genes showed significant differences in expression between the dietary treatments (Fig. 3). The transcription of *StAR, cox2*, *pla2* and *hsl* was significantly depressed in 2.5% DHA larvae ($p = 0.043$; 0.030, 0.018 and 0.0076 respectively) while an opposite significant effect of dietary DHA elevation was recorded in *gpx* and *i-fabp* expression ($p = 0.0218$ and 0.0002). Besides the DHA effect, the results of one-way ANOVA indicated, that larvae fed D6 differed significantly in *hsl* and *i-fabp* expression from D1, D2 and D3 treatments ($p = 0.0476$ and 0.0014 respectively). *I-fabp* expression was significantly upregulated in larvae fed diet D5 compared to D2 and D3 groups (Fig. 3a) ($p = 0.0014$), similarly *pla2* expression was higher in D6 than D1 treatment ($p = 0.0186$). Both DHA and ARA had a significant effect in *pparα*, *gr*, and *pge2* expressions. The transcription of these genes (*pparα*, *gr* and *pge2*) was significantly depressed with the dietary DHA increment ($p = 0.0004$; 0.0041; 0.003); a similar pattern of gene expression occurred in the lowest ARA-fed group compared to 1.2% ARA group ($p = 0.015$; 0.0011; 0.0251). Although no significant interactions were found, *gr* and *pparα* transcript levels were higher in D1-fed larvae compared with D4, D5 and D6 groups (Fig. 3a) (One-way ANOVA $p = 0.0083$; 0.0004), while D2 differed significantly from D6 treatment. Compared to D6, larvae fed D1, D2 and D4 displayed an increased transcript levels in *pge2* (Fig. 3b) ($p = 0.0135$). *Twist2* gene expression presented a significant interaction among DHA and ARA dietary content; pikeperch fed diet D6 showed the highest expression in *twist2* than larvae fed the other diets ($p = 0.0079$) (Fig. 3c). Furthermore, results from the two-way ANOVA regarding expression of *twist2* showed also a higher dietary effect of both DHA and ARA (p = 0.01 and 0.0043 respectively). Dietary ARA content had a clear effect on the expression of *5-lox* ($p = 0.0345$).

The large standard deviations in the expression of the rest of the genes studied (*fadsd6*, *elovl5*, *lta4h*, *cat*, *sod*, *sox9*, *mef2c*, *alp* and *pepck*) did not allow to find significant differences among the different treatments. However, larvae fed diet D3 showed approximately twice as high expression in lipid metabolism *elovl5* and *fadsd6* genes than D1 fed group (Fig. 3b). Likewise, *pepck* expression showed a tendency to up-regulation in larvae fed low DHA level (0.6%) ($p = 0.054$). A trend for an increased expression of *mef2c* gene with the dietary ARA elevation was observed ($p = 0.069$), while *alp* gene expression tended to decrease gradually with the dietary ARA supply (Fig. 3c; $p = 0.059$). No significant differences or specific tendencies were found in *cat*, *sod*, *sox9* and *lta4h* gene expressions.

3.3. Relationships between gene expression and larval fatty acid content

The relationships between the studied target genes and larval fatty acid profile explored through Pearson's correlation coefficient is illustrated in Fig. 4a. The strongest associations were found for the amount of ARA and DHA with eicosanoid metabolism, stress response and skeleton anomaly related genes ($p < 0.05$). Thus *mef2c*, *pparα, pla2*, *pge2* and *gr* were positively correlated with ARA, while negatively with the amounts of DHA. Significant correlation was found between *StAR*, *hsl* and *i-fabp* gene expressions and DHA. *Twist2* and *alp* showed a negative correlation with ARA level. Equally, 20:3n-3 and 18: 3n-6 displayed a similar correlation as ARA with *twist2* and *pparα*. The expression of specific antioxidant genes was significantly correlated with 18:3n-3 (*sod* and *gpx*) and 18:2n-6 (*gpx*). Those correlations were reinforced by principal component analysis (PCA) combined with co-inertia analysis and algorithm clustering results presented in Fig. 4b, which concomitantly illustrates the segregation of two clusters in both genes and fatty acid profile. Except 20:3n-3; n-3 LC-PUFA (EPA and DHA) and oleic acid were clustered together and separately than the other figured fatty acids. Furthermore, all eicosanoid metabolism genes were clustered with *mef2c* and stress response genes (*StAR*, *gr* and *pepck*) in the opposite direction of DHA level while positively linked to ARA level.



	DHA	ARA	DHA*ARA
cat	ns	ns	ns
sod	ns	ns	ns
gpx	*	ns	ns
twist	*	**	**
mef2c	ns	ns	ns
alp	ns	ns	ns
sox9	ns	ns	ns
StAR	*	ns	ns
pepck	ns	ns	ns
gr	**	**	ns
cox2	*	ns	ns
lta4h	ns	ns	ns
elovl5	ns	ns	ns
pla2	*	ns	ns
i-fabp	***	ns	ns
5-lox	ns	*	ns
pparα	***	*	ns
fadsd6	ns	ns	ns
pge2	**	*	ns
hsl	**	ns	ns

Fig. 3. Effects of dietary DHA and ARA on relative mRNA levels of genes involved in stress response (a), lipid metabolism pathways including LC-PUFA biosynthesis and eicosanoid metabolism (b), and skeleton anomaly related genes (c) in 40 dph pikeperch larvae as determined by qPCR. Results are normalized expression ratios (means±SEM; $n = 5$). Different superscript letters denote differences among treatments identified by one-way ANOVA. The inset table presents *p* values for the effect of DHA, ARA and their interaction on the relative gene expression.

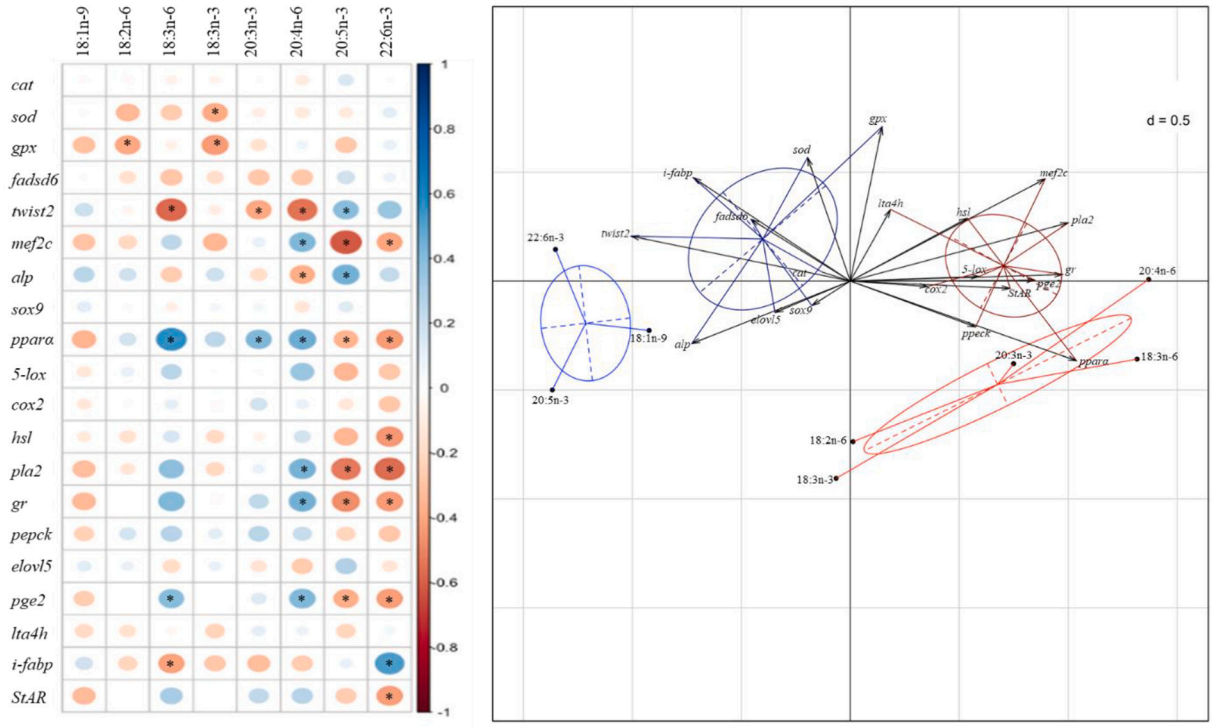


Fig. 4. Association between expression of target genes and selected larval fatty acid content. (a) Correlation matrix between gene expression and larval fatty acid content as presented by Pearson's product moment correlation coefficient. (b) Combined Principal component analysis (PCA) and co-inertia (CIA) of larval fatty acid data (%) and expression of target genes; the components scores were clustered according to RVAideMemoire package. Different color refers to the degree to which a pair of variables are linearly related as presented in the inset colored axis.

3.4. Skeleton anomaly evaluation

Overall, high incidence of lordosis and cephalic anomalies were observed in the present study (Table 7). Two-way ANOVA results indicated a significant increase in different skeletal anomaly typologies with dietary DHA intake increment, in particular anomalies of bone formed by direct ossification ($p = 0.012$). Higher incidence of opercular deformities was observed in larvae fed high DHA ($p = 0.043$), mainly governed by the higher branchiostegal ray anomaly observed in these larvae ($p = 0.001$). Similarly, the increase in DHA led to a higher incidence of dentary bone anomalies ($p = 0.001$) and pectoral element deformities ($p = 0.007$), in particular cleithrum anomaly ($p = 0.006$). Furthermore, despite the lack of significant interaction between DHA and ARA in the occurrence of deformities, one -way ANOVA results showed a significantly higher occurrence of maxillary bone and branchiostegal rays anomalies in D6 than in D2 fed-larvae ($p = 0.011$ and 0.008 respectively). No differences were found in the degree of mineralization according to the size of the larvae (data not shown).

3.5. Specific enzymatic activities

Pepsin activity was higher in the high DHA-fed groups (Table 8) ($p = 0.0127$), while no differences were observed at 32 and 40dph. Combined effect of DHA and ARA with significant interaction was found in trypsin activity at 27 and 40 dph ($p = 0.0003$ and 0.0017 respectively). Larvae fed diet 6 presented the highest trypsin activity at 27 dph ($p = 0.0003$);

Table 7
Occurrence of bone anomalies found at 40 dph in pikeperch fed the different experimental diets.

Diet	0.6% DHA			2.5% DHA			Two way ANOVA		
	1.2% ARA	0.6% ARA	0.3% ARA	1.2% ARA	0.6% ARA	0.3% ARA	DHA	ARA	DHA*ARA
Severe	64.63 ± 1.71	56.44 ± 7.39	67.56 ± 2.35	66.08 ± 6.06	74.22 ± 10.65	71.51 ± 4.76	ns	ns	ns
Lordosis	35.22 ± 5.97	37.23 ± 3.62	41.65 ± 7.30	38.23 ± 3.53	38.02 ± 11.32	25.83 ± 0.83	ns	ns	ns
Scoliosis	8.01 ± 5.03	5.80 ± 5.80	8.64 ± 2.93	7.21 ± 2.46	26.87 ± 14.25	6.07 ± 2.78	ns	ns	ns
Branchiostegal rays	5.32 ± 0.62b	7.80 ± 5.98b	3.96 ± 1.98b	10.33 ± 2.24ab	17.17 ± 2.64ab	24.51 ± 4.1a	**	ns	ns
Dentary	22.69 ± 1.85ab	12.28 ± 1.62b	16.56 ± 0.72ab	26.10 ± 5.42ab	25.93 ± 3.58ab	31.11 ± 3.09a	**	ns	ns
Maxillary	12.68 ± 2.45	9.86 ± 5.94	3.97 ± 2.31	14.56 ± 10.72	11.38 ± 7.49	11.94 ± 6.74	ns	ns	ns
Jaws	28.06 ± 3.27	20.81 ± 8.09	18.56 ± 1.84	27.38 ± 4.45	35.06 ± 7.53	39.85 ± 1.23	*	ns	ns
Cleithrum	5.43 ± 4.47	2.73 ± 1.37	1.32 ± 0.66	7.79 ± 2.22	10.07 ± 5.21	23.23 ± 7.97	**	ns	ns
Opercular	11.44 ± 5.50	10.70 ± 8.85	10.58 ± 5.43	14.18 ± 4.13	20.76 ± 2.76	28.15 ± 4.24	*	ns	ns
Pectoral elements	6.79 ± 5.82	3.37 ± 1.74	1.32 ± 0.66	8.44 ± 2.78	10.07 ± 5.21	23.23 ± 7.97	**	ns	ns
Direct ossification	11.42 ± 3.67	6.71 ± 1.62	5.29 ± 0.65	14.85 ± 4.41	18.60 ± 9.86	29.59 ± 8.79	*	ns	ns
Other cephalic anomalies	41.96 ± 2.74	31.66 ± 8.20	42.50 ± 8.52	36.367 ± 3.34	54.42 ± 13.91	48.14 ± 2.87	ns	ns	ns

Different superscript letters within a row denote significant differences among diets determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$).
* $p < 0.05$.
** $p < 0.01$.
** $p < 0.001$.

Table 8
Specific enzymatic activity (mU mg protein^{-1}) in pikeperch larvae fed different experimental diets.

Diet	0.6% DHA			2.5% DHA			Two way ANOVA		
	1.2% ARA	0.6% ARA	0.3% ARA	1.2% ARA	0.6% ARA	0.3% ARA	DHA	ARA	DHA*ARA
Specific enzymatic activity at 27 dph									
Trypsin	13.63 ± 1.1b	11.13 ± 1.4b	7.73 ± 1.6b	7.67 ± 2.9b	14.07 ± 2.4b	27.46 ± 1.9a	**	*	***
Pepsin	4.50 ± 1.6b	3.08 ± 0.8b	4.30 ± 0.9b	5.07 ± 1.5a	5.80 ± 1.1a	14.57 ± 3.0a	*	ns	ns
Aminopeptidase	5.03 ± 0.9	7.00 ± 0.8	8.44 ± 1.5	7.51 ± 2.6	2.64 ± 0.5	8.86 ± 0.5	ns	ns	ns
Alkaline phosphatase	60.52 ± 3.5	43.72 ± 7.6	60.79 ± 5.5	51.55 ± 3.2	52.94 ± 5.4	72.21 ± 16.6	ns	ns	ns
Specific enzymatic activity at 32 dph									
Trypsin	12.33 ± 1.0	10.67 ± 1.2	15.76 ± 2.2	11.62 ± 1.4	11.81 ± 1.5	9.62 ± 1.1	ns	ns	ns
Pepsin	164.57 ± 31.0	248.17 ± 52.4	242.55 ± 80.4	176.28 ± 68.7	242.10 ± 37.6	214.08 ± 59.2	ns	ns	ns
Aminopeptidase	5.63 ± 1.5	7.07 ± 1.2	7.42 ± 2.2	7.44 ± 2.6	9.03 ± 2.0	7.84 ± 2.5	ns	ns	ns
Alkaline phosphatase	21.90 ± 4.1	30.74 ± 7.6	36.25 ± 12.1	34.14 ± 12.7	39.52 ± 10.5	31.65 ± 5.9	ns	ns	ns
Specific enzymatic activity at 40 dph									
Trypsin	10.88 ± 0.4bcd	20.0 ± 2.0ab	26.03 ± 1.4a	8.43 ± 1.6 cd	14.74 ± 4.0bc	3.25 ± 1.2d	***	*	**
Pepsin	141.18 ± 22.6	123.93 ± 14.3	141.94 ± 22.4	145.25 ± 22.4	133.42 ± 9.3	136.03 ± 6.9	ns	ns	ns
Amonipeptidase	15.20 ± 3.0a	8.75 ± 0.5b	12.04 ± 0.4a	12.66 ± 0.9a	9.02 ± 0.6b	12.94 ± 0.5a	ns	**	ns
Alkaline phosphatase	56.52 ± 0.5a	39.32 ± 2.6b	45.85 ± 1.3b	66.39 ± 6.7a	40.71 ± 3.1b	50.29 ± 2.3b	ns	***	ns

Different superscript letters within a row denote significant differences among diets determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$).
* $p < 0.05$.
** $p < 0.01$.
** $p < 0.001$.

on the opposite, this treatment resulted in the lowest trypsin activity at 40dph ($p = 0.0017$). Brush border enzymes (alkaline phosphatase and aminopeptidase) displayed significant differences among the different dietary ARA levels ($p = 0.0005$ and 0.001 respectively) at 40 dph. On the other hand, no differences of alkaline phosphatase and aminopeptidase activities were recorded between treatments at 27 and 32 dph.

4. Discussion

To the best of our knowledge, there are no report so far on the expression level of genes associated with eicosanoid synthesis, lipid metabolism and stress response during early development of pikeperch larvae. The present study represents the first investigation on how dietary LC-PUFAs (DHA and ARA) affect immune/stress gene regulation and their putative implication in skeleton development.

Although no significant growth differences were observed among the different treatments, molecular biomarkers, biochemical and osteological endpoints investigated in the present study highlight the influence of both DHA and ARA and their interaction on pikeperch larval development. The increased dietary DHA up to 2.5%, led to the increment in incidence of skeletal deformities. This result is somewhat contradictory with the results of a recent study (Lund et al., 2018) in which a clear tendency towards decreasing prevalence of severe skeletal deformities was observed in pikeperch fed increased dietary levels of DHA. In fact, the positive effect of dietary DHA elevation recorded by Lund et al. (2018) on pikeperch skeletal anomalies was probably attributed to dietary phospholipid elevation applied in this experimental design, since the increased dietary PL reduced the prevalence of skeletal anomalies (Lund et al., 2018; Cahu et al., 2003; Boglione et al., 2013; Saleh et al., 2013). Thus, besides the fatty acid profile, lipid structure seems to be another important nutritional factor influencing the skeletal development in pikeperch larvae. In this respect Villeneuve et al. (2005) associated the increased skeletal anomaly occurrence with n-3 LC-PUFA (EPA and DHA) elevation in the neutral lipid fraction. Negative effects of excessive DHA intake on the occurrence of skeleton anomalies- especially dentary and maxillary deformities- were also reported in gilthead seabream *Sparus aurata* (Izquierdo et al., 2013). Same authors associated the increased oxidative stress with the endochondral bone anomalies. Consistently with this hypothesis, together with the increased oxidative status of pikeperch larvae – as presented by the higher expression of *gpx*- in the present study, the skull, especially the cranial structures such as dentary and operculum complex including the branchiostegal rays remind the most affected, when high DHA induced anomalies were detected. However, *sod* and *cat* expression showed no significant differences in transcription levels among the different groups. Jin et al. (2017) suggested no oxidative stress effects on antioxidant defense capability through Sod activation in juvenile black seabream (*Acanthopagrus schlegelii*) fed high DHA/EPA ratio. Interestingly, antioxidant enzyme mRNA expression levels increased concomitantly with the decrease of larval C18 fatty acid content, especially α-linolenic acid (ALA; 18:3n-3) which correlated negatively with *sod* and *gpx* expression levels and linoleic acid (LA; 18:2n-6) negatively correlated with *gpx* expression levels. High dietary LA also negatively impacted nonspecific immunity and antioxidant capacity in juvenile large yellow croakers (*Larimichthys crocea*) (Zuo et al., 2015). Previous studies demonstrated that ALA tended to be more prone to β-oxidation or excretion rather than to elongation into EPA and DHA (Fu and Sinclair, 2000). In spite of the different dietary and larval fatty acid contents (including DHA, EPA, ARA and their precursors ALA and LA), the expression of genes involved in desaturation (*fadsd6*) and elongation (*elovl5*) were not influenced. Indeed, the present results likely reflected an adaptation as a result of a negative feedback, especially in fish fed higher DHA level (diets: D4, D5, D6) permitting to maintain LC-PUFA and their metabolites within the required physiological levels.

The differences in larval fatty acid profiles were not limited to ARA, and DHA. The results of gene expression may reflect the combined actions of other fatty acids (EPA, oleic acid, LA and ALA). Accordingly, differential pattern of gene expression was recorded depending on the fatty acid larval content. *Alp* expression showed a negative correlation with ARA content. Alp is recognized as a biomarker of osteoblast differentiation and direct formation of bone via the intra-membranous ossification pathway (Hessle et al., 2002). However, a significant increase in anomalies of bone formed by direct ossification was observed with dietary DHA increment. Increase in DHA in lower ARA-fed group (diet D6) resulted in higher branchiostegal rays and dentary deformities. These fish presented the highest expression of *twist2*, a gene involved in osteoblast inhibition, but also displayed an antioxidant activity being involved in the control of reactive oxygen species (ROS) (Floc'h et al., 2013). Recent results showed a differential effect of dietary ARA on skeletal deformities depending on the EPA + DHA levels (El Kertaoui et al., 2019) pointing out the need of a balanced dietary n-3/n-6 ratio in this species. This is well known that prostaglandins are potent regulators of bone formation and bone resorption (Meghji et al., 1988; Raisz, 1995). Thus, an imbalance of n-3/n-6 -especially EPA/ARA ratio- may result in the prostaglandin imbalance and consequently, affects the production of PGs which can lead to an imbalance of bone formation and

resorption (Boglino et al., 2014), in particular, the PGE2 concentrations known to influence both bone formation and resorption (Berge et al., 2009). In Senegalese sole (*Solea senegalensis*) increased PGE2 production induced by dietary ARA supplementation resulted in the reduction in bone ossification (Boglino et al., 2013). ARA is the major precursor of eicosanoids in fish cells and usually considered as the major substrate for eicosanoid synthesis (Bell et al., 1994; Furuita et al., 2007). Thus, increased amounts of ARA led to an increased amount of substrate available for synthesis of ARA-derived eicosanoids. In this sense, our finding showed a clear response to ARA intake with the expression of eicosanoid metabolism related genes. On the other hand, as expected, the larval body fatty acid composition reflected dietary fatty acid profiles, especially DHA and ARA, which increased in the higher DHA and higher ARA fed groups respectively. This explains the positive correlation ($p < 0.05$) found between the larval ARA content and the expression of the eicosanoid metabolism genes in particular *pge2* and *pla2*. *5-lox* expression was mainly governed by ARA level. The present results are in agreement with those found in gilthead sea-bream, where changes in the expression of these genes were associated with ARA intake (Alves Martins et al., 2012). Meanwhile, despite the similar EPA concentrations among the experimental diets, EPA larval content was reduced significantly with larval ARA increment, indicating a preferential EPA metabolism, especially with the increase in dietary ARA. The strong negative correlation between the two fatty acids in the tissues was reported in other studies, suggesting the competition between these latter for inclusion in the tissues (Alves Martins et al., 2012; Izquierdo, 2005; Sargent et al., 1999; Van Anholt et al., 2004). The major mechanism of action for n-3 LC-PUFAs (EPA and DHA) is thought to block the formation of pro-inflammatory mediators via substrate competition with ARA for enzymes that generate several inflammatory mediators (Lands, 1987; Massaro et al., 2008; Sears and Ricordi, 2012). Furthermore, the EPA: ARA ratio is considered as a major determinant of eicosanoid production. Nonetheless, genes related to eicosanoid production showed the higher expression in low DHA fed fish, in particular *pla2*, *cox2* and *pge2*. DHA has been suggested to affect eicosanoid production (Nablone et al., 1990). Long chain n-3 PUFAs such as DHA and EPA exert also an anti-inflammatory action by inhibiting production of ARA-derived eicosanoids (Huang et al., 2018). In concordance with this finding, we hypothesize that the production of ARA-derived eicosanoids was decreased due to DHA elevation in this species.

An imbalance in eicosanoid profiles due to dietary LC-PUFAs supplementation can affect various metabolic pathways, including the corticosteroid production and thus the stress response/tolerance in fish (Van Anholt et al., 2004; Wales, 1988; Bessonart et al., 1999; Koven et al., 2003). PCA performed in the present study clustered the genes involved in stress response together with eicosanoid metabolism and *mef2c* transcript level whose were positively correlated with ARA larval content, whereas all oxidative stress and the skeleton anomaly related genes other than *mef2c*, were clustered together and positively associated to DHA larval content. The potential of ARA in the modulation of genes involved in stress response has been studied in gilthead sea-bream and Senegalese sole larvae (Alves Martins et al., 2012, 2013). Our results seemed to support this fnding; in this respect, the *pla2* up regulation in the present study reflected the ARA abundance (Hughes-Fulford et al., 2005; Yoshida et al., 2007) since phospholipase A2 is mostly responsible for catalyzing the release of ARA from phospholipids in cell membranes (Burke and Dennis, 2009). In addition, in vitro results have already proved the marked participation of COX and LOX metabolites on cortisol release mechanism in fish (Ganga et al., 2006, 2011). Effects of dietary ARA on cortisol response have been clearly demonstrated in Senegalese sole post-larvae, accompanied by an up regulation of *gr* by ARA dietary supplement (Alves Martins et al., 2011, 2013). Equally, our finding indicated that the *gr* gene responded positively to ARA supply. In fact, cortisol is the main endogenous GC hormones that regulates the expression of target genes through Gr located in the cytoplasm, signaling within cells including bone cells (Suarez-Bregua et al., 2018). The skeleton is one of the target organs of the stress hormones and physiological levels of GCs are vital for normal skeletogenesis (Suarez-Bregua et al., 2018; Zhou et al., 2013). Considering the down regulation of *gr* and the higher anomaly occurrence in pikeperch fed high fed DHA diets, the endogenous glucocorticoids action on bone metabolism might have also been responsible for the above-mentioned differences observed in skeletal anomalies in these larvae. In addition, previous study on pikeperch larvae reported a positive effect of high levels of DHA supplementation on stress tolerance, while no such effects were observed by high levels of dietary ARA (Lund et al., 2012, 2014). The present molecular results confirm those previous findings on DHA effect on stress sensitivity in pikeperch. Since the lower transcript levels of genes involved in stress response such as *StAR*, *gr*, *pla2* and *hsl* likely reflect an adaptation to increasing dietary amounts of LC-PUFA (Alves Martins et al., 2012). DHA is involved in processes that increase stress tolerance through the regulation of *StAR*, a key rate-limiting enzyme in steroidogenesis. Indeed, DHA acts as an inhibitor of the oxoeicosanoid receptor (OXE-R) in steroidogenic cells, reducing StAR protein levels and steroidogenesis (Cooke et al., 2013). Hormone-sensitive lipase are important enzymes involved in lipolysis, that reported to be enhanced under stress conditions (Ma et al., 2013; Nielsen and Møller, 2014). This latter (hsl) catalyzes the hydrolysis of cholesteryl esters and plays an essential role in the regulation of Dibutyryl cyclic AMP (Bt2cAMP) - induced steroidogenic acute regulatory protein (*StAR*) expression, hence, steroid biosynthesis (Manna et al., 2013).

Besides, the relative expression of the above-mentioned stress response genes (*pla2* and *gr*) as well as *pge2* were highly correlated with *pparα* expression, likely pointing out a common mechanism of dietary regulation in this case. Another mechanism by which LC-PUFAs and eicosanoids could be acting to regulate gene transcription is through ppar's pathway (Kersten et al., 2000), considering that PUFAs and their metabolites, in particular leukotriene B4 (LTB4), have been shown to activate pparα, being one of the main endogenous ligands (Lin et al., 1999; Choi and Bothwell, 2012). This provides an alternative explanation for the marked similarities observed in the expression pattern of these genes. *Pparα* has been suggested to regulate *gr* transcription, as one of the potent transcription factors adapting the expression of several genes involved in stress response and eicosanoid metabolism (Dichtl et al., 1999; Jia and Turek, 2005), and therefore we hypothesize that *pge2* and *pla2* were likely modulated by this transcription factor.

The expression of *i-fabp* gene has been considered as an indicator for assessing nutrient supply and represents a useful marker for intestinal development functional and the digestive system function in fish larvae diets (Pierce et al., 2000; Andre et al., 2000; Yamamoto et al., 2007; Overland et al., 2009; Venold et al., 2013; Lin et al., 2017), due to its crucial role in intracellular fatty acid trafficking and metabolism in fish gut (Her et al., 2004). Thus, the resulted higher expression levels of *i-fabp* gene in high DHA fish group may indicate the enhancement of fatty acid transfer rate and absorption (Baier et al., 1996; Levy et al., 2001; Storch and Thumser, 2010). On the other hand, a recent study in pikeperch larvae highlighted the potential involvement of ARA but not n-3 LC-PUFAs in the development of the digestive tract (El Kertaoui et al., 2019). Within the duration of the present study, the intestinal brush border digestive capacity was not significantly affected by DHA dietary content, but was significantly increased in fish fed intermediate ARA level (0.6%) at 40 dph. Such effect has been observed in tongue sole (*Cynoglossus semilaevis*) larvae (Yuan et al., 2015). The morphoanatomical development and maturation of the gut is known to be accompanied by an increase in activity of the brush border enzymes from the enterocytes (Zambonino-Infante and Cahu, 2007; Lazo et al., 2010). Concurrently, larval ARA content was positively correlated with transcript level of myocyte enhancer factor 2c (*mef2c*), this latter regulates the final step of chondrocyte maturation- chondrocyte hypertrophy. And as a chondrogenic marker gene, *mef2c* has been used to characterize the maturation process in fish (Ytteborg et al., 2010). Taking together the present finding and the above referred studies, we suggest ARA-sensitive

effect on the maturation process in pikeperch larvae.

In summary, considering the different endpoints investigated in the present study, our results suggest an antagonistic effect of ARA and DHA fatty acids on immune/stress response of pikeperch, and its influence on bone development and deformity occurrence.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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N.E.K. was in charge of the design of this study, she performed several analyses (digestive enzyme analyses, skeleton anomalies and gene expression) and wrote the main manuscript text. I.L. performed the experiment and was in charge of fatty acid analysis and contributed to the manuscript revision. M. B. assisted with gene expression analysis and she critically reviewed the manuscript. C.C. assisted on the statistical analysis. P.K. and D.M. were involved in the design of the study and in the final revision of the manuscript.

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