Teleost fish larvae adapt to dietary arachidonic acid supply through modulation of the expression of lipid metabolism and stress response genes

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Running title: Fatty acids affect fish gene expression

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Abstract

Dietary fatty acid supply can affect stress response in fish during early development. Although knowledge on the mechanisms involved in fatty acid regulation of stress tolerance is scarce, it is often hypothesised that eicosanoid profiles can influence cortisol production. Genomic cortisol actions are mediated by cytosolic receptors which may respond to cellular fatty acid signalling. An experiment was designed to test the effects of feeding gilthead seabream larvae with four microdiets, containing graded arachidonic acid (ARA) levels (0.4%, 0.8%, 1.5%, 3.0%), on the expression of genes involved in stress response (steroidogenic acute regulatory protein, glucocorticoid receptor, phosphoenolpyruvate carboxykinase), lipid and particularly eicosanoid metabolism (hormone-sensitive lipase, peroxisome proliferator-activated receptor alpha, phospholipase A_2, cyclooxygenase-2, and 5-lipoxygenase) as determined by real time Q-PCR. Fish fatty acid phenotypes reflected dietary fatty acid profiles. Growth performance, survival after acute stress, and similar whole-body basal cortisol levels suggested seabream larvae could tolerate a wide range of dietary ARA supply. Transcription of all genes analysed was significantly reduced at dietary ARA levels above 0.4%. Nonetheless, despite practical suppression of phospholipase A_2 transcription, higher leukotriene B_4 levels were detected in larvae fed 3.0% ARA, whereas a similar trend was observed regarding prostaglandin E_2 production. This study demonstrates that adaptation to a wide range of dietary ARA levels in gilthead seabream larvae involves the modulation of the expression of genes related with eicosanoid synthesis, lipid metabolism, and stress response. The role of ARA, other polyunsaturates and eicosanoids as signals in this process is discussed.

Introduction

Intensive fish aquaculture can negatively impact animal welfare, hence farming practices have developed with the intention of minimising stress below the threshold of prepathological manifestation, thereby avoiding diseases and mortality[1]. Stress resistance has been admitted as an important indicator of a fish's physiological condition and considered as a target for genetic improvement since stress can negatively affect relevant production traits[2,3]. The unavailability of essential nutrients, especially during delicate early life stages, may compromise normal development and survival. In fact, high mortalities (up to 99% in nature) are considered normal for marine teleost larvae. In face of a stressor, energetic resources must be diverted away from growth and other biological processes into a stress coping response. Therefore, it is important to provide fish larvae with nutrients that optimise their growth and survival, and that satisfy extra energy requirements inherent to intensive production. Dietary lipid in particular, strongly influences immunity and response to stress associated with handling and suboptimal environmental conditions[4-6].

Dietary lipids are a major source of energy and provide essential fatty acids and phospholipids, widely acknowledged as critical success factors for larval fish rearing[7,8]. In marine fish nutrition, major attention has been given to docosahexaenoic (DHA; 22:6n-3) and eicosapentaenoic acids (EPA; 20:5n-3) due to their predominance in fish tissues, particularly in cell membranes, but the potential of arachidonic acid (ARA; 20:4n-6) to affect growth, survival and stress resistance has also been recognised[9,10]. The dietary requirement for ARA during early larval development in gilthead seabream (*Sparus aurata*) has been linked to survival during the stressful events of metamorphosis, weaning, crowding, grading[11], and other handling procedures[12-14]. Various fatty acids and phospholipids have long been demonstrated to present stress resistance conferring properties in fish[15-19], although the mechanisms involved are still somewhat speculative.

Modulation of cellular membrane structure and/or function, through diet-induced changes in phosphoacylglyceride fatty acids, is likely responsible for major dietary outcomes on fish physiology[7,20], including stress-reducing effects[5]. The consequences of dietary supplementation in certain long-chain polyunsaturated fatty acids (LC-PUFA) on stress tolerance in fish are often suggested to be mediated by eicosanoids affecting corticosteroid production[21,9,13,14]. Most studies addressing this issue have focused on ARA-derived eicosanoids since these are generally considered the most abundant and bioactive, whereas those produced from EPA tend to be of lower efficacy[22,23]. Recent *in vitro* studies using gilthead seabream head kidney cells have clearly demonstrated the participation of cycloxygenase and lipoxygenase metabolites on cortisol release[24,25], as hypothesised in a model proposed for steroidogenesis regulation in mammals[26]. The regulation of the steroidogenic acute regulatory protein (StAR), a key rate-limiting enzyme in steroidogenesis, by ARA and its metabolites has still not been examined in fish. In mammalian research for example, cycloxygenase-2 inhibition, or 5-lipoxygenase- and epoxygenase-derived ARA metabolites have been reported to enhance StAR gene transcription and steroidogenesis[27-29]. Furthermore, fatty acids and eicosanoids serve as ligands for nuclear receptors which may affect the transcription of genes involved in lipid and energy homeostasis, including cholesterol metabolism[30,31] which is central in steroidogenesis. Indeed, PPARs have been reported to modulate genes involved in cholesterol uptake and transport[32], including StAR[33], hence affecting steroid production in mammals[34]. Similar interactions between PPARs and StAR have been recently implicated in Atlantic salmon (*Salmo salar*)[35].

Cortisol release from the interrenal cells may be affected by the relative abundance of fatty acids through other pathways, such as calcium messenger systems[26]. Enhancement of intracellular calcium levels by ARA or its metabolites, including leukotriene B_4[36-39], could play an additional role in steroidogenesis regulation.

Within target cells, cortisol signalling entails the activation of glucocorticoid receptors (GRs), their translocation into the nucleus and binding to the promoter of glucocorticoid responsive genes, hence

104 modulating their expression[40]. In fish, the existence of non-genomic pathways involving
105 membrane-bound proteins is still unclear[41,42]. A study in seabream larvae showed GR mRNA
106 abundance could be affected by dietary lecithin source[43]. Also, *in vitro* studies in fish[44] and
107 mammals[45,46] have shown dose-dependent suppression of GR binding by unsaturated fatty acids, a
108 mechanism possibly mediated *in vivo* by fatty acid binding proteins[47]. Despite the common use of
109 cortisol as a stress indicator, GRs are recognised to mediate actual physiological effects of this
110 hormone. Hence, studying the potential of dietary fatty acids to modulate these receptors is likely to
111 provide clues as to how lipid nutrition could affect the stress response in fish larvae.
112 The objective of this study was to advance our knowledge on the role of dietary fatty acids in
113 regulating metabolic pathways involved in the stress response in fish. Specifically, we have
114 examined potential effects of dietary ARA levels on cortisol production and the expression of genes
115 related to stress response in gilthead seabream larvae. These included StAR, GR, PPARα, and
116 eicosanoid synthesis enzymes. The transcription of hormone sensitive lipase (HSL), possibly
117 regulated by GRs[48,49], and phosphoenolpyruvate carboxykinase (PEPCK) were also analysed. The
118 production of ARA-derived eicosanoids (prostaglandin E_2 and leukotriene B_4) was determined, and
119 overall results were examined in light of larval fatty acid phenotypes.
120

Experimental methods

Larval rearing

Animal manipulations were carried out in compliance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals. Protocols were performed under license of Group-1 from the General Directorate of Veterinary (Ministry of Agriculture, Rural Development and Fisheries, Portugal).

Gilthead seabream eggs were obtained from INRB/IPIMAR EPPO facility (Olhão, Portugal) and the experiment conducted at the Centre of Marine Sciences (University of Algarve, Faro, Portugal). The larvae were distributed into 12 cylindro-conical tanks (100 litres), at a density of 100 larvae per litre. This tank system was supplied with constantly aerated seawater (18.4 ± 0.6 ^{o}C temperature, salinity about 35.6, and dissolved oxygen about 6 mg/l), kept under a photoperiod of 14 h light: 10 h dark until 16 days after hatch (DAH) and constant lighting conditions from thereon. Tank water renewal was 0.5 times daily initially and increased up to 8 times per day throughout the course of the experiment. During this period, the green water technique was applied to the rearing tanks with the addition of *Tetraselmis suecica* (clone *chuii*) and *Isochrysis galbana*. From 4 DAH, the larvae were fed rotifers (*Brachionus plicatilis*) previously enriched with a commercial product (Easy DHA Selco, INVE Aquaculture, Belgium), and gradual replacement with the experimental microdiets occurred from 9 to 25 DAH, after which the larvae were fed exclusively on microdiets. However, from 15 DAH the amount of rotifers supplied was nutritionally negligible (about 1 rotifer per ml) in order to stimulate larval feeding behaviour. Monitoring of water quality, tank maintenance and removal of mortalities were performed daily.

Experimental diets

147 Four microdiets were manufactured according to the method of microencapsulation by
148 emulsification and internal gelation[50] and presented graded ARA levels ranging from 3.7 to 30.0
149 g/kg feed. These doses were selected in order to vary from a relatively low ARA level commonly
150 found in larval microdiets to a level sufficiently high as to lower the dietary EPA/ARA ratio below
151 one. This was expected to highlight potential effects of ARA abundance in the tissues on the
152 expression of genes and other parameters studied. Dietary formulations are presented in table 1.
153 Sieving allowed the separation of microdiets into two size classes to be used according to larval
154 size: 80 to 200 µm, and 200 to 400 µm.
155
156 *Experimental design and sampling procedures*
157
158 The beginning of this experiment was considered to be at 16 DAH, when about 75 % of the larvae
159 were estimated to accept the microdiets, through microscopic observation of gut content. The
160 photoperiod was then changed to continuous lighting conditions and rotifer supply reduced
161 considerably. The microdiets were tested in triplicate and distributed by automatic feeders
162 (Fishmate, PETMATE, UK) 5 times daily from 9 to 16 DAH, and 8 times per day (every 3 h) from
163 16 DAH until 34 DAH.
164 At the end of the experiment, 30 larvae per tank were subjected to an acute stress consisting of 1
165 min gentle stirring, in a 1 litre beaker, and left to recover for 24 h at the end of which mortalities
166 were recorded and live larvae sampled for whole-body cortisol determination. Cortisol levels were
167 determined under basal conditions and at 24 h post-stress only, due to the shortage of larvae at the
168 end of the experiment and the large number of fish required for other biochemical analyses. The 24
169 h period was selected in order to evaluate also stress resistance (survival) one day after acute stress.
170 Initial average dry weight (16 DAH) was determined from pooled samples (200 larvae per tank),
171 which were stored at -20 °C until measurements could be conducted. At the final sampling, 50
172 larvae per tank were collected for individual dry weight assessment, and about 100 per tank were

stored at -80 °C for lipid and fatty acid composition analysis. Whole-body cortisol concentration was assessed, before (n = 15) and 24 h after stress (n = 15), in pooled larvae samples which were kept at -80 °C until analyses could be conducted. Furthermore, 50 larvae per tank were sampled for whole-body eicosanoid determination and stored in HBSS (Hank's balanced salt solution, Sigma), containing 15 % ethanol (v/v) and 5 % formic acid (2 N), at -20 °C. Finally, for gene expression analysis, 10 larvae per tank were preserved in RNAlater at 4 °C for 24 h and then at -20 °C. All larvae sampled were previously anaesthetised with an overdose of 2-phenoxyethanol and washed with distilled water before storage or measurements, with the exception of those intended for gene expression analysis, which were stored directly in RNAlater.

Analytical methods

The microdiets were analysed for proximate composition according to the following procedures: *DM* determined gravimetrically by drying in an oven at 105 °C for 24 h; crude ash by incineration in a muffle furnace at 500 °C for 12 h; crude protein (N * 6.25) assessed by a nitrogen determinator (LECO, FP-528); total lipid extracted with petroleum ether (Soxhlet 40 – 60 °C); and gross energy in an adiabatic bomb calorimeter (IKA C2000). For fatty acid composition analyses of the microdiets and larvae, acid-catalyzed transesterification[51] was performed, to produce fatty acid methyl esters (FAME) which were measured and quantified by GC in a Varian Star 3800 CP (Walnut Creek, CA) equipped with an auto-sampler and fitted with a flame ionisation detector at 250 °C. The separation was performed in a polyethylene glycol capillary column DB-WAX 30 m-length, 0.25 mm i.d., and 0.25 mm film thickness from J&W Scientific (Folsom, CA, USA). The column was subjected to a temperature program starting at 180 °C for 5 min, increasing by 4 °C/min for 10 min, and held at 220 °C for 25 min. The injector (split ratio 100:1) and detector temperatures were kept constant at 250 °C during the 40 min analysis. Fatty acid peaks were

identified by directly comparing retention times with those of a known standard ("PUFA 3"; SIGMA-ALDRICH®, USA) and quantified by means of the response factor to an internal standard (21:0) which was used at 5 ml/mg of sample.

Survival at the end of the experiment and at 24 h post-stress was determined by direct counting of individuals, relative to the initially stocked number of larvae, and excluding the 200 individuals sampled at 16 DAH. Individual determination of whole-body dry weight was performed in a Sartorius M5P balance (0.001 mg precision; Sartorius micro, Göttingen, Germany) after freeze-drying the samples for 24 h in a Savant SS31 (Savant Instruments Inc., Hokbrook, NY, USA).

Whole-body cortisol was determined in pooled larvae samples of about 150-300 mg per tank (wet weight), according to methodology which has been described previously[43], and using a commercial cortisol ELISA kit (Neogen Corporation, Lexington, KY, USA).

For the determination of whole-body eicosanoid concentration, samples were homogenised in the storage solution and centrifuged to remove debris. The supernatants were extracted using octadecyl silyl (C_{18}) "Sep-Pak" cartridges (Millipore), as described in detail by Bell *et al.*[52]. The extracts were dried under nitrogen, re-dissolved in 1 ml of methanol and stored in glass vials at –20 ^{o}C awaiting immunoassay analysis. Upon sample preparation for analysis, 500 µl of the methanol extracts were dried under nitrogen, re-dissolved in 2 ml of EIA buffer, and loaded onto the plate contained in the assay kit. Eicosanoids were quantified using enzyme immunoassay kits, namely, Prostaglandin E_2 EIA kit (Cayman, ref. 514010) and Leukotriene B_4 EIA kit (Cayman, ref. 520111), according to the manufacturer's instructions.

Total RNA from individual fish larvae (average weight 1.5 mg) was extracted using the QIAGEN RNeasy® Plus Mini Kit designed to purify RNA from small amounts of animal tissues (maximum 30 mg) allowing yields of up to 100 µg total RNA. Total body tissue was disrupted and homogenized using a rotor-stator homogenizer Ultra Turrax T8 (IKA®-Werke) and RTL plus buffer. The lysate was passed through a genomic DNA (gDNA) Eliminator spin column to remove all gDNA contamination. The sample was transferred into an RNeasy spin column where total RNA

224 bonded to a membrane and contaminants were washed away. Purified RNA was then eluted with 30
225 µl of RNase-free water. The quality and quantity of the RNA were assessed using the Bioanalyzer
226 2100 (Agilent Biosystem, Germany) and the RNA 6000 Nano kit, accurate to a qualitative range of
227 5 – 500 ng/µL. One nano chip carried up to 12 RNA samples of 1 µL each. Through electrophoresis
228 analysis of RNA with nano chips, two peaks were detected in well preserved samples (RNA
229 fragments 18S and 28S). After detection, the ratio of the fragment areas and the RNA integrity
230 number (RIN) were calculated. RNA was quantified spectrophotometrically at 260 nm using the
231 Eppendorf Biophotometer Plus and plastic Eppendorf UVettes® RNase free. The analysis was
232 performed with 5 µL per sample, diluted with 50 µL of DEPC water and the correction factor
233 automatically calculated. The cDNAs were synthesized from 500 ng of total RNA using the
234 qScript-cDNA synthesis kit (Quanta Bioscience™) according to the manufacturer's instructions, in
235 a Mastercycler® VapoProtec (Eppendorf, ProS).
236 Gene expression was analyzed by real time Q-PCR using the Mastercycler® ep Realplex^2 S system
237 (Eppendorf) and the procedure provided by the PerfeCTa SYBR Green kit (Quanta). Seabream
238 specific primers were used, with β-actin as the normalisation gene, in a final volume of 20 µL per
239 reaction well, using 12 ng of total RNA reverse transcribed to cDNA. The amount of cDNA per
240 reaction was established after *a priori* optimisation tests, considering the efficiency of the
241 amplification process and the regression fit to 6 serial 10-fold dilutions of cDNA. Moreover, each
242 primer pair annealing temperature and concentration were established in advance using the
243 temperature gradient function of the thermocycler. Each gene sample was analysed in triplicate. The
244 PCR conditions were: 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s,
245 and a final denaturing step from 60 °C to 95 °C during 20 min to check for primer-dimers and
246 spurious amplification products. The ΔΔCt method[53] was used to determine the relative mRNA
247 expression levels. For gilthead seabream specific primer design, nucleotide and EST GenBank
248 databases were searched for the following genes: PLA_2, COX-2, 5-LOX, StAR, GR, PPARα,

PEPCK, and HSL. Oligonucleotides were designed using the Primer3 program, and ordered HPLC-purified. Primer sequences and accession numbers for the mRNAs analysed are described in table 2.

Statistical analysis

Larval growth expressed as relative growth rate (RGR) was determined at the end of the experiment for all treatment groups, according to the following equation[54]: RGR = (e^{g}-1) x 100, with g = [(ln final weight – ln initial weight)/time]. A one-way ANOVA was used, with dietary treatment as the independent variable, for the statistical analysis of growth performance, whole-body fatty acid composition, eicosanoid, and gene expression data. For data not presenting variance homogeneity and normal distribution, the Kruskal-Wallis and Dunnett tests were performed. Cortisol data were analysed by a 2 * 2 mixed-design ANOVA to assess the effect of diet (between-subject variable) and sampling time (within-subjects variable, i.e. differences between basal values and levels detected 24 h post-stress). Differences were considered significant when $P < 0.05$. Tukey HSD multiple mean comparison test was used to identify differences between means. The relationships between fatty acid concentrations in diet and in fish can differ among fatty acids. Hence, Pearson's correlation coefficients and differences (Δ values) between the percentages of selected fatty acids in larval lipids and in dietary lipids were calculated (% total fatty acids). Pearson's coefficients were also used to explore correlations between cortisol levels and fatty acid content of the larvae. All statistical tests were conducted with the software package SPSS® 16.0 for Windows®.

Results

The proximate composition of the microdiets showed crude protein levels of 58 - 60 %, crude lipid content between 25 – 27 % and gross energy about 25 kJ/g of *DM* (table 1). Dietary fatty acid composition is presented in table 3. Total fatty acid content analysis showed values between 171 and 182 mg/g diet. Saturates represented 3.4 - 4.0 % of the diet, whereas monounsaturated fatty acids were about 4.3 - 4.5 %, mainly oleic acid (OA; 18:1n-9). Polyunsaturated fatty acid content ranged from 9.3 to 10.2 %. Among polyunsaturates, ARA increased from 0.4 (ARA0.4) to 3.0 % (ARA3.0), whereas linoleic (LA; 18:2n-6) and linolenic (LNA; 18:3n-3) acid concentrations decreased with ARA addition. However, EPA and DHA levels were relatively constant between dietary treatments. Thus, dietary EPA/ARA and DHA/ARA ratios were lowered with increasing dietary ARA inclusion, whereas the DHA/EPA ratio was maintained practically identical between diets.

Seabream initial dry weight was 71.3 ± 10.6 µg per larva and, despite slightly lower relative growth rates in the mid-range treatments at the end of the experiment, no significant differences were observed in growth parameters or survival between dietary groups (table 4). Overall, RGR values were approximately 3.6 - 6.0 % per day, whereas survival was determined between 4.5 % and 5.8 %. At 24 h after acute stress, this parameter varied between 84 - 90 % without statistically significant differences between experimental groups.

Whole-body fatty acid composition reflected dietary profiles, particularly regarding ARA which increased from 4.2 % total fatty acids in ARA0.4 groups to 11.6 % in ARA3.0 fed larvae (table 5). The Pearson correlation coefficient for ARA was 0.99 and Δ values indicated its preferential retention up to 1.5 % dietary content, whereas at the highest dietary concentration tested Δ values pointed to its preferential metabolism (table 6). The DHA content of the seabream was high (26.8 – 28.6 %) and Δ values suggested strong preferential deposition of this fatty acid in fish tissues. On the other hand, despite relatively similar EPA concentrations among the experimental microdiets,

295 larval levels were significantly reduced in groups fed ARA1.5 and ARA3.0 diets. The relationship
296 between larval contents in ARA and EPA was also analysed and Pearson's correlation coefficient (-
297 0.997) indicated a strong negative correlation between the two fatty acids in the tissues (Figure 1).
298 Furthermore, unlike ARA or DHA, EPA appeared to be preferentially metabolised in all
299 experimental groups, and more so as ARA levels increased in the larvae, as suggested by Δ values.
300 Regarding EPA/ARA and DHA/ARA ratios a significant decrease was noted as ARA deposition
301 increased in the larvae. In particular, EPA/ARA ratio was only above 1.0 in the group supplied with
302 the lowest ARA levels. The ratio DHA/EPA was highest in ARA3.0 fed groups. Other
303 polyunsaturates, LA and LNA, decreased significantly reflecting dietary differences, and seemed to
304 be preferentially metabolised by the larvae as well as OA. No statistically significant differences
305 regarding saturated and monounsaturated fatty acids were identified between groups. Finally,
306 palmitic (PA; 16:0) and stearic acids (SA; 18:0) were preferentially retained in the larval tissues in
307 all experimental groups and their respective Pearson's correlation coefficients were relatively low.
308 Whole-body basal cortisol levels (Figure 2) ranged between 12 and 18 ng/g larvae wet weight, and
309 levels at 24 h post-stress were between 20 and 30 ng/g. No statistical interaction was found between
310 diet and sampling time (P = 0.70). Overall, cortisol values did not differ between experimental
311 groups (P = 0.12) but significantly higher levels were found at 24 h post-stress than before stress (P
312 = 0.005).
313 Whole-body prostaglandin E_2 measurements did not show significant differences between
314 treatments (P = 0.21), whereas the highest leukotriene B_4 levels were determined in seabream larvae
315 fed the ARA3.0 diet (P = 0.04; Figure 3).
316 All genes studied showed significant differences in expression among dietary treatments (Figure 4).
317 Above the lowest dietary ARA level tested (0.4 %) the transcription of the eight analysed genes was
318 significantly depressed. In particular, a drastically reduced expression of PLA_2 was observed,
319 whereas other enzymes involved in eicosanoid synthesis (5-LOX and COX-2) showed decreased
320 mRNA levels to only about half in groups fed 0.8 % ARA and above. PPARα mRNA abundance

was also highest in the lowest ARA fed groups. The StAR gene, encoding for the rate-limiting enzyme in steroidogenesis, presented a similar trend. On the other hand, GR gene expression appeared to be gradually reduced as dietary ARA supply increased. HSL transcript levels were only slightly higher in ARA0.4 fed larvae as compared to other groups, while PEPCK gene expression was up to 6-fold higher in ARA0.4 fed fish than in other experimental groups.

Discussion

This study clearly shows that gilthead seabream larvae can tolerate a wide range of dietary ARA levels, as neither growth rates nor survival presented significant differences between experimental groups. Variation in responses obtained for other analysed parameters (eicosanoid levels, gene expression) did not appear to compromise the general growth performance of the fish for the duration of the experiment, and could be regarded as adaptive to the nutritional conditions tested. Relatively low survival was registered between 16 and 34 DAH which may well relate to the stress of weaning (performed at an early life stage) combined with the fairly small tank volumes used in the rearing system. Still, the survivals observed are within the normal range for the species, despite higher values which have been reported when microdiets were offered at a later stage[55,43]. Relative growth rates may have reflected the same type of constraints, as higher values have been reported for seabream larvae fed casein-based microencapsulated diets[56]. Nonetheless, seabream larvae more than doubled their initial weight during the experimental period and their fatty acid profiles clearly reflected the dietary fatty acid composition at the end of the study.

Besides the increase in ARA content in larval tissues from ARA0.4 to ARA3.0 groups, it is also important to note reduced LA and LNA deposition which mirrored dietary profiles. In contrast, EPA content in ARA3.0 fed fish was about 60 % that determined for ARA0.4 larvae, despite similar dietary supply levels. This reduction in EPA concomitant with increased ARA levels in larval tissues is a clear indication of competition between these fatty acids for inclusion into fish tissues by acyltransferases as reported in this[14] and other species[57]. The suggested displacement of EPA by increasing ARA competition is in accordance with results obtained from comparisons between dietary and larval fatty acid profiles (% TFA, see table 6), which indicated preferential EPA metabolism, especially with the increase in dietary ARA. In fact, whereas the EPA/ARA ratio was below 1.0 only in the ARA3.0 diet, in larval whole-body the same ratio was found equal or

lower than 1.0 in all groups receiving dietary ARA levels above 0.4 %. The EPA/ARA ratio is of particular interest since it is a major determinant of eicosanoid production and bioactivity.

An important shift in eicosanoid profiles can affect various metabolic pathways, including the stress response, as often proposed in fish[21,13,14,58]. Leukotriene B_4, an eicosanoid known for its pro-inflammatory properties[23], showed a clear increase in groups supplied with the highest dietary ARA levels. On the other hand, only such a trend could be identified regarding prostaglandin E_2. Preferential ARA metabolism was in fact suggested by comparison between dietary and larval fatty acid profiles (% TFA). Nonetheless, genes related to eicosanoid production showed highest expression in ARA0.4 fed fish, in particular PLA_2. However, it is well known that PLA_2 is not strictly required for ARA release from cellular stores since other enzymes, like acyl-CoA synthetase 4 and acyl-CoA-thioesterase, may undertake this role[59]. Despite nearly complete suppression of PLA_2 gene expression and significant downregulation of COX-2 and 5-LOX genes, high dietary ARA supply (3 %) or low EPA/ARA ratios in larval whole body (0.3) caused significantly higher LTB_4 production. It is possible that reduced transcription of these genes was an adaptation, as a result of a negative feed-back mechanism, to a transient increase in ARA-derived eicosanoids in groups supplied with dietary EPA/ARA ratios ≤ 2.5, in order to maintain these metabolites within "normal" physiological concentrations.

A study in 28 DAH seabream larvae showed cortisol peaks around 20-40 min past a similar type of stress [14]. All groups seemed able to cope with the acute stress imposed as survival past 24 h was 84 - 90 %, overall, and cortisol levels did not differ significantly between groups at this time. Together, these data suggest larval resistance to the stress test was not affected by the diets, which further supports the idea that seabream larvae could adapt to changes induced by different fatty acid supply. Various studies in seabream larvae have reported positive effects of ARA on growth, survival[9] or stress resistance[12,14], although results may depend much on the nature of the stressor applied, larval stage[13,14], feed type, rearing conditions, genetic factors, as well as on the relative abundance of other fatty acids (n-3 LC-PUFA). Hence, a direct comparison between studies conducted in

different labs may be difficult. For instance, an effect of EPA supply was reported previously in seabream larvae survival to air exposure and temperature shock[60], whereas present results showed no trend in stress resistance despite the reduction in larval EPA as ARA increased. However, the maintenance of elevated DHA levels in all groups (27-29% total fatty acids) compared to previously cited studies could have masked effects potentially induced by ARA or other fatty acids on stress resistance. Studies in larvae of other marine fish species also support the superior role of DHA as an essential fatty acid relative to EPA and ARA in terms of growth and resistance to a vitality test[61, 62]. In face of a stressor, efficient ATP production is required to satisfy the extra energy demand. It is possible that larval cardiorespiratory performance for example, could be affected by dietary DHA which is structurally important for cardiolipin, a phospholipid found abundantly in fish mitochondrial membranes[20].

This experiment evidenced effects of fatty acid supply on the modulation of the expression of various genes in undisturbed seabream larvae. The ability of metabolic factors to activate PPARs allows for these transcription factors to alter gene expression in response to the nutritional status of the animal[63]. LTB_4 and ARA, but also C_{18} unsaturates, are important ligands for PPARα[64] and therefore may have affected its transcriptional activity. This could potentially involve the regulation of the StAR gene[33] and, in fact, the expression pattern of the two genes among experimental groups presented striking similarities. The implications of LC-PUFAs and their derivatives in steroidogenesis, particularly cortisol synthesis, are numerous and complex, and have been addressed in recent years in seabream studies[24,25]. Still, differences in StAR expression among groups did not affect basal cortisol levels. In fact, the control of StAR activity even following acute stress or ACTH signalling in fish may be exerted at the post-transcriptional level[65-67], involving steps such as StAR protein phosphorylation for the activation of the enzyme[68].

Glucocorticoid receptors are central in mediating the genomic actions of cortisol and this study clearly demonstrated a downregulation of the GR gene expression with increasing dietary ARA supply. Various GR transcription factors which are sensitive to fatty acid signalling may mediate

403 this effect. In mammals, NF-κB and AP-1, for example, can be directly activated by fatty acids such
404 as LA and EPA, or by PPARs to regulate GR transcription[69-71]. As previously noted, differences in
405 larval fatty acid profiles were not limited to ARA and it is likely that gene expression results
406 reflected the combined actions of other fatty acids (EPA, LA, LNA). In seabream larvae fed similar
407 microdiets varying in lecithin source, upregulation of the GR gene in soybean lecithin fed fish was
408 associated with higher LA and slightly reduced ARA levels in the larvae polar and total lipid[43]. In
409 comparison, the present results showed higher variation in the expression of the GR gene which
410 could be due to larger discrepancies between LA and ARA contents between groups. Therefore,
411 clear evidence exist that GR gene expression in seabream can be affected by dietary LA and/or
412 ARA supply.
413 Slightly increased HSL expression associated with higher GR expression was found in ARA0.4 fed
414 groups relative to other treatments, as reported previously[43]. In addition, highest PEPCK transcript
415 levels were found in these fish. The two genes are known to respond to GR activity in mammals,
416 although to our knowledge a corticosteroid responsive element has not been identified in fish HSL
417 promoter[48,49]. Both enzymes are responsible for the release of energy substrates into the blood
418 stream. Given the differences in expression between groups under basal conditions, particularly of
419 the PEPCK gene, it would be interesting to assess their expression in response to an acute stress.
420 In summary, this study demonstrated that gilthead seabream larvae adaptation to a wide range of
421 dietary ARA levels involves changes in the expression of genes associated with eicosanoid
422 synthesis, lipid metabolism and stress response. All genes analysed were significantly
423 downregulated in seabream larvae presenting whole body EPA/ARA ratios ≤ 1, but also lower LA
424 and LNA levels relative to ARA0.4 groups. Therefore, observed effects may not be due to the
425 increase in ARA supply alone. Fatty acids and their derivatives can signal nuclear receptors and
426 transcription factors (such as PPARs), interact with StAR and GRs, indirectly modulate metabolic
427 pathways related with energetic metabolism (HSL, PEPCK), and ultimately affect stress coping
428 ability. It is possible that high larval DHA levels in all experimental groups contributed to the

absence of differences in growth and survival after an acute stress challenge, masking potential effects of deficient/excessive ARA supply or of an inadequate dietary ARA/EPA ratio. Considering the increasing interest in promoting animal welfare under intensive farming conditions, and within the current context of research for alternative dietary lipid sources in aquaculture, this information may be valuable for the optimisation of feeds containing vegetable oils rich in C_{18} fatty acids but lacking in LC-PUFAs.

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455 D.A.M. and F.R. performed the statistical analysis; all authors contributed for manuscript writing,
456 and D.A.M. had primary responsibility for the final content. All authors read and approved the
457 manuscript.

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634 Table 1. Formulation and proximate composition of the experimental microencapsulated diets, prepared by
635 internal gelation, for gilthead seabream larvae

	Diet			
	ARA0.4	ARA0.8	ARA1.5	ARA3.0
Ingredients (g/kg)				
Fish meal[*]	50.0	50.0	50.0	50.0
Fish hydrolysate[†]	100.0	100.0	100.0	100.0
Cuttlefish meal[‡]	420.0	420.0	420.0	420.0
Casein[§]	50.0	50.0	50.0	50.0
Sodium alginate[‖]	70.0	70.0	70.0	70.0
Dextrine[¶]	13.0	9.0	7.0	4.0
Soybean lecithin[**]	50.0	50.0	50.0	50.0
Linseed oil[††]	35.0	30.0	30.0	-
Sunflower oil[‡‡]	34.0	30.0	10.0	-
Olive oil[§§]	20.0	20.0	20.0	20.0
ARASCO[‖‖]	7.0	21.0	44.0	88.0
DHASCO[¶¶]	40.0	40.0	40.0	40.0
Incromega[***]	31.0	30.0	29.0	28.0
Vitamin premix[†††]	20.0	20.0	20.0	20.0
Vitamin C[‡‡‡]	30.0	30.0	30.0	30.0
Vitamin E[§§§]	10.0	10.0	10.0	10.0
Mineral premix[‖‖‖]	20.0	20.0	20.0	20.0
Proximate composition				
DM (%)	97.4	96.8	96.9	97.1
Protein (% DM)	58.0	59.5	59.5	57.9
Lipid (% DM)	27.3	25.0	25.4	27.3

Ash (% DM)	3.8	3.5	4.0	3.7
Carbohydrates (% DM) ¶¶¶	10.9	11.9	11.1	11.0
Energy (kJ/g DM)	25.4	25.1	25.2	25.8

636 * AgloNorse Microfeed, Norsildmel Innovation AS, Bergen, Norway.

637 † CPSP-90, Soprepêche, France.

638 ‡ Squid Powder 0278, Rieber & Søn ASA, Bergen, Norway.

639 § VWR International, Fontenay sous Bois, France.

640 ‖ MP Biomedicals 154724, LLC, Illkirch, France.

641 ¶ Commercial grade type I, MP Biomedicals, LLC, Illkirch, France.

642 ** Lecithin Soy Refined, MP Biomedicals, LLC, Illkirch, France.

643 †† Commercial linseed oil, Biolasi Productos Naturales, S.L., Ordizia, Guipúzcoa, Spain.

644 ‡‡ Commercial sunflower oil, Ibarrasol, Aceites Ybarra S.A., Dos Hermanas, Spain.

645 §§ Commercial olive oil, Hacendado, Sovena Iberica de Aceites S.A., Brenes, Spain.

646 ‖ ‖ Vegetable oil from fungi, approximately 40 % ARA, Martek life enriched TM, Martek
647 Biosciences Corporation.

648 ¶¶ Vegetable oil from microalgae, approximately 40 % DHA, Martek life enriched TM, Martek
649 Biosciences Corporation.

650 *** Incromega TG7010 SR, Croda, USA.

651 ††† Vitamin premix supplied the following (per kg of diet): retinol/cholecalciferol 500/100, 1000
652 mg; cholecalciferol 500, 40 mg; α-tocopherol acetate, 3000 mg; menadione 23 %, 220 mg; thiamine
653 HCl, 50 mg; riboflavine 80, 250 mg; d-Ca pantothenic acid, 1100 mg; nicotinamide, 500 mg;
654 pyridoxine, 150 mg; Pteroylglutamic acid, 50 mg; cyanocobalamin 0.1, 500 mg; biotin 20, 38 mg;
655 ascorbic acid poliphosphate 35 %, 57.2 g; choline chloride 60 %, 100 g; myo-inositol, 15 g;
656 antioxidants, 1.25 %.

657 ‡‡‡ Sodium, calcium ascorbyl-2-phosphate, Rovimix STAY-C 35, DSM Nutritional Products, Inc.

658 §§§ DL-alpha-tocopherol acetate, MP Biomedicals 100555, LLC, Illkirch, France.

659 ‖ ‖ ‖ Mineral premix supplied the following (per kg of diet): monocalcium phosphate, 35.2 %;
660 calcium carbonate, 11.5 %; sodium chloride, 20 %; potassium chloride, 26 %; copper sulphate,
661 0.024 %; magnesium sulphate, 5 %; ferrous sulphate, 0.6 %; manganous sulphate, 0.81 %; zync
662 sulphate, 0.17 %; potassium iodide, 0.0031 %; sodium selenite, 0.6 %.
663 ¶¶ Carbohydrates = 100 - (protein + lipid + ash)

664 Table 2. Sequences of forward and reverse primers (5'- to 3') for real-time Q-PCR of seabream
665 genes and amplification product size

Primer	Forward	Reverse	Product size (bp)	Accession number
β-actin	TCTTCCAGCCATCCTTCCTCG	TGTTGGCATACAGGTCCTTACGG	108	X89920
StAR	ACGCAGGTGGACTTTGCCAAC	TGAGTGCACGGTGCCAAAGC	115	EF640987
GR	GATGACCACCCTCAACAGGT	TTAGGAAGAGCCAGGAGCAC	134	DQ486890
PPARα	ACCGCAACAAGTGCCAGTA	TTCTCCACCACCTTTCGTTC	133	AY590299
PLA_2	CCAGACCATCTTCACCATCC	CACCCAATCCACAGGAGTTC	114	AF427868
COX-2	CGTCTGCAATAACGTGAAGG	CCTGAGTGGGACGTGCTC	105	AM296029
5-LOX	CCTGGCAGATGTGAACTTGA	CGTTCTCCTGATACTGGCTGA	100	FP334124
HSL	CGGCTTTGCTTCAGTTTACC	ACCCTTCTGGATGATGTGGA	115	EU254478
PEPCK	AGAGCCATCAACCCTGAGAA	CTCCCACCACACTCCTCCAT	144	AF427868

666 StAR, steroidogenic acute regulatory protein; GR, glucocorticoid receptor; PPARα, peroxisome
667 proliferator-activated receptor alpha; PLA_2, phospholipase A_2; COX-2, cycloxygenase-2; 5-LOX, 5-
668 lipoxygenase; HSL, hormone-sensitive lipase; PEPCK, phosphoenolpyruvate carboxykinase.

669 Table 3. Total fatty acid content (mg/g diet DM) and fatty acid composition (g/100 g diet DM) of
670 the experimental diets

	Diet			
	ARA0.4	ARA0.8	ARA1.5	ARA3.0
Total FAMEs	182.2	171.7	171.3	179.3
Fatty acid				
16:0	2.2	2.0	2.1	2.4
18:0	0.6	0.6	0.7	0.9
SAFA	3.5	3.4	3.5	4.0
16:1*n*-7	0.1	0.1	0.1	0.1
18:1*	4.2	3.9	4.0	4.0
20:1*n*-9	0.2	0.2	0.2	0.2
MUFA	4.5	4.3	4.3	4.3
18:2*n*-6	3.4	2.8	1.9	1.5
20:4*n*-6	0.4	0.8	1.5	3.0
n-6 PUFA	3.8	3.6	3.6	4.7
18:3*n*-3	1.4	1.1	1.1	0.1
20:5*n*-3	2.0	1.9	1.8	1.8
22:6*n*-3	2.7	2.6	2.6	2.8
n-3 PUFA	6.3	5.9	5.7	4.8
PUFA	10.2	9.5	9.3	9.6
DHA/EPA	1.3	1.4	1.4	1.5
EPA/ARA	5.4	2.5	1.2	0.6
DHA/ARA	7.3	3.5	1.7	0.9

671 * Includes 18:1*n*-7 and 18:1*n*-9.

672

673 Table 4. Growth performance* and survival at 24 h after stress of seabream larvae fed the

674 experimental diets containing graded ARA levels (Mean values with their standard deviations)

675

	ARA0.4		ARA0.8		ARA1.5		ARA3.0	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dry weight (µg/larva)	186.8	39.3	160.8	5.8	159.2	9.6	196.4	62.6
RGR (%/day, 16-34DAH)	5.8	1.7	5.0	0.0	3.6	1.0	6.0	1.7
Survival (%, 16-34DAH)	4.8	0.5	5.8	1.4	5.6	1.0	4.5	1.7
Survival at 24 h (%)	86.3	3.9	89.8	6.3	89.4	15.3	83.7	8.5

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682 * Initial dry weight, 71.3 ± 10.6 µg/larva.

683 RGR, relative growth rate.

684 Absence of letters means no statistical differences (ANOVA, $P > 0.05$).

685

686 Table 5. Whole-body total fatty acid content (mg/g sample) and profile (g/100 g total fatty acids) of seabream
687 larvae fed graded ARA levels
688 (Mean values with their standard deviations)

	ARA0.4		ARA0.8		ARA1.5		ARA3.0	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total FAME	71.1	1.3	70.3	12.8	71.1	2.0	65.3	2.0
Fatty acid								
14:0	0.9	0.0	0.9	0.1	0.8	0.0	0.8	0.1
16:0	17.8	0.9	17.0	1.4	17.4	0.4	17.4	0.4
18:0	10.1	0.3	9.6	0.4	10.1	0.2	10.4	0.5
SAFA	30.1	1.0	29.3	1.5	29.9	0.5	30.2	0.9
16:1*n*-9	1.0	0.3	1.5	0.6	1.1	0.3	1.1	0.4
18:1*n*-9	13.3	0.1	13.4	0.4	13.2	0.1	12.7	0.3
20:1*n*-9	0.8	0.0	0.8	0.0	0.8	0.0	0.8	0.0
MUFA	17.1	0.5	18.3	1.8	17.0	0.7	16.5	1.1
18:2*n*-6	7.5^{a}	0.0	6.3^{b}	0.3	4.6^{c}	0.2	3.4^{d}	0.2
20:4*n*-6	4.2^{a}	0.2	5.5^{a}	0.8	8.7^{b}	0.7	11.6^{c}	1.2
n-6 PUFA	12.4^{a}	0.1	12.9^{a}	1.2	$14.4^{a,b}$	0.6	16.2^{b}	0.7
18:3*n*-3	1.3^{a}	0.0	$1.1^{a,b}$	0.0	1.0^{b}	0.1	0.2^{c}	0.1
18:4*n*-3	0.3^{a}	0.1	0.3^{a}	0.0	$0.4^{a,b}$	0.0	0.5^{b}	0.1
20:4*n*-3	0.4	0.1	0.4	0.1	0.4	0.1	0.4	0.1
20:5*n*-3	5.7^{a}	0.1	5.2^{a}	0.2	4.2^{b}	0.1	3.4^{b}	0.4
22:6*n*-3	28.6	1.0	27.1	0.9	27.2	0.4	26.8	0.9
n-3 PUFA	37.7^{a}	0.8	35.6^{b}	0.5	34.7^{b}	0.4	32.7^{c}	0.3

PUFA	50.4	0.7	48.7	1.7	49.4	1.0	49.2	0.9
n-3 PUFA/*n*-6 PUFA	3.0[a]	0.1	2.8[a,b]	0.2	2.4[b]	0.1	2.0[c]	0.1
DHA/EPA	5.0[a]	0.1	5.2[a]	0.2	6.5[a,b]	0.0	7.9[b]	0.1
EPA/ARA	1.4[a]	0.2	1.0[b]	0.4	0.5[c]	0.1	0.3[c]	0.2
DHA/ARA	6.7[a]	0.1	5.0[b]	0.6	3.1[c]	0.2	2.3[c]	0.2

Different letters mean statistically significant differences (ANOVA, $P < 0.05$).

Table 6. Pearson correlation coefficients (r) and slopes of linear regressions between selected fatty acid content in the microdiets and larvae, and differences (Δ) between fatty acid levels in larvae and in the corresponding experimental diets (% total fatty acids)*

Fatty acid	r	Slope	Δ ARA0.4	Δ ARA0.8	Δ ARA1.5	Δ ARA3.0
16:0	0.2412	0.13	5.77	5.33	5.35	4.44
18:0	0.7593	0.39	6.57	5.94	5.93	5.50
18:1*n*-9	0.9775	0.52	-8.41	-8.16	-8.06	-7.68
18:2*n*-6	0.9981	0.39	-10.77	-9.48	-6.36	-4.60
20:4*n*-6	0.9884	0.53	2.23	1.21	0.16	-4.48
18:3*n*-3	0.9984	0.16	-6.27	-5.36	-5.22	-0.52
20:5*n*-3	0.9965	2.04	-5.26	-5.49	-5.97	-6.45
22:6*n*-3	0.0412	0.10	13.95	12.00	12.55	12.41

*Negative values indicate lower fatty acid percentage in larval tissue total lipid than in dietary lipid (preferential metabolism), whereas positive values indicate accumulation in the larvae relative to diet (preferential retention).

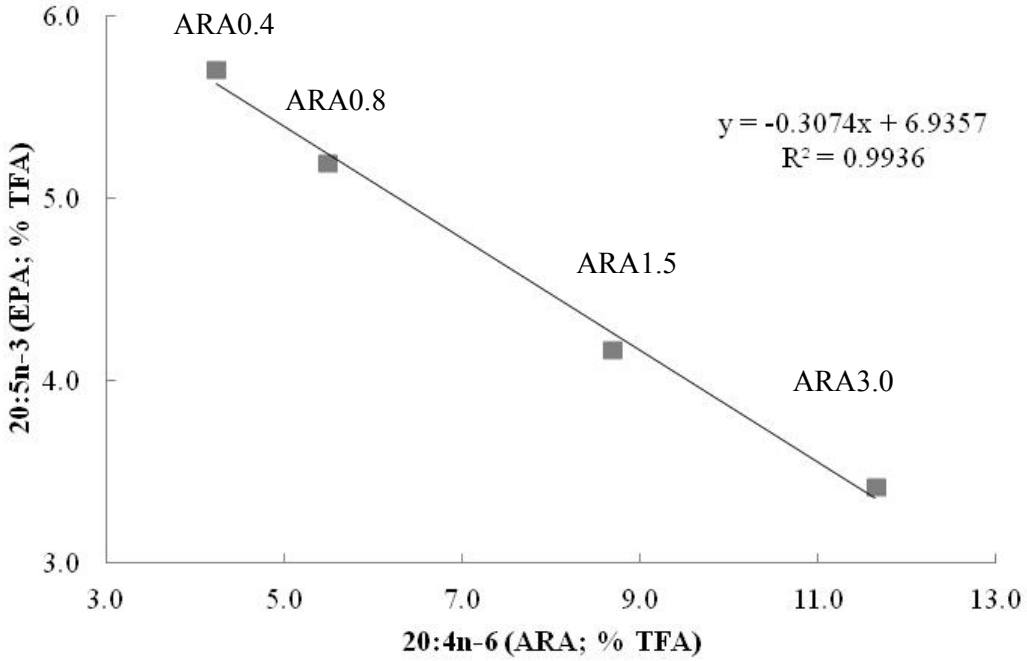

Figure 1. Correlation between EPA and ARA levels (% total fatty acids) in the whole-body of seabream larvae fed graded ARA levels.

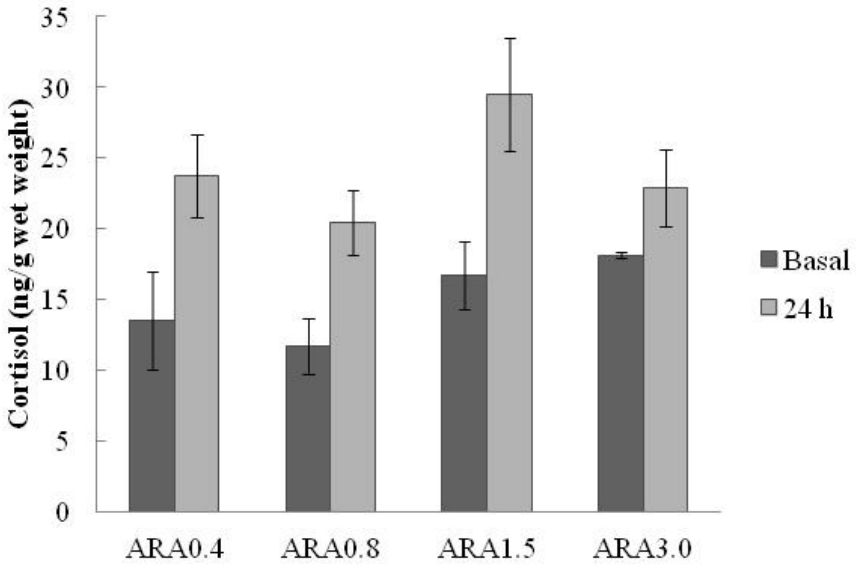

Figure 2. Whole-body cortisol levels in seabream larvae fed different ARA levels before and 24 h after a handling stress (1 min stirring). Values represented are treatment means with standard errors represented by vertical bars. Absence of letters denotes no statistical differences between dietary treatments within sampling times (ANOVA, $P > 0.05$). A significant effect of stress was found (ANOVA, $P < 0.05$).

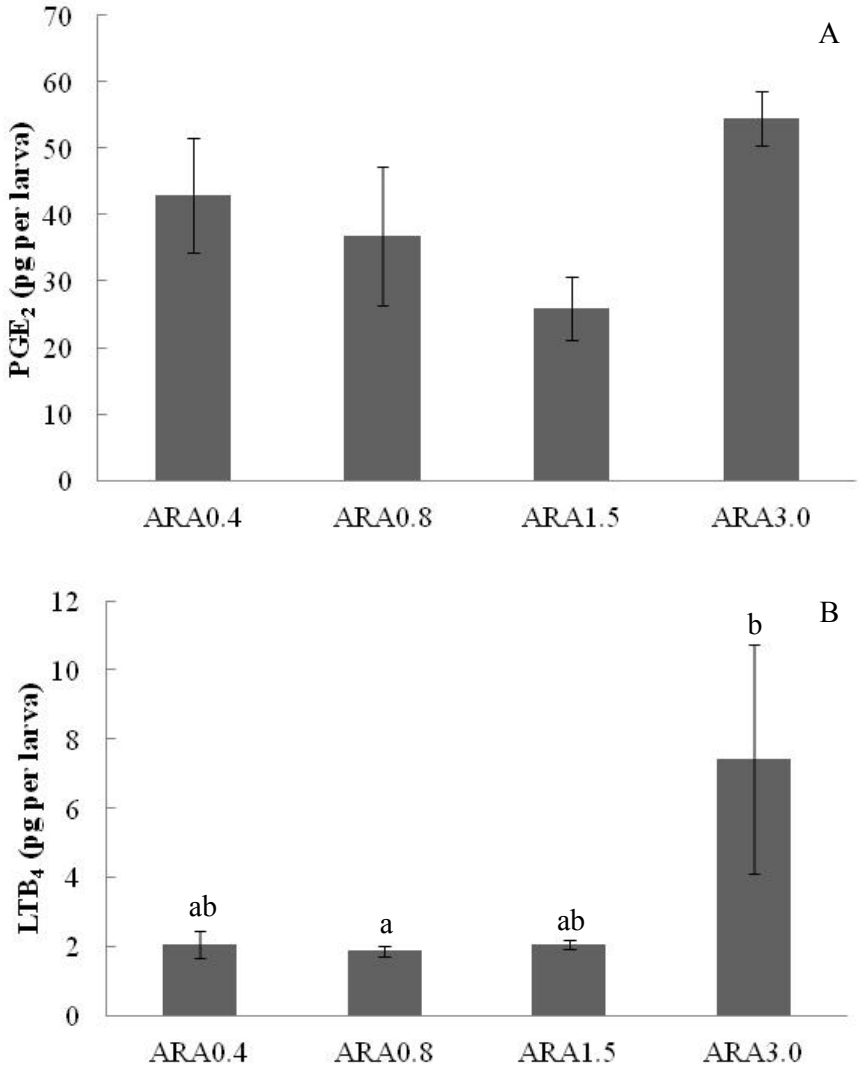

Figure 3. Whole-body prostaglandin E_2 (A) and leukotriene B_4 (B) concentrations in seabream larvae fed different ARA levels. Values represented are treatment means with standard errors represented by vertical bars. Different letters mean statistical differences between treatments (ANOVA, $P < 0.05$).

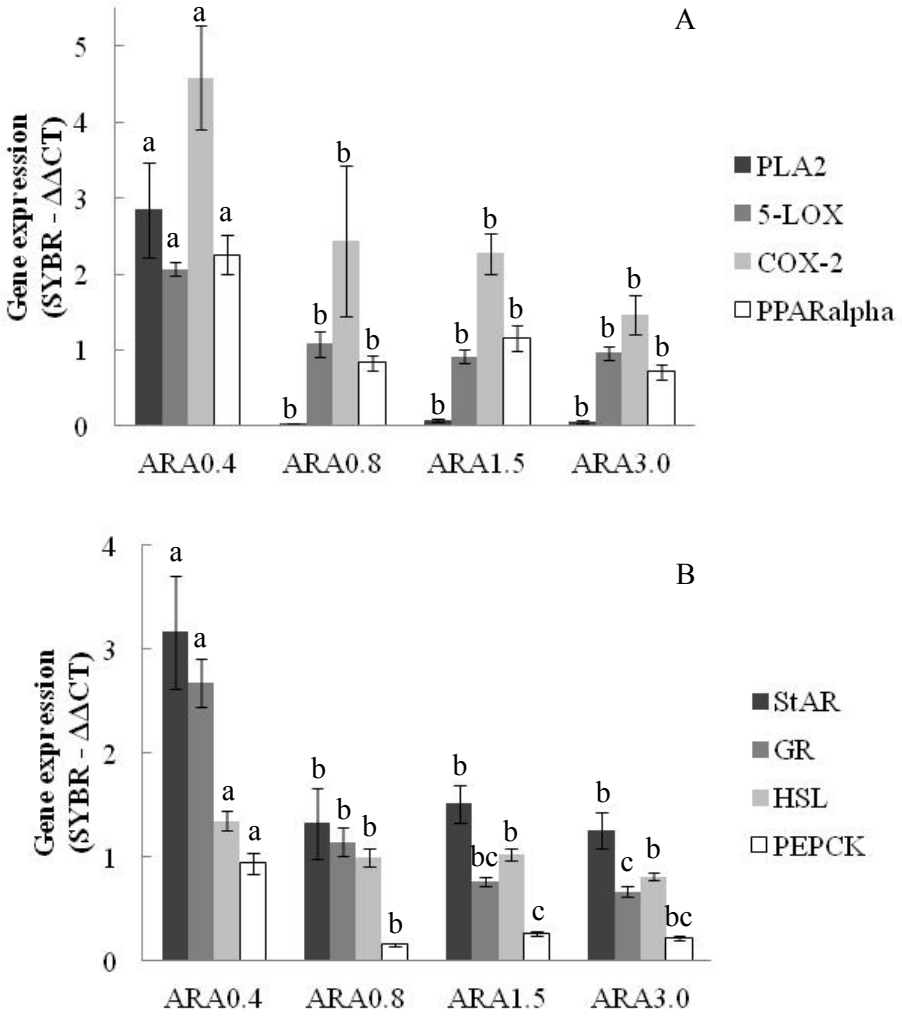

Figure 4. Whole-body expression of genes in seabream larvae fed different ARA levels. Part A shows results relative to phospholipase A_2 (PLA_2), 5-lipoxygenase (5-LOX), cycloxygenase-2 (COX-2), and peroxisome-proliferator activated receptor alpha (PPARalpha). Part B shows results relative to steroidogenic acute regulatory protein (StAR), glucocorticoid receptor (GR), hormone-sensitive lipase (HSL), and phosphoenolpyruvate carboxykinase (PEPCK). Values represented are treatment means with standard errors represented by vertical bars. Different letters mean statistical differences between treatments (ANOVA, $P < 0.05$).