

# 1 Dietary DHA and ARA level and ratio affect the occurrence of skeletal anomalies in pikeperch 2 larvae (*Sander lucioperca*) through a regulation of immunity and stress related gene expression

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## 16 17 18 **Abstract**

19  
20 Several causative factors have been proposed for the occurrence of skeletal anomalies in fish larvae,  
21 among which we quote nutritional factors, such as LC-PUFAs. This study aimed to investigate the  
22 effect of different dietary DHA and ARA level and ratio on pikeperch (*Sander lucioperca*) larval  
23 development and performance, digestive capacity, fatty acids composition, skeleton anomalies and  
24 molecular markers of oxidative stress status (*sod*, *gpx*, and *cat*), stress response (*StAR*, *gr*, *ppara*, *hsl*  
25 and *pepck*), fatty acid synthesis (*fadsd6*, *elovl5*), eicosanoids synthesis (*pla2*, *cox2*, *lox5*, *pge2*, and  
26 *lta4h*), and bone development (*twist*, *mef2c*, *sox9*, and *alp*). Pikeperch larvae were fed six microdiets  
27 containing two different dietary levels of DHA (0.5 % and 3.5 %) combined with three levels of ARA  
28 (1.2 %, 0.6 %, and 0.3 %). Dietary fatty acid changes did not affect growth performance but  
29 significantly influenced enzymatic activities. A significant increase in skeletal anomalies with DHA  
30 intake increment was recorded. *StAR*, *cox2*, *pla2* and *hsl* expression were significantly depressed in  
31 2.5 % DHA larvae. An opposite effect of dietary DHA elevation was recorded in *gpx* expression.  
32 Both DHA and ARA had a significant effect on *ppara*, *gr*, and *pge2* expressions. Although no  
33 significant interactions were found, *pge2*, *gr*, and *ppara* displayed a differential pattern of expression  
34 between the different treatments. A strong association was found for the larval tissue amount of ARA  
35 and DHA with eicosanoid metabolism, stress response and skeleton anomaly related genes. These

36 results denoted the effects of dietary LC-PUFAs on immune/stress gene regulation and their potential  
37 implication in skeleton development.

38

39 **List of abbreviations**

40

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
Ppara $\alpha$	Peroxisome proliferator-activated receptor
Sod	Superoxide dismutase
Sox9	Transcription factor Sox9
StAR	Steroidogenic acute regulatory protein

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42

## 43 **Introduction**

44

45 Pikeperch (*Sander lucioperca*) is recognized as one of the main freshwater species with a great  
46 potential for the expansion of the EU aquaculture industry mainly because the good flesh quality and  
47 the high market value (Alexi et al., 2018). The major bottlenecks for further expansion of pikeperch  
48 culture today include low larval survival and high incidence of skeletal anomalies (Kestemont et al.,  
49 2015). Pikeperch larvae are very stress sensitive to lack or low levels of n-3 dietary essential long  
50 chain polyunsaturated fatty acids (LC-PUFA, n-3) causing lower performance, higher mortality;  
51 deficiency syndroms and deformities (Lund and Steinfeldt 2011; Lund et al., 2014). Thus, recent  
52 studies suggested requirements similar to those of marine carnivorous fish larvae for both  
53 phospholipids and LC-PUFAs (Hamza et al., 2015; Lund et al., 2019). Moreover, at a physiological  
54 level, oxidative risk is particularly high in the fast-growing larvae due to the high metabolic rate,  
55 oxygen consumption and water content in the larval tissues (Betancor et al., 2012). Fish have an  
56 endogenous antioxidant defense system with a wide range of antioxidant mechanisms to maintain an  
57 adequate oxidative balance (Filho et al., 1993). Among them, various antioxidant enzymes such as  
58 catalase (cat), superoxide dismutase (sod) and glutathione peroxidase (gpx) (Bell et al., 1987).

59 Glucocorticoids (GCs) are central steroid hormones on endocrine stress response modulation and  
60 whole-body homeostasis in vertebrates, well known to affect glucose metabolism, immune system,  
61 reproduction as well as bone metabolism regulation (Subramaniam et al., 1992; Sapolsky et al., 2000;  
62 Suarez-Bregua et al., 2018). Endogenous GC hormones regulate the expression of target genes  
63 through glucocorticoid receptor (*gr*) signaling within bone cells, and affecting skeletal development  
64 and metabolism (Suarez-Bregua et al., 2018). Also, *gr* is considered as an indicator of lipid nutrition  
65 effect on stress response in fish (Alves Martins et al., 2012). In trout, it has been shown that  
66 unsaturated fatty-acids inhibit glucocorticoid receptor-binding of hepatic cytosol (Lee and Struve,  
67 1992). Previous studies report possible regulation by *gr* of the transcription of hormone-sensitive  
68 lipase (*hsl*) (Alves Martins, et al. 2012; Le et al., 2005; Lampidonis et al., 2008). Furthermore, the  
69 gene expression of lipolytic enzymes such as *hsl* were regulated by dietary modifications (Turchini  
70 et al., 2003; Ma et al. 2013; Peng et al., 2014). In this respect, Alves Martins et al., (2012) suggested  
71 that fatty acids and their derivatives can-indirectly- modulate metabolic pathways related to energetic  
72 metabolism (*hsl* and phosphoenolpyruvate carboxykinase *pepck*).

73 LC-PUFAs are important ligands for nuclear receptors and transcription factors such as peroxisome  
74 proliferator-activated receptor (*ppar*) (Lin et al., 1999). Beside the regulation of the expression of

75 genes that participate in fatty acid oxidation, transcription factor *ppara* have been reported to  
76 modulate genes involved in cholesterol uptake and transport (Xie et al., 2002) which is central in  
77 steroidogenesis. Previous studies have reported the implications of LC-PUFAs and their derivatives  
78 in steroidogenesis in sea-bream (*Sparus aurata*) (Ganga et al., 2006; 2011). Interactions between  
79 *Ppara* and steroidogenic acute regulatory protein mitochondrial (StAR) have been addressed in  
80 Atlantic salmon (*Salmo salar*) (Pavlikova et al., 2010).

81 On the other hand, the ratio among dietary fatty acids, such as eicosapentaenoic (EPA),  
82 docosahexaenoic (DHA) and arachidonic (ARA) acids constitutes a critical factor for broodstock and  
83 larval performance due to competitive interaction among them (Bell and Sargent, 2003; Izquierdo,  
84 2005). Hence, regardless of the need to study the optimum absolute dietary values for LC-PUFAs in  
85 this species, optimum dietary ratios must be defined. In fact, LC-PUFAs (specially EPA and ARA)  
86 are precursors for highly bioactive eicosanoids. These PUFA-derived mediators (eicosanoids and  
87 resolvins), are recognized of high importance in signalling molecules playing roles in biological  
88 processes such as inflammation (Kremmyda et al., 2011). Eicosanoids are involved in a great variety  
89 of physiological functions and are produced in response to stressful situations. The major precursor  
90 of eicosanoids in fish is ARA, while eicosanoids formed from EPA are less biologically active than  
91 those formed from ARA (Tocher, 2003). Initially, eicosanoids production is catalyzed by  
92 phospholipases (pla), mainly cpla2. The free ARA can undergo several possible enzymatic pathways  
93 to create bioactive eicosanoids, among them cyclooxygenase – governed by cytochrome c oxidase  
94 subunit (coxs) such as cox2 that mediate the production of prostaglandins -including prostaglandin E  
95 synthase 2 (pge2); and lipoxygenase pathway which consists of arachidonate 5-lipoxygenase (lox5)  
96 enzymes as well as their products such as leukotrienes -including leukotriene A(4) hydrolase (lta4h)  
97 (Kremmyda et al., 2011; Hannah and Hafez, 2018). Furthermore, cox2 seems to play a key role in  
98 osteogenic differentiation (Kirkham and Cartmell, 2007).

99 Initially a multifactorial approach was used to investigate the effects of various dietary nutrients (fatty  
100 acids, vitamins and minerals). Results of this screening experiment showed a significant interaction  
101 between EPA+DHA and ARA in pikeperch larvae, especially on deformity occurrence, suggesting  
102 the importance of a balanced n-3 HUFA/n-6 HUFA ratio in this species (El Kertaoui and Lund et al.,  
103 2019). Based on this result, the present experiment was carried out in the facilities of DTU Aqua  
104 (Dannmark). The objective of the present study is to understand how dietary DHA/EPA/ARA ratios  
105 affect tissue fatty acid profiles and antioxidant and stress response capacity, as well as the relationship  
106 between the deformity occurrence and the stress status in pikeperch. In this sense, the present data

107 evaluated -particularly- larval development and performance, digestive capacity, skeleton deformities  
108 and molecular markers of oxidative stress status including: Sod, Gpx, and Cat; stress response  
109 including: StAR, Gr, Ppara, Hsl and Pepck; fatty acid synthesis such as fatty acid desaturase 2/acyl-  
110 coa 6-desaturase 6 (Fadsd6) and elongation of very long chain fatty acids protein 5 (Elov15);  
111 eicosanoids synthesis such as Pla2, Cox2, Lox5, Pge2 and Lta4h: status and bone development such  
112 as twist related protein (Twist), myocyte enhancer factor 2C (Mef2c), transcription factor Sox9  
113 (Sox9) and alkaline phosphatase (Alp).

## 114 **Materials and methods**

115

### 116 *Ethical standards*

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

124

### 125 *Larvae and rearing conditions*

126 Newly hatched larvae were obtained from AQUPRI Innovation, Egtved, Denmark and transferred to  
127 DTU Aqua at North Sea Research Centre, Denmark, where the experiment was carried out. Larvae  
128 were distributed into conical tanks (0,7 m in height and a diameter of 0.3 m), and from 3 dph larvae  
129 were fed on unenriched *Artemia* nauplii (AF and EG strains) (INVE, Dendermond, Belgium) until  
130 they reached 14 dph, followed by a co-feeding period from 15 to 17 dph using *Artemia* nauplii and a  
131 mixture of the experimental diets. The experiment was carried out in a triplicate set-up with 3 tanks  
132 per diet. Pikeperch larvae (initial body weight  $3.15 \pm 1.08$  mg) were randomly distributed into 18  
133 experimental conical tanks (50 L) at a density of 1300 larvae per tank in a flow through system with  
134 adjustable light and temperature control. Oxygen concentration and temperature were monitored daily  
135 by a hand-held Oxyguard meter from Oxyguard, Birkerød, Denmark. During the experiment, oxygen  
136 saturation was kept at a mean saturation of  $74.8 \pm 3.0$  % for all tanks with no significantly difference  
137 between treatments ( $P \geq 0.480$ ), and temperature was kept at  $20.6 \pm 0.7$  °C. Larvae in each tank were fed

138 with one of six experimental diets. Feed was administered by automatic feeders from 8 am to 6 pm.  
139 To ensure feed availability, daily feed supply was maintained at app. 15-20 % of larval wet biomass  
140 per tank during the first week (particles of 200-400  $\mu\text{m}$  /400-700  $\mu\text{m}$ ) and 10-15 % per tank biomass  
141 (particles of 400-700  $\mu\text{m}$ ) during the rest of the experimental period approximately every 20-30 min.  
142 Daily, bottom of tanks were vacuum cleaned to remove feed waste. Photoperiod was kept at 12h light:  
143 12h dark.

144

#### 145 *Experimental diets*

146 Two different dietary levels of DHA were formulated: 0.5 % (low) and 3.5 % (high) combined with  
147 three levels of ARA 1.2 %, 0.6 % and 0.3 % (Table 1). Therefore, six isonitrogenous and isolipidic  
148 diets were formulated and fabricated by SPAROS S.A. (Portugal) as cold extruded feed pellets of  
149 200-400  $\mu\text{m}$  and 400-700  $\mu\text{m}$ . Experimental diets were formulated using a mix of oils as sources of  
150 EPA, DHA and ARA to reach the required fatty acid content and to equalize the lipid content in each  
151 diet. Moisture (A.O.A.C. 1995), crude protein (A.O.A.C. 1995) and crude lipid (Folch, Lees &  
152 Sloane-Stanley 1957) contents of diets were analyzed. The proximate composition of the main  
153 nutrients is shown in table 1. Feeds were tested according to DHA, ARA and DHA/ARA ratios  
154 respectively.

155

#### 156 *Samplings, husbandry variables and analyses*

157 Final survival was calculated by individually counting all living larvae in each tank at the end of the  
158 experiment, and expressed as the percentage of the initial numbers of larvae. Representative samples  
159 of pikeperch larvae were sampled at 27, 32 and 40 dph for wet weight, and digestive enzymatic assays.  
160 Specific growth rate (SGR) was calculated according to the formula ( $\text{SGR} = (\ln w.w. f - \ln w.w. i) \times$   
161  $100) / t$ , Where  $\ln w.w. f, i$  = the natural logarithm of the final and initial wet weight,  $t$  = time (days)).  
162 A random subsample of 10 larvae per replicate was used for FA composition at 32 and 40 dph.  
163 Additional 50 larvae per tank were also taken at the end of the experimental period for skeleton  
164 morphogenesis and mineralization by staining. These larvae were sedated by an overdosis of clove  
165 oil, fixed and stored in 10 % phosphate buffered formaldehyde until analysis. Finally, for the  
166 molecular study 10 larvae per replicate were similarly sedated and stored in RNA later overnight at  
167 4 °C and then frozen at -80 °C until analysis.

168

#### 169 *Fatty acid analysis*

170 FA analysis of feeds and larvae was done according to previously described method (Lund et al.,  
171 2014). Lipids were extracted by a chloroform/methanol mixture, (2:1 (v/v) (Folch et al., 1957) and  
172 40  $\mu\text{l}$  (1  $\text{mg mL}^{-1}$ ) of an internal 23:0 FAME standard from Sigma-Aldrich (Denmark A/S) was added.  
173 A fixed amount of each feed (2-3mg) was weighed and for larval samples (10 larvae per tank) were  
174 weighed and homogenized by a Tissue Tearor probe diameter 4.5 mm, Biospec Products, Inc;  
175 Bartlesville, USA. Samples were allowed standing for 24 h in  $-20^{\circ}\text{C}$  followed by centrifugation. The  
176 supernatant was subsequently transferred to clean GC vials and allowed drying out in a Pierce, reacti-  
177 therm heating module at  $60^{\circ}\text{C}$ , under a continuous flow of nitrogen. Trans esterification of the lipids  
178 was done by addition of 1 mL of acetyl chloride in methanol (40:50:10, HPLC quality) at  $95^{\circ}\text{C}$ . The  
179 fatty acid methyl esters were analyzed by gas chromatography–mass spectrometry (GC–MS). Peaks  
180 on a given chromatogram were identified by comparison with the retention time of a commercial mix  
181 of a known FAME standard, SUPELCO 18919 (4:0–24:0), from Sigma-Aldrich (St. Louis, MO,  
182 USA). Peaks were quantified by means of the target response factor of the fatty acids and 23:0 as  
183 internal standard. Fatty acid concentrations were calculated (MSD Chemstation Data Analysis,  
184 G1710FA) based on the quantified peaks of the standard series and the samples as well of dry weight  
185 of prey and larvae and expressed as  $\text{ng sample}^{-1}$ .

186

#### 187 *RNA extraction and reverse-transcriptase quantitative PCR*

188 Samples were homogenized in 1 ml of TriReagent® (Sigma-Aldrich, Danmark A/S) RNA extraction  
189 buffer using a bead tissue disruptor (Bio Spec, Bartlesville, Oklahoma, USA). Total RNA was  
190 isolated following manufacturer's instructions and quantity and quality determined  
191 by spectrophotometry using a Nanodrop ND-1000 (Labtech Int., East Sussex, UK), and  
192 electrophoresis using 200 ng of total RNA in a 1 % agarose gel. cDNA was synthesized using 2  $\mu\text{g}$   
193 of total RNA and random primers in 20  $\mu\text{l}$  reactions and the high capacity reverse transcription kit  
194 without RNase inhibitor according to the manufacturer's protocol (Applied Biosystems, Warrington,  
195 UK). Gene expression was determined by qPCR of candidate genes: *ppara*, *fadsd6*, *elovl5*, *pepck*,  
196 *hsl*, *gr*, *StAR*, *pge2*, *pla2*, *lta4h*, *cox2*, *5-lox*, *gpx*, *sod*, *cat*, *twist*, *mef2c*, *sox9*, *alp*, and intestinal fatty-  
197 acid binding protein (*i-fabp*), Elongation factor-1 $\alpha$  (*elf1a*) and  $\beta$ -actin ( *$\beta$  actin*) were used as reference  
198 genes. The cDNA was diluted 20-fold with milliQ water. The efficiency of the primers for each gene  
199 was previously evaluated by serial dilutions of cDNA pooled from the samples to guarantee it was >  
200 90 % for all primer pairs. qPCR was performed using a Biometra TOptical Thermocycler (Analytik  
201 Jena, Goettingen, Germany) in 96-well plates in duplicate 20  $\mu\text{l}$  reaction volumes containing 10  $\mu\text{l}$  of

202 Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific, Hemel Hempstead, UK), 1 µl of the  
203 primer corresponding to the analyzed gene (10 pmol), 3 µl of molecular biology grade water and 5 µl  
204 of cDNA (1/20 diluted). In addition, amplifications were carried out with a systematic negative  
205 control (NTC, no template control) containing no cDNA. Standard amplification parameters  
206 contained an UDG pre-treatment at 50 °C for 2 min, an initial denaturation step at 95 °C for 10 min,  
207 followed by 35 cycles: 15 s at 95 °C, 30 s at the annealing T<sub>m</sub> and 30 s at 72 °C. Primer sequences  
208 for genes are given in table 3. Data obtained were normalized and the Livak method (2<sup>-ΔΔCt</sup>) used  
209 to determine relative mRNA expression levels. Sequence alignment was done and conserved domains  
210 obtained were used to design primers with Primer3 (v. 0.4.0) program and subsequent sequencing of  
211 PCR products and BLAST of them. Sequences of genes encoding for *ppara*, *fadsd6*, *elovl5*, *pepck*,  
212 *hsl*, *gr*, *i-fabp*, *StAR*, *pge2*, *pla2*, *lta4h*, *cox2*, *5-lox*, *gpx*, *sod* and *cat* were obtained by identifying the  
213 sequences from Sequence Read Archives (SRA) SRX1328344 and SRX1385650. The set of  
214 contiguous sequences were assembled using CAP3 (Huang and Madan, 1999) and identity of the  
215 deduced aa sequences confirmed using the BLASTp sequence analysis service of the NCBI.  
216 Sequences for *alp*, *twist22*, *mef2c* and *sox9* were available for the species of interest (Lund et al.,  
217 2019, Lund and El Kertaoui et al., 2018). Pikeperch specific gene primers were designed after  
218 searching the NCBI nucleotide database and using Primer3. Detailed information on primer  
219 sequences is presented in table 3.

220

### 221 *Skeleton anomalies*

222 To determine the presence of skeletal anomalies, 50 larvae per tank were fixed and stored in buffered  
223 (10 % phosphate) formalin at the end of the experiment. Staining procedures with alizarin red and  
224 alcian blue were conducted to evaluate skeletal anomalies following a modified method from previous  
225 studies (Izquierdo et al., 2013). Classification of skeletal anomalies was conducted according to  
226 Boglione et al. (2001). Anomalies were expressed as frequency of total severe anomalies and specific  
227 anomalies, such as jaw deformities, scoliosis, lordosis, cleithrum and branchiostegal rays within each  
228 dietary group (Fig. 1).

229

### 230 *Digestive enzyme activities*

231 The head and tail of 10 pikeperch larvae were dissected on a glass maintained on ice to isolate the  
232 digestive segment, and the stomach region was separated from the intestinal segments. Pooled  
233 samples from each tank were homogenized in 10 volumes (v/w) cold distilled water. Alkaline



234 phosphatase (AP) and aminopeptidase (N), two enzymes of brush border membrane, were assayed  
235 according to Bessey et al. (1946) and Maroux et al. (1973) using p-nitrophenyl phosphate (Sigma-  
236 Aldrich) and L-leucine p-nitroanalide (Sigma-Aldrich) as substrates, respectively. Pepsin was  
237 assayed by the method of Worthington (1982) modified by Cuvier-Péres and Kestemont (2002).  
238 Trypsin activity was assayed according to Holm et al. (1988), such as described by Gisbert et al.  
239 (2009). Protein was determined using the Bradford (1976) procedure. Enzyme activities are expressed  
240 as specific activities (U or mU mg protein<sup>-1</sup>).

241

## 242 *Statistics*

243 Data are expressed as the mean  $\pm$  standard error (SEM). Kolmogorov and Smirnov's test was used to  
244 assess the normality of data sets ( $p < 0.05$ ) and Bartlett's test was conducted to evaluate variance  
245 homogeneity ( $p < 0.05$ ). Two-way ANOVA was used to compare the different endpoints using DHA  
246 and ARA dietary levels as fixed factors. The statistical analyses were performed using the JMP 12.1  
247 software (SAS Institute Inc., North Carolina, USA). A Tukey HSD test was used to determine  
248 significance of mean differences ( $P < 0.05$ ) between the treatment groups where applicable. If no  
249 interaction between factors (DHA and ARA dietary levels) in the outcome of the two-way ANOVA,  
250 a further one-way ANOVA and Tukey's HSD test were used to determine any significant differences  
251 according to the DHA/ARA ratio effect. Data with no normality and/or homogeneity of variances  
252 were tested with Kruskal-Wallis tests and post-hoc pair-wise Wilcoxon comparison test. The  
253 relationship between the expression of the target genes and larval fatty acid profiles were performed  
254 using the R software; first association between paired samples was checked using one of Pearson's  
255 product moment correlation coefficient, the correlation matrix was generated using corrplot package  
256 and the significance levels (p-values) was generated using lattice package. Then, multivariate  
257 principal component analysis (PCA) combined with co-inertia analysis (CIA) were applied to the  
258 cross-platform comparison of gene-expression and fatty acid content datasets. Component scores  
259 were further clustered according to RVAideMemoire package. PCA and co-inertia analyses were  
260 performed with ADE-4 package. All statistical computations were considered significant when  
261 resulting p-values were:  $< 0.05$ .

262

## 263 **Result**

### 264 *Growth and survival*

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

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

273

#### 274 *Larval fatty acid composition and gene expression*

275 Fatty acid compositions of 32 dph and 40 dph pikeperch larvae are presented in tables 5 and 6. Higher  
276 levels of DHA, 18: 3n-3, total n-3 LC-PUFA and total n-3 larval contents were found in 40 dph larvae  
277 fed diets 4, 5 and 6 ( $p = 0.0005, 0.0375, 0.0153$  and  $0.0219$  respectively) as a consequence of higher  
278 dietary DHA levels, while no significant differences were detected at 32 dph except for 18: 3n-3 ( $p =$   
279  $0.0001$ ). The group of larvae fed a higher dietary ARA content showed an increase in their ARA body  
280 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  
281 contrast, monounsaturated acid content was significantly higher in larvae fed 0.3 % ARA ( $p = 0.0442,$   
282  $0.0005$  respectively at 32 dph and 40 dph) principally due to a higher percentage of oleic acid (18:  
283 1n-9) in these larvae ( $p = 0.0334$  and  $0.0087$  respectively at 32 dph and 40 dph). Similarly, a decrease  
284 in dietary ARA resulted also in graded increase in EPA/ARA ratio ( $p = 0.0001, 0.0005$  at 32 dph and  
285 40 dph respectively) and EPA larval content ( $p = 0.047, 0.0001$  at 32 dph and 40 dph respectively)  
286 was negatively correlated with ARA larval content (Fig. 2;  $R = 0.9708$  at 40dph). Significant  
287 interactions between DHA and ARA were found in DHA/ARA and n-3 LC-PUFA/ n-6 LC-PUFA  
288 ratios in 40 dph larvae ( $p = 0.00978$  and  $0.039$  respectively), while the total content of saturated fatty  
289 acids (SFA) was similar among larvae fed the different experimental diets.

290

291 Among the 20 studied genes, 11 target genes showed significant differences in expression between  
292 the dietary treatments (Fig. 3). The transcription of *StAR*, *cox2*, *pla2* and *hsl* was significantly  
293 depressed in 2.5 % DHA larvae ( $p = 0.043; 0.030, 0.018$  and  $0.0076$  respectively) while an opposite  
294 significant effect of dietary DHA elevation was recorded in *gpx* and *i-fabp* expression ( $p = 0.0218$  and  
295  $0.0002$ ). Besides the DHA effect, the results of one-way ANOVA indicated, that larvae fed D6  
296 differed significantly in *hsl* and *i-fabp* expression from D1, D2 and D3 treatments ( $p = 0.0476$  and

297 0.0014 respectively). *I-fabp* expression was significantly upregulated in larvae fed diet D5 compared  
298 to D2 and D3 groups (Fig. 3a) ( $p=0.0014$ ), similarly *pla2* expression was higher in D6 than D1  
299 treatment ( $p= 0.0186$ ). Both DHA and ARA had a significant effect in *ppara*, *gr*, and *pge2*  
300 expressions. The transcription of these genes (*ppara*, *gr* and *pge2*) was significantly depressed with  
301 the dietary DHA increment ( $p= 0.0004$ ;  $0.0041$ ;  $0.003$ ); a similar pattern of gene expression occurred  
302 in the lowest ARA-fed group compared to 1.2 % ARA group ( $p= 0.015$ ;  $0.0011$ ;  $0.0251$ ). Although  
303 no significant interactions were found, *gr* and *ppara* transcript levels were higher in D1-fed larvae  
304 compared with D4, D5 and D6 groups (Fig. 3a) (One-way ANOVA  $p = 0.0083$ ;  $0.0004$ ), while D2  
305 differed significantly from D6 treatment. Compared to D6, larvae fed D1, D2 and D4 displayed an  
306 increased transcript levels in *pge2* (Fig. 3b) ( $p = 0.0135$ ). *Twist2* gene expression presented a  
307 significant interaction among DHA and ARA dietary content; pikeperch fed diet D6 showed the  
308 highest expression in *twist2* than larvae fed the other diets ( $p = 0.0079$ ) (Fig. 3c). Furthermore, results  
309 from the two-way ANOVA regarding expression of *twist2* showed also a higher dietary effect of both  
310 DHA and ARA ( $p= 0.01$  and  $0.0043$  respectively). Dietary ARA content had a clear effect on the  
311 expression of *5-lox* ( $p= 0.0345$ ).

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

320

#### 321 *Relationships between gene expression and larval fatty acid content*

322 The relationships between the studied target genes and larval fatty acid profile explored through  
323 Pearson's correlation coefficient is illustrated in figure 4a. The strongest associations were found for  
324 the amount of ARA and DHA with eicosanoid metabolism, stress response and skeleton anomaly  
325 related genes ( $p < 0.05$ ). Thus *mef2c*, *ppara*, *pla2*, *pge2* and *gr* were positively correlated with ARA,  
326 while negatively with the amounts of DHA. Significant correlation was found between *StAR*, *hsl* and  
327 *i-fabp* gene expressions and DHA. *Twist2* and *alp* showed a negative correlation with ARA level.  
328 Equally, 20:3n-3 and 18: 3n-6 displayed a similar correlation as ARA with *twist2* and *ppara*. The

329 expression of specific antioxidant genes was significantly correlated with 18:3n-3 (*sod* and *gpx*) and  
330 18:2n-6 (*gpx*). Those correlations were reinforced by principal component analysis (PCA) combined  
331 with co-inertia analysis and algorithm clustering results presented in figure 4b, which concomitantly  
332 illustrates the segregation of two clusters in both genes and fatty acid profile. Except 20:3n-3; n-3  
333 LC-PUFA (EPA and DHA) and oleic acid were clustered together and separately than the other  
334 figured fatty acids. Furthermore, all eicosanoid metabolism genes were clustered with *mef2c* and  
335 stress response genes (*StAR*, *gr* and *pepck*) in the opposite direction of DHA level while positively  
336 linked to ARA level.

337

### 338 *Skeleton anomaly evaluation*

339 Overall, high incidence of lordosis and cephalic anomalies were observed in the present study (Table  
340 7). Two-way ANOVA results indicated a significant increase in different skeletal anomaly typologies  
341 with dietary DHA intake increment, in particular anomalies of bone formed by direct ossification ( $p$   
342 = 0.012). Higher incidence of opercular deformities was observed in larvae fed high DHA ( $p$  = 0.043),  
343 mainly governed by the higher branchiostegal ray anomaly observed in these larvae ( $p$  = 0.001).  
344 Similarly, the increase in DHA led to a higher incidence of dentary bone anomalies ( $p$  = 0.001) and  
345 pectoral element deformities ( $p$  = 0.007), in particular cleithrum anomaly ( $p$  = 0.006). Furthermore,  
346 despite the lack of significant interaction between DHA and ARA in the occurrence of deformities,  
347 one -way ANOVA results showed a significantly higher occurrence of maxillary bone and  
348 branchiostegal rays anomalies in D6 than in D2 fed-larvae ( $p$  = 0.011 and 0.008 respectively). No  
349 differences were found in the degree of mineralization according to the size of the larvae (data not  
350 shown).

351

### 352 *Specific enzymatic activities*

353 Pepsin activity was higher in the high DHA-fed groups (Table 8) ( $p$  = 0.0127), while no differences  
354 were observed at 32 and 40dph. Combined effect of DHA and ARA with significant interaction was  
355 found in trypsin activity at 27 and 40 dph ( $p$  = 0.0003 and 0.0017 respectively). Larvae fed diet 6  
356 presented the highest trypsin activity at 27 dph ( $p$  = 0.0003); on the opposite, this treatment resulted  
357 in the lowest trypsin activity at 40dph ( $p$  = 0.0017). Brush border enzymes (alkaline phosphatase and  
358 aminopeptidase) displayed significant differences among the different dietary ARA levels ( $p$  = 0.0005  
359 and 0.001 respectively) at 40 dph. On the other hand, no differences of alkaline phosphatase and  
360 aminopeptidase activities were recorded between treatments at 27 and 32 dph.

361

## 362 **Discussion**

363

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

369 Although no significant growth differences were observed among the different treatments, molecular  
370 biomarkers, biochemical and osteological endpoints investigated in the present study highlight the  
371 influence of both DHA and ARA and their interaction on pikeperch larval development. The  
372 increased dietary DHA up to 2.5 %, led to the increment in incidence of skeletal deformities. This  
373 result is somewhat contradictory with the results of a recent study (Lund and El Kertaoui et al., 2018)  
374 in which a clear tendency towards decreasing prevalence of severe skeletal deformities was observed  
375 in pikeperch fed increased dietary levels of DHA. In fact, the positive effect of dietary DHA elevation  
376 recorded by Lund and El Kertaoui et al. (2018) on pikeperch skeletal anomalies was probably  
377 attributed to dietary phospholipid elevation applied in this experimental design, since the increased  
378 dietary PL reduced the prevalence of skeletal anomalies (Lund and El Kertaoui et al. 2018; Cahu et  
379 al., 2003; Boglione et al., 2013; Saleh et al., 2013). Thus, besides the fatty acid profile, lipid structure  
380 seems to be another important nutritional factor influencing the skeletal development in pikeperch  
381 larvae. In this respect Villeneuve et al. (2005) associated the increased skeletal anomaly occurrence  
382 with n-3 LC-PUFA (EPA and DHA) elevation in the neutral lipid fraction. Negative effects of  
383 excessive DHA intake on the occurrence of skeleton anomalies- especially dentary and maxillary  
384 deformities- were also reported in gilthead seabream *Sparus aurata* (Izquierdo et al., 2013). Same  
385 authors associated the increased oxidative stress with the endochondral bone anomalies. Consistently  
386 with this hypothesis, together with the increased oxidative status of pikeperch larvae – as presented  
387 by the higher expression of *gpx*- in the present study, the skull, especially the cranial structures such  
388 as dentary and operculum complex including the branchiostegal rays remind the most affected, when  
389 high DHA induced anomalies were detected. However, *sod* and *cat* expression showed no significant  
390 differences in transcription levels among the different groups. Jin et al. (2017) suggested no oxidative  
391 stress effects on antioxidant defense capability through Sod activation in juvenile black seabream  
392 (*Acanthopagrus schlegelii*) fed high DHA/EPA ratio. Interestingly, antioxidant enzyme mRNA

393 expression levels increased concomitantly with the decrease of larval C18 fatty acid content,  
394 especially  $\alpha$ -linolenic acid (ALA; 18:3n-3) which correlated negatively with *sod* and *gpx* expression  
395 levels and linoleic acid (LA; 18:2n-6) negatively correlated with *gpx* expression levels. High dietary  
396 LA also negatively impacted nonspecific immunity and antioxidant capacity in juvenile large yellow  
397 croakers (*Larimichthys crocea*) (Zuo et al., 2015). Previous studies demonstrated that ALA tended to  
398 be more prone to  $\beta$ -oxidation or excretion rather than to elongation into EPA and DHA (Fu and  
399 Sinclair, 2000). In spite of the different dietary and larval fatty acid contents (including DHA, EPA,  
400 ARA and their precursors ALA and LA), the expression of genes involved in desaturation (*fadsd6*)  
401 and elongation (*elovl5*) were not influenced. Indeed, the present results likely reflected an adaptation  
402 as a result of a negative feedback, especially in fish fed higher DHA level (diets: D4, D5, D6)  
403 permitting to maintain LC-PUFA and their metabolites within the required physiological levels.  
404 The differences in larval fatty acid profiles were not limited to ARA, and DHA. The results of gene  
405 expression may reflect the combined actions of other fatty acids (EPA, oleic acid, LA and ALA).  
406 Accordingly, differential pattern of gene expression was recorded depending on the fatty acid larval  
407 content. *Alp* expression showed a negative correlation with ARA content. *Alp* is recognized as a  
408 biomarker of osteoblast differentiation and direct formation of bone via the intra-membranous  
409 ossification pathway (Hessle et al., 2002). However, a significant increase in anomalies of bone  
410 formed by direct ossification was observed with dietary DHA increment. Increase in DHA in lower  
411 ARA-fed group (diet D6) resulted in higher branchiostegal rays and dentary deformities. These fish  
412 presented the highest expression of *twist2*, a gene involved in osteoblast inhibition, but also displayed  
413 an antioxidant activity being involved in the control of reactive oxygen species (ROS) (Floc'h et al.,  
414 2013). Recent results showed a differential effect of dietary ARA on skeletal deformities depending  
415 on the EPA+DHA levels (El Kertaoui and Lund et al., 2019) pointing out the need of a balanced  
416 dietary n-3/n-6 ratio in this species. This is well known that prostaglandins are potent regulators of  
417 bone formation and bone resorption (Meghji et al., 1988; Raisz, 1995). Thus, an imbalance of n-3/n-  
418 6 -especially EPA/ARA ratio- may result in the prostaglandin imbalance and consequently, affects  
419 the production of PGs which can lead to an imbalance of bone formation and resorption (Boglino et  
420 al., 2014), in particular, the PGE2 concentrations known to influence both bone formation and  
421 resorption (Berge et al., 2009). In Senegalese sole (*Solea senegalensis*) increased PGE2 production  
422 induced by dietary ARA supplementation resulted in the reduction in bone ossification (Boglino et  
423 al., 2013). ARA is the major precursor of eicosanoids in fish cells and usually considered as the major  
424 substrate for eicosanoid synthesis (Bell et al., 1994; Furuita et al., 2007). Thus, increased amounts of

425 ARA led to an increased amount of substrate available for synthesis of ARA-derived eicosanoids. In  
426 this sense, our finding showed a clear response to ARA intake with the expression of eicosanoid  
427 metabolism related genes. On the other hand, as expected, the larval body fatty acid composition  
428 reflected dietary fatty acid profiles, especially DHA and ARA, which increased in the higher DHA  
429 and higher ARA fed groups respectively. This explains the positive correlation ( $p < 0.05$ ) found  
430 between the larval ARA content and the expression of the eicosanoid metabolism genes in particular  
431 *pge2* and *pla2*. *5-lox* expression was mainly governed by ARA level. The present results are in  
432 agreement with those found in gilthead sea-bream, where changes in the expression of these genes  
433 were associated with ARA intake (Alves Martins et al., 2012). Meanwhile, despite the similar EPA  
434 concentrations among the experimental diets, EPA larval content was reduced significantly with  
435 larval ARA increment, indicating a preferential EPA metabolism, especially with the increase in  
436 dietary ARA. The strong negative correlation between the two fatty acids in the tissues was reported  
437 in other studies, suggesting the competition between these latter for inclusion in the tissues (Alves  
438 Martins et al., 2012; Izquierdo, 2005; Sargent et al., 1999; Van Anholt et al., 2004). The major  
439 mechanism of action for n-3 LC-PUFAs (EPA and DHA) is thought to block the formation of pro-  
440 inflammatory mediators via substrate competition with ARA for enzymes that generate several  
441 inflammatory mediators (Lands, 1987; Massaro et al., 2008, Sears and Ricordi, 2012). Furthermore,  
442 the EPA: ARA ratio is considered as a major determinant of eicosanoid production. Nonetheless,  
443 genes related to eicosanoid production showed the higher expression in low DHA fed fish, in  
444 particular *pla2*, *cox2* and *pge2*. DHA has been suggested to affect eicosanoid production (Nablone et  
445 al., 1990). Long chain n-3 PUFAs such as DHA and EPA exert also an anti-inflammatory action by  
446 inhibiting production of ARA-derived eicosanoids (Huang et al., 2018). In concordance with this  
447 finding, we hypothesize that the production of ARA-derived eicosanoids was decreased due to DHA  
448 elevation in this species.

449 An imbalance in eicosanoid profiles due to dietary LC-PUFAs supplementation can affect various  
450 metabolic pathways, including the corticosteroid production and thus the stress response/tolerance in  
451 fish (Van Anholt et al., 2004; Wales, 1988; Bessonart et al. 1999; Koven et al., 2003). PCA performed  
452 in the present study clustered the genes involved in stress response together with eicosanoid  
453 metabolism and *mef2c* transcript level whose were positively correlated with ARA larval content,  
454 whereas all oxidative stress and the skeleton anomaly related genes other than *mef2c*, were clustered  
455 together and positively associated to DHA larval content. The potential of ARA in the modulation of  
456 genes involved in stress response has been studied in gilthead sea-bream and Senegalese sole larvae

457 (Alves Martins et al., 2012; 2013). Our results seemed to support this finding; in this respect, the *pla2*  
458 up regulation in the present study reflected the ARA abundance (Hughes-Fulford et al., 2005; Yoshida  
459 et al., 2007) since phospholipase A2 is mostly responsible for catalyzing the release of ARA from  
460 phospholipids in cell membranes (Burke and Dennis, 2009). In addition, in vitro results have already  
461 proved the marked participation of COX and LOX metabolites on cortisol release mechanism in fish  
462 (Ganga et al., 2006; 2011). Effects of dietary ARA on cortisol response have been clearly  
463 demonstrated in Senegalese sole post-larvae, accompanied by an up regulation of *gr* by ARA dietary  
464 supplement (Alves Martins et al., 2011; 2013). Equally, our finding indicated that the *gr* gene  
465 responded positively to ARA supply. In fact, cortisol is the main endogenous GC hormones that  
466 regulates the expression of target genes through Gr located in the cytoplasm, signaling within cells  
467 including bone cells (Suarez-Bregua et al., 2018). The skeleton is one of the target organs of the stress  
468 hormones and physiological levels of GCs are vital for normal skeletogenesis (Suarez-Bregua et al.,  
469 2018; Zhou et al., 2013). Considering the down regulation of *gr* and the higher anomaly occurrence  
470 in pikeperch fed high fed DHA diets, the endogenous glucocorticoids action on bone metabolism  
471 might have also been responsible for the above-mentioned differences observed in skeletal anomalies  
472 in these larvae. In addition, previous study on pikeperch larvae reported a positive effect of high levels  
473 of DHA supplementation on stress tolerance, while no such effects were observed by high levels of  
474 dietary ARA (Lund et al., 2012; 2014). The present molecular results confirm those previous findings  
475 on DHA effect on stress sensitivity in pikeperch. Since the lower transcript levels of genes involved  
476 in stress response such as *StAR*, *gr*, *pla2* and *hsl* likely reflect an adaptation to increasing dietary  
477 amounts of LC-PUFA (Alves Martins et al., 2012). DHA is involved in processes that increase stress  
478 tolerance through the regulation of *StAR*, a key rate-limiting enzyme in steroidogenesis. Indeed, DHA  
479 acts as an inhibitor of the oxoeicosanoid receptor (OXE-R) in steroidogenic cells, reducing *StAR*  
480 protein levels and steroidogenesis (Cooke et al., 2013). Hormone-sensitive lipase are important  
481 enzymes involved in lipolysis, that reported to be enhanced under stress conditions (Ma et al., 2013;  
482 Nielsen and Møller, 2014). This latter (*hsl*) catalyzes the hydrolysis of cholesteryl esters and plays an  
483 essential role in the regulation of Dibutyryl cyclic AMP (Bt2cAMP) - induced steroidogenic acute  
484 regulatory protein (*StAR*) expression, hence, steroid biosynthesis (Manna et al., 2013).  
485 Besides, the relative expression of the above-mentioned stress response genes (*pla2* and *gr*) as well  
486 as *pge2* were highly correlated with *ppara* expression, likely pointing out a common mechanism of  
487 dietary regulation in this case. Another mechanism by which LC-PUFAs and eicosanoids could be  
488 acting to regulate gene transcription is through *ppar*'s pathway (Kresten et al., 2000), considering that



489 PUFAs and their metabolites, in particular leukotriene B4 (LTB4), have been shown to activate  $ppar\alpha$ ,  
490 being one of the main endogenous ligands (Lin et al., 1999, Choi et al., 2012). This provides an  
491 alternative explanation for the marked similarities observed in the expression pattern of these genes.  
492 *Ppara* has been suggested to regulate *gr* transcription, as one of the potent transcription factors  
493 adapting the expression of several genes involved in stress response and eicosanoid metabolism  
494 (Dichtl et al., 1999; Jia and Turek, 2005), and therefore we hypothesize that *pge2* and *pla2* were likely  
495 modulated by this transcription factor.

496 The expression of *i-fabp* gene has been considered as an indicator for assessing nutrient supply and  
497 represents a useful marker for intestinal development functional and the digestive system function in  
498 fish larvae diets (Pierce et al., 2000; Andre et al., 2000; Yamamoto et al., 2007; Overland et al., 2009;  
499 Venold et al., 2013; Lin et al., 2018), due to its crucial role in intracellular fatty acid trafficking and  
500 metabolism in fish gut (Her et al., 2004). Thus, the resulted higher expression levels of *i-fabp* gene  
501 in high DHA fish group may indicate the enhancement of fatty acid transfer rate and absorption (Baier  
502 et al., 1996; Levy et al., 2001; Storch and Thumser, 2010). On the other hand, a recent study in  
503 pikeperch larvae highlighted the potential involvement of ARA but not n-3 LC-PUFAs in the  
504 development of the digestive tract (El Kertaoui and Lund et al., 2019). Within the duration of the  
505 present study, the intestinal brush border digestive capacity was not significantly affected by DHA  
506 dietary content, but was significantly increased in fish fed intermediate ARA level (0.6 %) at 40 dph.  
507 Such effect has been observed in tongue sole (*Cynoglossus semilaevis*) larvae (Yuan et al., 2015).  
508 The morphoanatomical development and maturation of the gut is known to be accompanied by an  
509 increase in activity of the brush border enzymes from the enterocytes (Zambonino-Infante and  
510 Cahu, 2007; Lazo et al., 2010). Concurrently, larval ARA content was positively correlated with  
511 transcript level of myocyte enhancer factor 2c (*mef2c*), this latter regulates the final step of  
512 chondrocyte maturation- chondrocyte hypertrophy. And as a chondrogenic marker gene, *mef2c* has  
513 been used to characterize the maturation process in fish (Ytteborg et al., 2010). Taking together the  
514 present finding and the above referred studies, we suggest ARA-sensitive effect on the maturation  
515 process in pikeperch larvae.

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

519

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521

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528

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535

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537

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873 **Figures**

874

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

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

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

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

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896 **Tables**

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898 **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
<b>Ingredients</b>	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
<b>Proximate composition(%)</b>						
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

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905 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

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921 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	TACTGAGGAGGGCAACTG	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	GCGTCGTGTCGTAAGTATT	
gpx	Forward	ACACCCAGATGAACGAGCTT	0.93
	Reverse	TCCACTTTCTCCAGGAGCTG	
hsl	Forward	CAGTTCAGTCCAGGCATTCTG	0.84
	Reverse	TTCTGCCCCCTCTCAACTCTG	
pepck	Forward	CGAACACATGCTGATCCTGG	0.89
	Reverse	CGGGAGCAACACCAAAGAAA	
ppar	Forward	GCCCCAGTCAGAGAAGCTAA	0.87
	Reverse	TTTGCCACAAGTGTCTGCTC	
fadsd6	Forward	GGTCATTTGAAGGGAGCGTC	0.90
	Reverse	TGTTGGTGGTGTATAGGGCAT	
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	GCTGTCCGATCCCAGTGTA	0.99
	Reverse	CCAGTCTCTGTCCACACTGT	
twist2	Forward	CCCCTGTGGATAGTCTGGTG	0.85
	Reverse	GACTGAGTCCGTTGCCCTCTC	
elfla	Forward	TGATGACACCAACAGCCACT	0.81
	Reverse	AAGATTGACCGTCGTTCTGG	

b-actin	Forward	CGACATCCGTAAGGACCTGT	0.93	922
	Reverse	GCTGGAAGGTGGACAGAGAG		

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926 Table 4. Effects of dietary treatments on specific growth rate, individual weight, apparent mortality rate, cannibalism and survival rate.  
 927 Data are presented as mean  $\pm$  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 <sup>-1</sup> )	13.05 $\pm$ 0.35	13.32 $\pm$ 0.33	13.02 $\pm$ 0.45	12.45 $\pm$ 0.67	12.99 $\pm$ 0.75	12.82 $\pm$ 0.55	ns	ns	ns
Apparent mortality (%)	25.62 $\pm$ 1.63	27.10 $\pm$ 9.40	31.28 $\pm$ 5.19	35.03 $\pm$ 8.88	34.31 $\pm$ 3.91	31.85 $\pm$ 7.86	ns	ns	ns
Survival (%)	22.69 $\pm$ 4.46	21.13 $\pm$ 3.07	22.46 $\pm$ 3.09	26.64 $\pm$ 4.10	21.15 $\pm$ 5.87	19.54 $\pm$ 6.02	ns	ns	ns
Cannibalism (%)	51.69 $\pm$ 6.09	51.77 $\pm$ 7.84	46.26 $\pm$ 5.81	38.30 $\pm$ 7.52	44.54 $\pm$ 2.58	48.62 $\pm$ 2.09	ns	ns	ns
Weight at 27dph (mg)	17.40 $\pm$ 0.83	14.41 $\pm$ 0.86	16.23 $\pm$ 1.60	16.08 $\pm$ 1.58	15.38 $\pm$ 1.38	16.68 $\pm$ 3.14	ns	ns	ns
Weight at 32 dph (mg)	23.10 $\pm$ 1.77	21.64 $\pm$ 2.21	24.74 $\pm$ 3.12	22.42 $\pm$ 0.99	23.47 $\pm$ 1.14	22.25 $\pm$ 4.49	ns	ns	ns
Weight at 40 dph (mg)	63.46 $\pm$ 5.09	67.48 $\pm$ 5.07	63.13 $\pm$ 6.65	55.59 $\pm$ 8.23	63.16 $\pm$ 10.37	60.41 $\pm$ 7.63	ns	ns	ns

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

Diet	0.6%DHA			2.5%DHA			Two way ANOVA			949
	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	950
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	951
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	952
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	953
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±0.05cd	12.13±0.62bc	10.13±1.10d	***	ns	**	954
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	955
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	956
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	957
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	958
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	959
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	960

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

963 \*  $p < 0.05$

964 \*\*  $p < 0.01$

965 \*\*\*  $p < 0.001$

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975 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±0.06cd	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±0.09cd	1.68±0.05b	0.62±0.04de	1.18±0.17c	2.39±0.42a	ns	***	*

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

977 \* p < 0.05

978 \*\* p < 0.01

979 \*\* p < 0.001

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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*	
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	988
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	989
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	990
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	991
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	992
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	993
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	994
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	995
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	996
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	997
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	998
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	999

1011 Different superscript letters within a row denote significant differences among diets determined by one-way ANOVA with Tukey's comparison test (p <0.05).  
 1012 \* p < 0.05  
 1013 \*\* p < 0.01  
 1014 \*\* p < 0.001  
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1026 Table 8. Specific enzymatic activity (mU mg protein<sup>-1</sup>) 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*	
Specific enzymatic activity at 27 dph										1027
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	**	*	***	1028
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	1029
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	1030
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	1031
Specific enzymatic activity at 32 dph										1032
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	1033
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	1034
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	1035
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	1036
Specific enzymatic activity at 40 dph										1037
Trypsin	10.88±0.4bcd	20.0±2.0ab	26.03±1.4a	8.43±1.6cd	14.74±4.0bc	3.25±1.2d	***	*	**	1038
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	1039
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	1040
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	1041

1053 Different superscript letters within a row denote significant differences among diets determined by one-way ANOVA with Tukey's comparison test (p <0.05).

1054 \* p < 0.05

1055 \*\* p <0.01

1056 \*\* p <0.001

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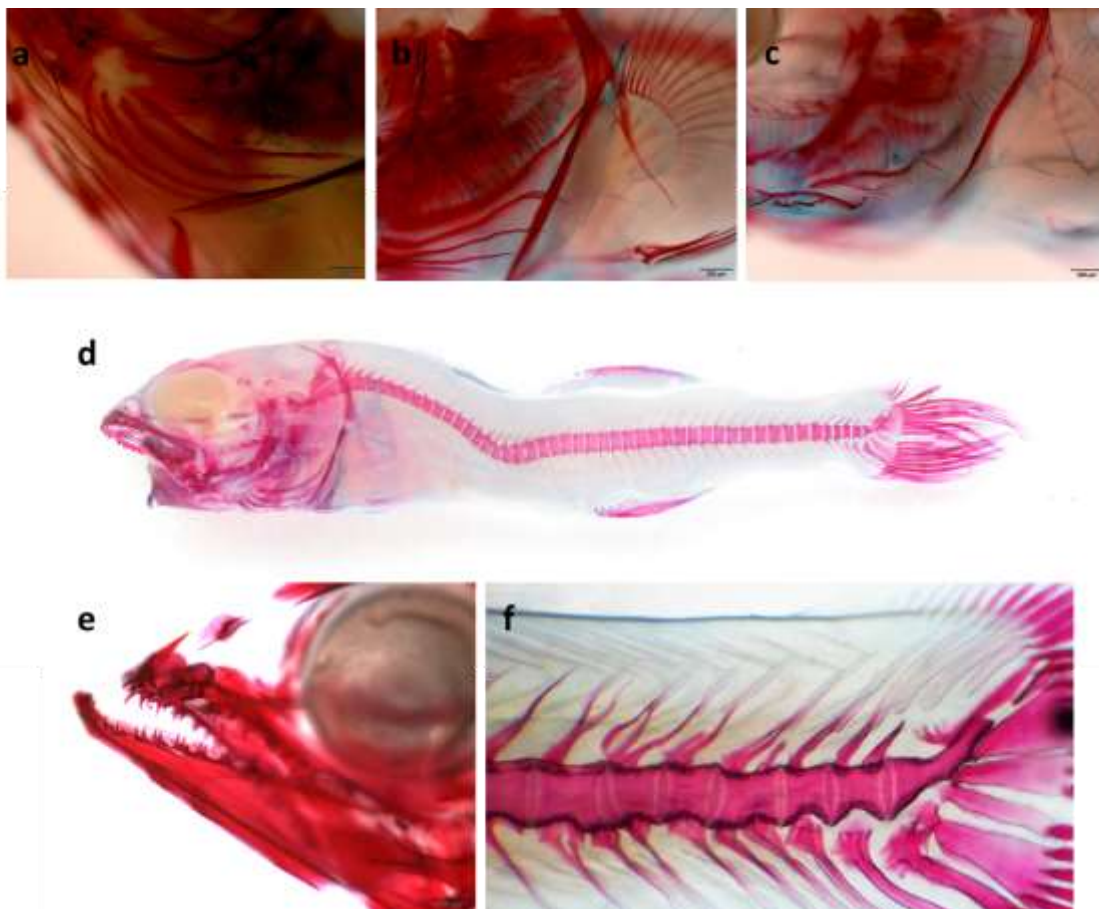
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1062 **Fig.1.**



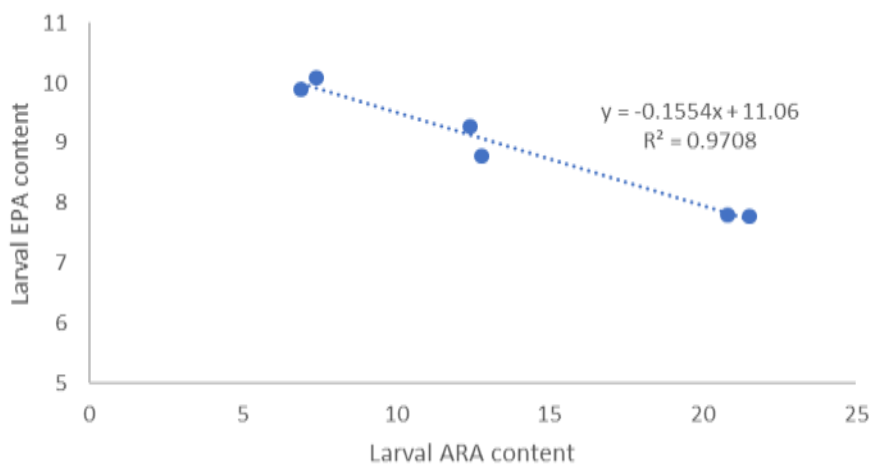
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1067 **Fig. 2.**

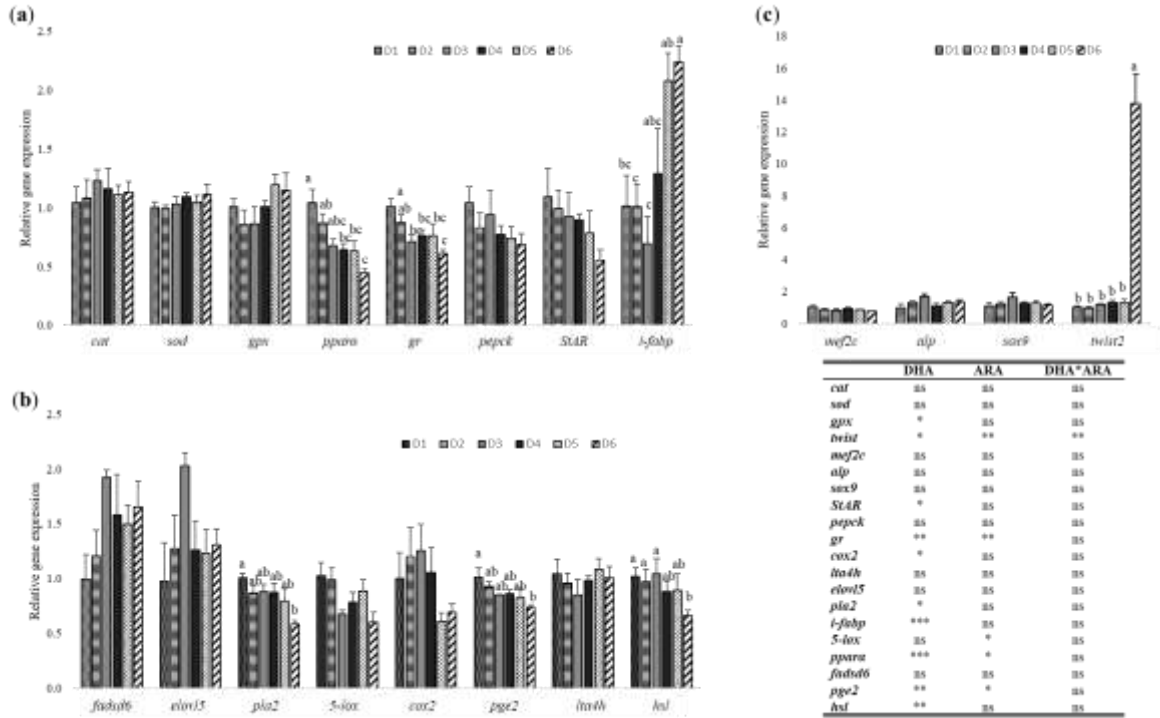


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1071 **Fig. 3.**



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1074 \* p < 0.05  
 1075 \*\* p < 0.01  
 1076 \*\*\* p < 0.001  
 1077 ns, not significant differences

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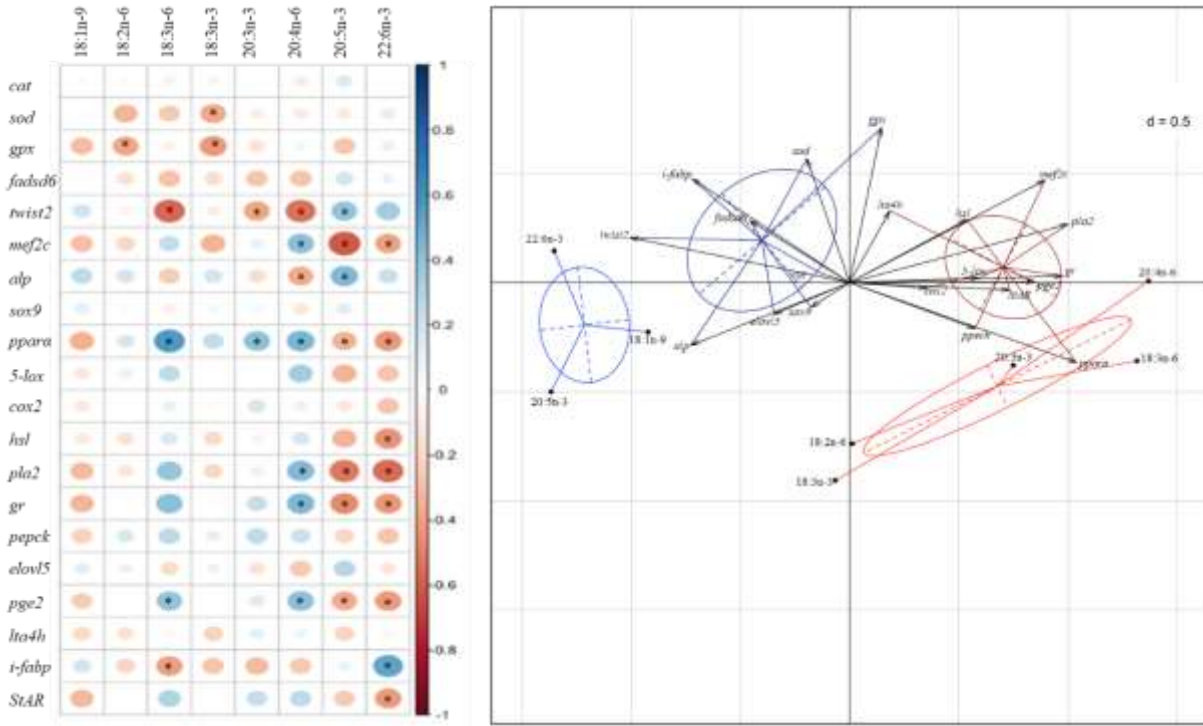
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1103 **Fig. 4.**



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\*  $p < 0.05$  Significant correlation