# Modulation of the expression of components of the metabolic stress response by dietary arachidonic acid in European sea bass (*Dicentrarchus labrax*) larvae

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Running Title: Arachidonic acid and stress metabolism in sea bass larvae

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**Abbreviations**

ACTH: Adrenocorticotropic hormone

ARA: Arachidonic acid (20:4n-6)

CAT: Catalase

COX: Cycloxygenase

CYP11β: cythochrome 11β- hydroxylase

DHA: Docosahexaenoic acid (22:6n-3)

EPA: Eicosapentaenoic acid (20:5n-3)

GH: Growth Hormone

GLC: Gas Liquid chromatography

GPX: Glutathione peroxidase

GR: Glucocorticoid receptor

HSP: Heat Shock Protein

HPI: Hypothalamic-pituitary-Interrenal

IGF: Insuline growth factor

LC-PUFA: Long chain polyunsaturated fatty acid

MW: Molecular weight

PIn: Peroxidation index

PGE: Prostaglandin E

RT-PCR: Real time polymerase chain reaction

SGR: Specific growth rate

SOD: Superoxide dismutase

StAR: Steroidogenic acute regulatory protein.

**Abstract**

This study reports for the first time in European sea bass, *Dicentrarchus labrax* (L.), larvae, the effect of different levels of dietary arachidonic acid (ARA; 20:4n-6) on the expression of genes related to the fish stress response. Copies of mRNA from genes related to steroidogenesis (StAR (steroidogenic acute regulatory protein), c-Fos, and CYP11β (11β-hydroxylase gene)), glucocorticoid receptor complex (GR (glucorticoid receptor) and HSP (heat shock proteins) 70 and 90) and antioxidative stress (catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX)) were quantified. Eighteen day-old larvae were fed for 14 days with three experimental diets with increasing levels of ARA (0.3, 0.6 and 1.2% d.w.) and similar levels of docosahexaenoic (DHA; 22:6*n*-3) and eicosapentaenoic (EPA; 20:5*n*-3) acids (5 and 3%, respectively). The quantification of stress-related genes transcripts was conducted by One-Step TaqMan real time RT-PCR with the standard curve method (absolute quantification). Increase dietary levels of ARA induced a significantly ($p<0.05$) down-regulation of genes related to cortisol synthesis, such as StAR and CYP11β and up-regulated genes related to glucocorticoid receptor complex, such as HSP70 and GR. No effects were observed on antioxidant enzymes gene expression. These results revealed the regulatory role of dietary ARA on the expression of stress-related genes in European sea bass larvae.

## Introduction

Stress response has been widely studied in fish within the last years [1,2,3,4,5,6]. The rapid elevation of plasma corticosteroid levels in response to stressor challenges is a constant within the different vertebrates. The stress response in vertebrates is initiated by the activation of the hypothalamic–pituitary–interrenal (HPI) axis, leading to the increase of plasma levels of cortisol, which is the main corticosteroid in teleost [1]. Fish interrenal cells located at the head kidney synthesize cortisol from cholesterol through a series of isomerizations and hydroxylations, including a final step catalyzed by the P450 11β-hydroxylase enzyme (11β). This enzyme belongs to the cytochrome P450 (CYP) family proteins, and is encoded by the CYP11β gene [7]. Another protein, the steroidogenic acute regulatory protein (StAR), is involved in the transport of cholesterol through the mitochondrial membrane of the steroidogenic cells to be used as substrate for steroids synthesis [8].

Following the stimulation by the adrenocorticotropic hormone (ACTH), cortisol is released into the blood and enters into the cells by passive diffusion. Cortisol effects in the cells are mediated by the intracellular glucocorticoid receptors (GR), which are members of the nuclear receptor superfamily and act as ligand-dependent transcription factors to control and regulate gene expression [4,9]. Glucocorticoid receptor is part of a multiprotein heterocomplex, which includes several heat shock proteins (HSP) such as HSP70 and HSP90, whose functions are the assembly, functionality and transport of GR [10]. HSP90 is associated to the GR until a hormone signal induces a conformation with lower affinity for HSP90. This action enables the GR to target sites of transcriptional activation [10]. HSP90 and HSP70 play a fundamental role on the folding and assembling of other cellular proteins and they are involved in regulation of kinetic partitioning between folding,

101 translocation and aggregation as well as having a wide role in relation to the immune,
102 apoptotic and inflammatory processes [11]. Furthermore, HSP molecules help organisms
103 to survive during conditions of stress, by playing a critical role on the host defenses
104 against neoplasia and chronic pathogens [11,12].

105 Dietary lipids have been found to be involved in the regulation of the stress
106 response in fish [13,14,15,16,17,18,19,20,21]. However, most studies have focused on the
107 effect of dietary lipids on the ability of fish to cope with stressful conditions and very little is
108 known about the specific role of particular fatty acids on the activation of the HPI axis.

109 Watanabe and co-authors [22] showed the essentiality of docosahexaenoic acid
110 (DHA, 22:6n-3) for the stress response in marine fish larvae. These authors found
111 progressive lower survival rates of red sea bream, *Pagrus major*, larvae after air exposure
112 when DHA decrease in the diet. Dietary arachidonic acid (ARA, 20:4n-6) has been also
113 shown to increase survival and resistance to stress in gilthead sea bream, *Sparus aurata*
114 L., larvae [15,16,23, 24], whereas Liu and co-authors [25] demonstrated the essentiality of
115 EPA for gilthead sea bream larvae to cope with different types of stress (air exposure,
116 salinity and temperature shocks) even when DHA and ARA requirements were covered for
117 this species. For juvenile fish, n-3 long chain – polyunsaturated fatty acids (n-3 LC-PUFA)
118 have been shown to alter both basal and post-stress plasma cortisol concentration [14]
119 acting as modulators of the *in vitro* ACTH-induced release of cortisol from interrenal cells
120 [26]. On the other hand, ARA seems to regulate stress response in juvenile fish, since Van
121 Anholt and co-authors [17] found that gilthead sea bream juveniles fed a ARA-
122 supplemented diet (containing 2.4% of total fatty acids) showed lower plasma cortisol
123 levels after an acute stress (5 min of confinement in a submerged dip-net) as compared to
124 those fed a low ARA diet (0.9% of total fatty acids). This is in agreement with the
125 regulatory role of ARA on the ACTH-induced release of cortisol described *in vitro* for

gilthead sea bream by Ganga and co-authors [25] and for European sea bass by Montero and co-authors [21].

Like in mammals, there are some reported evidences on the role of ARA on the expression of stress-related genes in fish [27]. Indeed, ARA has been reported to have an effect on the expression of StAR gene in Senegalese sole whole post-larvae [27], but a correlation between dietary levels of ARA and the expression of this gene was not found. In another study, Montero and co-authors [21] found no effect of ARA in the expression of the steroidogenic gene StAR, in European sea bass head kidney but demonstrated a direct effect of ARA on the *in vitro* mRNA levels of another steroidogenic gene, the CYP11β.

Arachidonic acid has also been described as a potent modulator of HSPs in a dose dependent manner in human respiratory epithelial-like cells [29], but also in fish [21]. Furthermore, a direct effect of dietary lipids and in particular of ARA on the expression of GR gene has been demonstrated in fish [28,30].

Accordingly, the aim of this study was to evaluate the effect of different dietary levels of ARA on basal expression levels of stress-related genes in European sea bass, *Dicentrarchus labrax* (L.), at larval stage in order to elucidate how ARA is affecting stress response at early stages of development of this species. European sea bass is one of the most important species of Mediterranean aquaculture and it has been reported to have a high susceptibility to stressful conditions [4].

## Materials and methods

All the experimental conditions and sampling protocols have been approved by the Animal welfare and bioethical committee of the University of Las Palmas de Gran Canaria (Ref 007/2012 CEBA ULPGC).

151 Experimental diets

152 Three isonitrogenous and isolipidic (64/19 protein/lipid) experimental microdiets (Pellet
153 size < 250 µm) with similar DHA and EPA content (5 and 3%, respectively), with constant
154 EPA/DHA ratio, and with increasing ARA levels (0.3, 0.6 and 1.2 %) were formulated using
155 EPA50, DHA50 (CRODA, East Yorkshire, England, UK) and ARA44 (Polaris, Pleuven,
156 France) as sources of EPA, DHA and ARA in triglyceride form, respectively. Composition
157 of the diets and their fatty acid content are shown in Table 1 and 2, respectively.

158

159 Experimental fish

160 European sea bass larvae were obtained from natural spawning at Ecloserie Marine de
161 Gravelines, Gravelines, France and the experiment was carried out at the Aquaculture
162 facilities of University of Las Palmas de Gran Canaria (Telde, Las Palmas, Canary Islands,
163 Spain). Larvae (standard length 6.77 ± 0.71 mm (mean ± SD), mean dry body weight 350
164 ± 21µg), previously fed a commercial microdiet until they reached 18 days old, were
165 randomly distributed over the experimental tanks at a density of 1200 larvae tank-1 and
166 fed one of the experimental diets tested in triplicates (3 tanks/diet) for 14 days, at a water
167 temperature of 19.6 to 20.9 ºC. All tanks (170 L light grey color cylinder fibreglass tanks)
168 were supplied with filtered seawater (37 ppm salinity) at an increasing rate of 0.4 - 1.0 L
169 $min^{-1}$ to assure optimal water quality along the whole trial. Water entered the tank from the
170 bottom and flow out from the top to maintain high water quality, which was tested daily.
171 Water was continuously aerated (125 ml/min) attaining 5-8 g $L^{-1}$ of dissolved $O_2$ and 60-
172 80% of saturation in all tanks. Photoperiod was kept at 12h light: 12h dark. Feeds were
173 manually supplied; fourteen times per day every 45 min from 9:00-19:00. Daily feed
174 supplied 2.0 and 2.5 g $tank^{-1}$ during the first and second week of feeding respectively.

175

176 Sampling procedures

177 Final survival was determined by counting live larvae at the beginning and end of the
178 experiment. Growth was determined by measuring the dry body weight (105°C, 24 h) and
179 the total length (Profile Projector V-12A; Nikon, Tokyo, Japan) of 30 fish tank-1 at the
180 beginning, in the middle, and at the end of the trial. The specific growth rate (SGR) was
181 determined according to the equation: SGR= (ln final body weight)-(ln initial body weight) x
182 100/t, where t is days of the feeding period. In addition, at the end of the trial and after 12
183 h of starvation, all the larvae in each tank were washed with distilled water, sampled and
184 kept at -80˚C for biochemical composition analysis. In addition, at the end of the
185 experiment, twenty larvae (32 days old) from each tank were sampled randomly, quickly
186 frozen in liquid nitrogen and then kept at -80ºC until gene expression analysis.

187 After the end of the experiment, an activity test was conducted by handling 20 larvae per
188 tank out of the water in a scoop net for 1min. After this, the larvae were placed in another
189 tank supplied aeration to determine survival rate after 24h of activity test.

190 Biochemical composition analysis

191 The moisture [31], protein [31] and crude lipid [32] contents of larvae and diets were
192 analyzed. Fatty acid methyl esters were obtained by transmethylation of crude lipids as
193 described by Christie [33]. Fatty acid methyl esters were separated by GLC (GC-14A;
194 Shimadzu, Tokyo, Japan) in a Supercolvax-10-fused silica capillary column (length: 30m;
195 internal diameter: 0.32mm; Supelco, Bellefonte, PA, USA) using helium as a carrier gas.
196 The column temperature was set at 180°C for the rest 10 min, increasing to 215 °C at a
197 rate of 2.5°C$min^{-1}$, and then held at 215°C for 10 min. Fatty acid methyl esters were
198 quantified by FID (GC- 14A; Shimadzu) following the conditions described in Izquierdo and
199 co-authors [34] and identified by comparison with previously characterized standards (EPA
200 28, Nippai, Ltd. Tokyo, Japan) and GLC-MS. The peroxidation index (PIn) was used to

estimate the susceptibility of lipids to oxidation and was calculated by the formula: PIn= 0.025 × (percentage of monoenoics) + 1 × (percentage of dienoics) + 2 × (percentage of trienoics) + 4 × (percentage of tetraenoics) + 6 × (percentage of pentaenoics) + 8 × (percentage of hexaenoics) [35].

Quantitative real-time RT-PCR

*Preparation of total RNA*

Total RNA was extracted from all the sampled whole larvae using PureYield RNA Midiprep System (Promega, Italy), following the protocol described in PureYield™ RNA Midiprep System Technical Manual #TM279, available online at: www.promega.com/tbs.

The quantity of the extracted RNA was calculated using the absorbance at 260 nm, whereas the integrity of RNA was assessed by agarose gel electrophoresis. Crisp 18S and 28S bands, detected by ethidium bromide staining were indicator of the intact RNA.

*Generation of in vitro-transcribed mRNAs for standard curves*

The approach used for the real-time quantification of our target genes expression relied on the standard curve method for target mRNA quantification. The target genes were c-Fos, StAR, CYP11β, HSP70 and HSP90, SOD, CAT, and GPX. Following this method, the number of each gene transcript copies could be quantified by comparing them with a standard graph constructed using the known copy number of mRNAs of each target gene. The first step in this direction is the generation of standards of mRNAs by *in vitro*-transcription. As an example, in the case of c-Fos, a forward and a reverse primer were designed based on the mRNA sequences of *D. labrax* c-Fos we have previously identified [36] (Genebank accession nº. DQ838581). This primer pair was used to create templates for the *in vitro* transcription of mRNAs for c-Fos: the forward primer was engineered to

contain a T3 phage polymerase promoter gene sequence to its 5' end (5'- *caattaaccctcactaaaggg*TCTCACAGAGCTCACCCCTA- 3') and used together with the reverse primer (5'- TGGTCTCCATTACTCCTTCCC -3') in a conventional RT-PCR of total sea bass head kidney RNA. RT-PCR products were then checked on a 2.5 % agarose gel stained with ethidium bromide, cloned using pGEM®-T cloning vector system (Promega, Italy) and subsequently sequenced in the SP6 direction.

*In vitro* transcription was performed using T3 RNA polymerase and other reagents supplied in the Promega RiboProbe *In Vitro* Transcription System kit according to the manufacturer's protocol.

The molecular weight (MW) of the *in vitro*-transcribed RNA for c-Fos was calculated according to the following formula:

c-Fos MW = [129(n° of A bases) x 329.2) + 69 (n° of U bases) x 306.2) + 66(n° of C bases) x 305.2) + 98(n° of G bases) x 345.2) ] + 159. The result was 126182.2. Spectrophotometry at 260 nm gave a concentration of 132.8 ng/μl for c-Fos. Therefore, the concentration of the final working solution was 6.34 x $10^{11}$ molecules/μl.

The same aforementioned approach was used for the *in vitro* transcription of the other target genes such as StAR, CYP11β, GR, HSP90, HSP70, CAT, SOD, and GPX. The primers used are shown in Table 3.

The MW of the *in vitro*-transcribed RNAs calculated according to the aforementioned formula were 117433.8 for HSP70; 73451.4 for StAR, and 96414.6 for CYP11β. Spectrophotometry at 260 nm gave a concentration of 33.7 ng/μl for HSP70; 201.1 for CYP11β, and 104.0 for StAR. Therefore, the concentration of the final working solutions were 1.73 x $10^{11}$ molecules/μl for HSP70, 1.26 x $10^{12}$ for CYP11β, and 8.53 x $10^{11}$ molecules/μl for StAR.

The mRNAs of target genes produced by *in vitro* transcription were used as quantitative standards in the analysis of experimental samples. Defined amounts of mRNAs of each gene, at 10-fold dilutions, were subjected to real-time PCR using One-Step TaqMan EZ RT-PCR Core Reagents (Life Technologies, Italy), including 1x Taqman buffer, 3 mM MnOAc, 0.3 mM dNTP except dTTP, 0.6 mM dUTP, 0.3 μM forward primer, 0.3 μM reverse primer, 0.2 μM FAM-6 (6-carboxyfluorescein-labeled probe), 5 units *rTH* DNA polymerase, and 0.5 units AmpErase UNG enzyme in a 30 μl reaction. The RT- PCR conditions were: 2 min at 50°C, 30 min at 60°C, and 5 min at 95°C, followed by 40 cycles consisting of 20 s at 92°C, 1 min at 62°C. The Ct values obtained by amplification were used to create standard curves for target genes.

*Quantification of mRNAs by One-Step TaqMan real time RT-PCR*

A hundred nanograms of total RNA extracted from the experimental samples was subjected, in parallel to 10-fold-diluted, defined amounts of standard mRNAs, to real-time PCR under the same experimental conditions. Real-time Assays-by-Design$^{SM}$ PCR primers and gene-specific fluorogenic probes were designed by Life Technologies. Primer sequences and Taqman® probes of the four target genes are shown in Table 4.

TaqMan® PCR was performed on a StepOne Real Time PCR System (Life Technologies). To reduce pipetting errors, master mixes were prepared to set up duplicate reactions (2 x 30 μl) for each sample.

Data from Taqman® PCR runs were collected with StepOne Real Time Sequence Detector Program. Cycle threshold ($C_T$) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The Ct values were used to create standard curves to serve as a basis for calculating the absolute amounts of mRNA in total RNA.

*Statistical analysis*

The qPCR data were analysed by one way analysis of variance (ANOVA) and each time point was analysed separately. A post hoc test was applied (Tukey). We used statistics package was IBM SPSS Statistics 21 (SPSS Inc., Chicago, IL, USA). The other data were statistically compared using one-way ANOVA followed by a Tukey multiple comparison of means (SPSS Inc., Chicago, IL, USA). To model the relationships between ARA inclusion levels and other studied parameters multiple linear regression analysis was used. The level of statistical significance was set at $P< 0.05$.

## Results

*Larval culture performance*

Fish accepted well all the experimental diets, regardless the ARA content in the diet. Increase in dietary ARA significantly ($R^2$=0.9739, p= 0.0002) reduced the PIn in the larvae (Fig.1a), despite similar PIn values were found in the diet. Raising dietary ARA levels also increased ($R^2$=0.9963, p=0.0001) incorporation of ARA into larval tissues (Fig.1b), which in turn were also correlated ($R^2$=0.9905, p=0.1619) with slight increase in larval survival (Fig.1c). Nevertheless, final survival rate was not significantly different among larvae fed the different affected ARA levels. Larval growth in terms of SGR was also significantly (p<0.05) increased by dietary ARA levels (4.03±0.38, 4.71±0.62 and 5.26±0.24 for 0.3, 0.6 and 1.2 %ARA respectively), and was highly correlated ($R^2$=0.9908, p=0.0096) with the ARA content in larval whole body (Fig. 1d). Whole body lipid content of the larvae was not significantly affected by the experimental diets (data not showed) and fatty acid composition of total lipids from whole larvae reflected the dietary fatty acid profiles. Thus,

299 increased dietary ARA levels were followed by increased 18:0, 18:2n-6, and ARA and
300 reduced monounsaturated and n-9 fatty acids, and 18:1n-9 (Table 5).
301 *StAR, c-Fos, CYP11β, GR, HSP70 and HSP90 mRNA copies in sea bass larvae fed*
302 *different levels of dietary ARA*
303 Larvae fed low levels of ARA in the diet (0.3%) showed significantly higher ($p<0.05$) mRNA
304 copies of StAR gene, in comparison to larvae fed high levels of dietary ARA (1.2%) (Fig
305 2a). Moreover, elevation of dietary ARA was negatively correlated ($R^2=0.9929$, $p=0.0001$)
306 with the expression of StAR gene. In the same way, mRNA copies of CYP11β gene were
307 significantly higher ($p<0.05$) in larvae fed on the 0.3ARA diet in comparison to those fed on
308 0.6ARA and 1.2ARA diets, and was also negatively correlated ($R^2=0.4433$, $p=0.4592$) to
309 dietary ARA although in a lower extent than StAR gene expression (Fig. 2c). Arachidonic
310 acid levels in the diet did not influence the mRNA copy number of c-Fos, as no differences
311 were found in the expression levels of this gene in larvae fed different diets (Fig.2b).
312 The mRNA levels of GR gene increased in larvae with the increase of ARA in their diet.
313 Indeed, larvae fed with 1.2ARA showed the highest ($p<0.05$) copies of this gene mRNAs,
314 with a value of (5.80E+01), 2 folds higher than that found in larvae fed 0.3ARA diet
315 (2.93E+01) (Fig. 3a). Thus, a significant ($R^2=0.9952$, $p=0.0008$) positive correlation was
316 observed among the mRNA copies of GR gene and dietary ARA. The lowest ARA content
317 in the diet induced the expression of HSP90 (Fig. 3b). Indeed the mRNA abundance of
318 this gene was significantly higher ($p<0.05$) in larvae fed 0.3ARA than in those fed 0.6 and
319 1.2ARA (Fig. 3b), and therefore, a positive correlation ($R^2=0.9996$, $p=0.1500$) was found
320 with the increased dietary ARA levels (Fig. 3b). An opposite trend was recorded for
321 HSP70: the highest mRNA copies of this gene were found in sea bass larvae fed on the
322 1.2 ARA diet and the lowest in larvae fed on 0.3 and 0.6 ARA (Fig. 3c), with a significant
323 correlation ($R^2=0.7427$, $p=0.0009$) among both parameters. Moreover, survival of larvae

after a handling stress was correlated ($R^2$=0.4006, p=0.1541) to the increased mRNA copies of GR gene (Fig 3d).

Regarding the expression of oxidative stress enzymes, there were no significant differences on mRNA copies of CAT, SOD, or GPX genes (Fig. 4a,b,c). Nevertheless, a negative correlation was found among SOD and CAT gene expression with dietary ARA (Fig. 4a,b). Thus a high positive correlation ($R^2$=0.9915, p=0.0001) was found among the CAT gene expression and the PIn in the larvae (Fig. 4d).

## Discussion

As it occurs in other species [17,37,38,39,40], in the present study dietary ARA levels included in microdiets significantly improved European sea bass growth and were positively correlated to final larval survival ($R^2$ = 0.9997), suggesting the importance of ARA to promote European sea bass performance along larval development. Accordingly, higher Insuline Growth Factor-1 (IGF-1) and growth hormone (GH) gene expressions were found in larvae fed higher levels of ARA (data not shown) denoting the role of this fatty acid as a growth promoter. Fatty acids seem to increase IGF-1 gene expression through the effect of arachidonic acid- cycloxygenase (COX)-derived prostaglandin E (PGE) [41, 42]. These growth factors are also related to stress and stress-mediated cortisol release, being IGF-I expression reduced by cortisol in sea bass juveniles [43] and in silver sea bream (*Sparus sarba* Forsskål) hepatocytes [44]. In the present study, fish fed higher dietary ARA levels showed higher growth-related genes expression and low expression of CYP11β, suggesting a combined effect of dietary ARA on growth and resistance to stress in European sea bass larvae.

The mRNA levels of genes related to cortisol synthesis (StAR and CYP11β) were reduced in larvae fed the highest ARA level, in agreement with studies conducted in

gilthead sea bream larvae [40]. Since increased abundance of CYP11β mRNA has been related to cortisol synthesis, this result suggests lower basal cortisol levels when ARA increases in diet [45]. Furthermore, ARA is a preferred substrate for COX to produce prostaglandins [46] that mediate a rapid ACTH-induced release of corticosteroid stored in microvesicules [47] and regulate steroidogenesis, inhibiting StAR gene expression and decreasing progesterone production in a dose-dependent manner [47]. Indeed, the effect of ARA levels on *in vitro* ACTH-induced release of cortisol from gilthead sea bream interrenal cells depends, at least in part, on COX activity [26]. Moreover, there is a dose-dependent effect of fatty acids on the *in vitro* ACTH-induced release of cortisol by interrenal cells in fish [26]. Accordingly, in the present study, increased dietary ARA would enhance COX activity and prostanoids synthesis down-regulating StAR gene expression that would reduce basal cortisol release.

Dietary ARA did not seem to have an effect on the other steroidogenic gene studied, c-Fos, which is a member of the activation protein one (AP-1) response elements [49]. The role of the AP-1 response elements on the steroidogenesis has been described in mammals [50]. In fish, we have previously found an effect of DHA but not of ARA on the mRNA levels of c-Fos [21] that could explain the lack of an effect of dietary ARA levels on c-Fos mRNA levels in the present study, since DHA levels were equal in both diets and whole larvae. On the other hand, it could also be feasible that c-Fos regulation occurs at protein level, as opposed to transcriptional level of mRNA expression.

A positive relationship between dietary ARA and larval GR mRNA copies was found in the present study, whereas a negative relationship was found in the case of CYP11β, which is involved in cortisol synthesis. These results are in agreement with the high correlation between basal GR expression levels and ARA content in Senegalese sole larvae [28]. Besides, the type of dietary oils and, hence, dietary essential fatty acids

markedly affects GRs genes expression in different tissues of Senegalese sole [30]. Thus, significantly higher levels of GR expression were found after a chasing stress in Senegalese sole fed a fish oil-based diet (with high ARA levels) in comparison to animals fed a vegetable oil-based diet (with low ARA levels) [30]. In previous studies in juvenile European sea bass reared at high density, a reduction of GR mRNA levels was found with increased blood cortisol concentration [4]. Similar relationship between cortisol and GR mRNA levels has been also described in mammals [51]. On the contrary, a previous *in vitro* study in rainbow trout (*Oncorhynchus mykiss*) hepatocytes found an up-regulation of GR mRNA abundance by cortisol [52], and similar results were also obtained *in vivo* [53] when trout were treated with cortisol mimicking stressed physiologically elevated plasma cortisol concentration. The authors of both studies suggested a negative feedback regulation of GR transcripts, through inhibition of the translational machinery and/or post-translational modifications resulting in enhanced GR breakdown [53]. However, this apparent controversy may be due to species-specific differences and the effect of different stressors, taking into account that our results targeted cortisol receptor basal levels, rather than ACTH-induced level of expression. Besides, whole body larvae have been used in this study, and ARA effects have been shown to regulate activation of GR complexes in a dose-tissue-dependent manner [54].

In this way, a dose-dependent relationship has been also described between ARA and the transcription levels of chaperone proteins associated to GR, the heat shock proteins, via acquisition of DNA-binding activity and phosphorylation of heat shock factor [55]. In the present study, larvae fed with highest ARA level showed the highest HSP70 basal expression, together with the highest GR basal expression, pointing out the important function of HSP70 in GR assembly and maintenance. Besides, larvae with highest expression of HSP70 had the lowest transcript levels of CYP11β, which agrees

399 with previous studies that have demonstrated that cortisol reduces the HSP70 expression
400 [56, 57] . In our previous studies in Senegalese sole, dietary vegetable oils (low in ARA)
401 reduced the expression of HSP90 gene in muscle and that of HSP70 in intestine, in
402 comparison to fish fed a fish oil based diet (high in ARA) [30]. PUFAs and, particularly,
403 ARA have been shown to increase the heat induced stress response in rainbow trout
404 leukocytes [58].

405 Although the stress response has been linked to the activity of the antioxidant
406 enzymes in fish [59, 60], there were no significant differences on CAT, SOD and GPX
407 transcripts. We can thus exclude an indirect effect on stress-related gene expression due
408 to changes in antioxidant enzymes. Nevertheless, it must be noted that the peroxidation
409 index, a parameter that measures the susceptibility to oxidation, was similar in tissues of
410 larvae fed three different diets. This fact could explain why antioxidant enzymes gene
411 expression did not differ among sea bass larvae fed increasing dietary ARA contents. In
412 addition, the oxidative potential of ARA is known to be not as high as that of other n-3 LC-
413 PUFA such as EPA or DHA [61]. In this sense, a recent study in zebrafish (*Danio rerio*)
414 demonstrated diverging GPX3 and GPX4b gene expression in liver of fish fed diets with
415 high or low peroxidation index generated by increasing DHA dietary levels [63].

416 Dietary and larval ARA contents were also found to be correlated to survival after
417 activity test in European sea bass larvae [23]. The relation between dietary ARA and
418 resistance to stress in fish larvae has been discussed in several studies [37]. Dietary
419 supplementation with ARA at 6–12% promoted the adaptive physiological responses to
420 hypersalinity stress and hypo-osmoregulatory ability in black sea bass (*Centropristis*
421 *striata*) larvae [63]. Gilthead sea bream larvae fed ARA enriched rotifers prior to an acute
422 handling stress showed significantly reduced accumulated mortality following tank transfer
423 [15]. However, the same enrichment in Artemia had no effect on larvae survival when fed

after transfer stress [15]. Moreover, in red sea bream [64] dietary ARA levels similar to those used in gilthead sea bream [15] did not affect handling stress resistance, whereas higher ARA levels markedly reduced it. Overall, these studies suggest the difference in quantitative ARA requirements among species, the dose-dependant effect of this fatty acid and the interrelations with other fatty acids [65]. Indeed, in the present study, ARA was increased in microdiets maintaining constant levels of DHA and EPA, whereas when gilthead sea bream was fed on Artemia [15], ARA supplementation reduced both EPA and DHA in this live prey. Thus the positive relationship between ARA and whole body cortisol found in gilthead sea bream larvae [15] could have been also related to the reduction in other essential fatty acids, such as DHA. Therefore, ARA effects on stress resistance seem to depend on ARA doses, species or type of stress, and on the dietary ratios with other fatty acids such as EPA and DHA, since these fatty acids are also essential for stress resistance [13,22,25].

In conclusion, 1.2% of ARA in the diet seems to have beneficial effects on European sea bass larvae, when EPA and DHA requirements are fulfilled, by optimizing the basal levels of stress-related genes. By decreasing cortisol-synthesis related CYP11β gene expression, basal (unstressed) circulating cortisol concentration is expected to be lower, avoiding the negative effects of chronic high levels of glucocorticoids in the blood. Besides, by increasing glucocorticoid receptor complex-related genes (GR and HSP70) basal expression, tissues are expected to be better prepared to cope with a stress-related increase of circulating glucocorticoids, optimizing thus the ability of these animals to cope with a stressful situation and enhancing larvae welfare.

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## References

1. Wendelaar Bonga SE (1997) The stress response in fish. Physiol rev 77: 591- 625.
2. Barton BA (2002) Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. Integ Comp Biol 42: 517-525.
3. Iwama GK, Alfonso LOB, Vijayan MM (2006) Stress in Fish. In: The Physiology of Fishes (Evans DH, Claiborne JB eds.), 3rd edn, pp. 319-342, CRC Press, Boca Raton, Florida.
4. Terova G, Gornati R, Rimoldi S, Bernardini G, Saroglia M (2005) Quantification of a glucocorticoid receptor in sea bass (*Dicentrarchus labrax*, L.) reared at high stocking density. Gene 357:144-151.
5. Aluru N, Vijayan MM (2009) Stress transcriptomics in fish: A role for genomic cortisol signaling. Gen Comp Endocrinol 164: 142-150.
6. Tort L. (2011) Stress and immune modulation in fish. Dev. Comp. Immunol. 35: 1366-1375.
7. Jiang JQ, Young G, Kobayashi T, Nagahama Y (1998) Eel, *Anguilla japonica*, testis 11beta-hydroxylase gene is expressed in interrenal tissue and its product lacks aldosterone synthesizing activity. Mol Cel Endocrinol 146: 207-211.
8. Stocco DM, Wang X, Jo Y, Manna PR (2005) Multiple signaling pathways regulation steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. Mol Endocrinol 19, 2647-2659
9. Filby AL, Tyler CR (2007) Cloning and characterization of cDNAs for hormones and/or receptors of growth hormone, insulin-like growth factor-I, thyroid hormone,

and corticosteroid and the gender-, tissue-, and developmental-specific expression of their mRNA transcripts in fathead minnow (*Pimephales promelas*). Gen Comp Endocrinol 150: 151-63.

10. Pratt WB (1993) The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. J Biol Chem 268: 21455-21458.
11. Roberts, R.J., Agius, C., Saliba, C., Bossier, P. & Sung, Y.Y. (2010) Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review.J Fish Dis 33: 789-801.
12. Srivastava PK (2002) Roles of heat shock proteins in innate and adaptative immunity. Nat Rev Immunol 2: 184-194.
13. Kanazawa A (1997) Effects of docosahexaenoic acid and phospholipids on stress tolerance of fish. Aquaculture 155: 129-134.
14. Montero D, Tort L, Izquierdo MS, Robaina L, Vergara JM (1998) Depletion of serum alternative complement pathway activity in gilthead sea bream caused by [alpha]-tocopherol and n-3 HUFA dietary deficiencies. Fish Physiol Biochem 18: 399-407.
15. Koven W, Barr Y, Lutzky S, Ben-Atia I, Weiss R, Harel M, Behrens P, Tandler A (2001) The effect of dietary arachidonic acid (20:4n-6) on growth, survival and resistance to handling stress in gilthead sea bream (*Sparus aurata*) larvae. Aquaculture 193: 107-122.
16. Koven W, Van Anholt R, Lutzky S, Ben Atia I, Nixon O, Ron B, Tandler A (2003) The effect of dietary arachidonic acid on growth, survival, and cortisol levels in different-age gilthead sea bream larvae (*Sparus aurata*) exposed to handling or daily salinity change. Aquaculture 228: 307-320.

497 17. Van Anholt RD, Koven WM, Lutzky S, Wendelaar Bonga SE (2004) Dietary
498 supplementation with arachidonic acid alters the stress response of gilthead sea
499 bream (*Sparus aurata*) larvae. Aquaculture 238: 369-383.
500 18. Montero D, Izquierdo MS (2010) Welfare and health of fish fed vegetable oils as
501 alternative lipid sources to fish oil. In. Fish Oil Replacement and Alternative Lipid
502 Sources in Aquaculture Feeds. pp: 439-486 (G Turchini, W Ng, D Tocher, editors)
503 CRC Press, Cambridge, UK.
504 19. Oxley A, Jolly C, Eide T, Jordal AEO, Svardal A, Olsen RE (2010) The combined
505 impact of plant-derived dietary ingredients and acute stress on the intestinal
506 arachidonic acid cascade in Atlantic salmon (*Salmo salar*). Br J Nutr 103:851–861.
507 20. Ganga R, Bell JG, Montero D, Atalah E, Vraskou Y, Tort L, Fernández A, Izquierdo
508 MS (2011) Adrenocorticotrophic hormone-stimulated cortisol release by the head
509 kidney inter-renal tissue from sea bass (*Sparus aurata*) fed with linseed oil and
510 soybean oil. Br J Nutr 105:238–247
511 21. Montero D, Terova G, Rimoldi S, Tort L, Negrin D, Zamorano MJ, Izquierdo MS
512 (2015) Modulation of ACTH-induced expression of stress-related genes by
513 polyunsaturated fatty acids in interrenal cells from European sea bass,
514 *Dicentrarchus labrax*. Journal of Nutritional Science 4, DOI: 10.1017/jns.2015.6
515 22. Watanabe T, Izquierdo MS, Takeuchi T, Satoh S, Kitajima C (1989). Comparison
516 between eicosapentaneoic and docosahexaenoic acids in terms of essential fatty
517 acids efficacy in larval red sea bream. Nippon Suisan Gakkaishi 55: 635–1640.
518 23. Bessonart, M, Izquierdo, MS, Salhi, M, Hernández-Cruz, CM, González, MM,
519 Fernández-Palacios H (1999) Effect of dietary arachidonic acid levels on growth
520 and survival of gilthead sea bream *Sparus aurata* L.) larvae. Aquaculture 179: 265–
521 275.

522 24. Atalah E, Hernández-Cruz CM, Ganuza E, Benítez-Santana T, Ganga R, Roo J,
523 Montero D, Izquierdo MS (2011) Importance of dietary arachidonic acid for survival,
524 growth and stress resistance of larval European sea bass (*Dicentrarchus labrax*)
525 fed high dietary docosahexaenic and eicosapentaenoic acids. Aquac Res 42: 1261-
526 1268.
527 25. Liu J, Caballero MJ, Izquierdo MS, El-Sayed Ali T, Hernández-Cruz CM, Valencia
528 A, Fernández-Palacios H (2002) Necessity of dietary lecithin and eicosapentaenoic
529 acid for growth, survival, stress resistance and lipoprotein formation in gilthead sea
530 bream *Sparus aurata*. Fish Sci 68: 1165-1172.
531 26. Ganga R, Tort L, Acerete L, Montero D, Izquierdo MS (2006) Modulation of ACTH-
532 induced cortisol release by polyunsaturated fatty acids in interrenal cells from
533 gilthead sea bream, *Sparus aurata*. J Endocrinol 190: 39- 45.
534 27. Wang X, Shen CL, Dyson MT, Yin X, Schiffer RB, Grammas P, Stocco MD (2006)
535 The involvement of epoxygenase metabolites of arachidonic acid in cAMP-
536 stimulated steroidogenesis and streoidogenic acute regulatory protein gene
537 expression. J Endocrinol 190, 871-878.
538 28. Alves Martins D, Rocha F, Castanheira F, Mendes A, Pousao-Ferreira P, Bandarra
539 N, Coutinho J, Morais S, Yúfera M, Conceiçao L, Martínez-Rodriguez G (2013)
540 Effects of dietary arachidonic acid on cortisol production and gene expression in
541 stress response in Senegalese sole (Solea senegalensis) post larvae. Fish Physiol
542 Biochem 39, 1223-1238.
543 29. Shah NG, Tulapurkar ME, Singh IS, Shelhamer JH, Cowan MJ, Hasday JD (2010)
544 Prostaglandin E2 Potentiates Heat Shock-Induced Heat Shock -induced heat shock
545 protein 72 expression in A549 cells. Prostaglandins Other Lipid Mediat 93, 1–7

30. Benitez-Dorta V, Caballero MJ, Izquierdo MS, Manchado M, Infante C, Zamorano MJ, Montero D (2013) Total substitution of fish oil by vegetable oils in Senegalese sole (Solea senegalensis) diets: effects on fish performance, biochemical composition, and expression of some glucocoticoid receptor-related genes. Fish Physiol Biochem 39: 335-349.
31. AOAC (1995) Official Methods of Analysis of the Association Analytical Chemist. AOAC, Arlington,VA, USA1018pp.
32. Folch J, Lees M, Sloane-Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226: 497-509.
33. Christie W.W. (1982) Lipid Analysis. Pergamon Press, Oxford, UK.
34. Izquierdo M, WatanabeT, Takeuchi T, Arakawa T, Kitajima C (1990) Optimum EFA levels in Artemia to meet the EFA requirements of red seabream (Pagrus major). In: The Current Status of Fish Nutrition in Aquaculture (ed. by M Takeda T Watanabe), pp. 221-232. Tokyo University Fisheries,Tokyo, Japan.
35. Witting LA, Horwitt MK (1964) Effect of Degree of Fatty Acid Unsaturation in Tocopherol Deficiency-induced Creatinuria. J Nutr 82:19-33.
36. Rimoldi S, Terova G, Brambilla F, Bernardini G, Gornati R, Saroglia M (2009) Molecular characterizaton and expression analysis of Na+/H+ exchanger NHE-1 and c-Fos genes in sea bass *Dicentrarchus labrax*, L. exposed to acute and chronic hypercapnia. J Exp Mar Biol Ecol 375: 32-40.
37. Izquierdo MS (1996) Essential fatty acid requirements of cultured marine fish larvae. Aquac Nutr 2: 183-191.
38. Bessonart M, Izquierdo MS, Salhi M, Hernandez-Cruz CM, Gonzalez MM, Fernandez-Palacios H (1999) Effect of dietary arachidonic acid levels on growth

570 and survival of gilthead sea bream (*Sparus aurata* L.) larvae. Aquaculture 179: 265-
571 275.
572 39. Boglino A, Darias MJ, Estevez A, Andree KB, Gisbert E (2012) The effect of dietary
573 arachidonic acid during the Artemia feeding period on larval growth and
574 skeletogenesis in Senegalese sole, Solea senegalensis. J. Applied Ichthyology 28,
575 411-418.
576 40. Alves Martins D, Rocha F, Martínez-Rodriguez G, Bell G, Morais S, Castanheira F,
577 Bandarra N, Coutinho J, Yúfera M, Conceiçao LEC (2012) Teleost fish larvae adapt
578 to dietary arachidonic acid supply through modulation of the expression of lipid
579 metabolism and stress genes. Br J Nutr 108, 864-874.
580 41. Li Y, Seifert MF, Ney DM, Grahn M, Grant AL, Allen KGD, Watkins BA (1999)
581 Dietary conjugated linoleic acids alter serum IGF-I and IGF binding protein
582 concentrations and reduce bone formation in rats fed (n-6) or (n-3) fatty acids. J
583 Bone Min Res 14: 1153-1162.
584 42. Celil AB, Campbell PG (2005) BMP-2 and insulin-like growth factor-I mediate
585 osterix (Osx) expression in human mesenchymal stem cells via the MAPK and
586 protein kinase D signaling pathways. J Biol Chem 280: 31353-31359
587 43. Carnevali O, de Vivo L, Sulpizio R, Gioacchini G, Olivotto I, Silvi S, Cresci A (2006)
588 Growth improvement by probiotic in European sea bass juveniles (*Dicentrarchus*
589 *labrax* L.), with particular attention to IGF-1, myostatin and cortisol gene expression.
590 Aquaculture 258: 430-438.
591 44. Leung LY, Kwong AKY, Man AKY, Woo NYS (2008) Direct actions of cortisol,
592 thyroxine and growth hormone on IGF-I mRNA expression in sea bream
593 hepatocytes. Comp Biochem Physiol - A Mol Int Physiol 151: 705-710.

45. Aluru N, Vijayan MM (2006) Aryl hydrocarbon receptor activation impairs cortisol response to stress in rainbow trout by disrupting the rate-limiting steps in steroidogenesis. Endocrinol 147:1895-1903.

46. Rowley AF, Pettitt TR, Secombes CJ, Sharp GJE, Barrow SE, Vickers PJ (1995) Eicosanoids and their role in immune modulation in fish -A brief overview. Fish Shellfish Immunol 5: 63-105.

47. Mohn CE, Fernandez-Solari J, De Laurentiis A, Prestifilippo JP, de la Cal C, Funk R, Bornstein SR, McCann SM, Rettori V (2005) The rapid release of corticosterone from the adrenal induced by ACTH in mediated by nitric oxide acting by prostaglandin E2. Proc Nat Acad Sci Un St Am 102: 6213- 6218.

48. Wang X, Dyson MT, Jo Y, Stocco DM (2003) Inhibition of Cyclooxygenase-2 Activity Enhances Steroidogenesis and Steroidogenic Acute Regulatory Gene Expression in MA-10 Mouse Leydig Cells. Endocrinol 144: 3368-3375.

49. Karin M, Liu Z, Zandi E (1997) Ap-1 function and regulation. Curr Opin Cell Biol 9, 240-246.

50. Okimoto DK, Blaus A, Schmidt M, Gordon K, Dent GW, Levine S (2002) Differential expression of c-Fos and tyrosine hydroxylase mRNA in the adrenal gland of the infant rat: evidence for an adrenal hyporesponsive period. Endocrinol 143, 1717-1725.

51. Yudt MR, Cidlowski JA (2002) The glucocorticoid receptor: Coding a diversity of proteins and responses through a single gene. Mol Endocrinol 16: 1719-1726.

52. Sathiyaa R, Vijayan MM (2003) Autoregulation of glucocorticoid receptor by cortisol in rainbow trout hepatocytes. Am J Physiol Cell Physiol 284: C1508 – C1515.

617 53. Vijayan MM, Raptis S, Sathiyaa R (2003) Cortisol treatment affects glucocorticoid
618 receptor and glucocorticoid-responsive genes in the liver of rainbow trout. Gen
619 Comp Endocrinol 132: 256-263.
620 54. Ranhotra HS, Sharma R (2004) Polyunsaturated fatty acids inhibit mouse hepatic
621 glucocorticoid receptor activation in vitro. Ind J Biochem Biophys 41: 246-249.
622 55. Jurivich DA, Sistonen L, Kevin D, Sarge KD, Morimoto RI (1994) Arachidonate is a
623 potent modulator of human heat-shock gene-transcription. Proc Natl Acad Sci USA
624 91: 2280-2284.
625 56. Basu N, Kennedy CJ, Iwama GK (2003) The effect of stress on the association
626 between HSP70 and the glucocorticoid receptor in rainbow trout. Comp Biochem
627 Physiol -A, Mol Integ Physiol 134: 655-663.
628 57. Basu N, Todgham AE, Ackerman MA, Bibeau MR, Nakano K, Schulte PM, Iwama
629 GK (2002) Heat shock protein genes and their functional significance in fish. Gene,
630 295: 173-183.
631 58. Samples BL, (1999) Polyunsaturated fatty acids enhance the heat induced stress
632 response in rainbow trout (Oncorhynchus mykiss) leukocytes. Comp Biochem
633 Physiol Part B: Biochem Mol Biol 123, 389-397.
634 59. Perez-Sanchez J, Borrel M, Bermejo-Nogales A, Benedito-Palos L, Saera-Vila A,
635 Calduch-Giner JA, Kaushik S (2013) Dietary oils mediate cortisol kinetics and the
636 hepatic mRNA expression profile of stress-responsive genes in gilthead sea bream
637 (*Sparus aurata*) exposed to crowding stress. Implications on energy homeostasis
638 and stress susceptibility. Comp Biochem Physiol Part D: Genom Proteom 8: 123-
639 130.

60. Sanz A, Furne E, Trenzado CE, De Haro C, Sanchez-Muros MJ (2012) Study of the Oxidative State, as a Marker of Welfare, on Gilthead Sea Bream, *Sparus aurata*, Subjected to Handling Stress. J World Aquac Soc 43: 707-715

61. Leonardi F, Attorri L, Di Benedetto R, Di Biase A, Sanchez M, Nardini M, Salvati S (2005) Effect of arachidonic, eicosapentaenoic and docosahexaenoic acids on the oxidative status of C6 glioma cells. Free Radical Res 39: 865-874.

62. Betancor MB, Almaida-Pagán PF, Sprague M, Hernández A, Tocher DR. Roles of selenoprotein antioxidant protection in zebrafish, *Danio rerio*, subjected to dietary oxidative stress. Fish Physiol. Biochem. DOI 10.1007/s10695-015-0040-2.

63. Carrier JK, Watanabe WO, Harel M, Rezek TC, Seaton PJ, Shafer TH (2011) Effects of dietary arachidonic acid on larval performance, fatty acid profiles, stress resistance, and expression of Na+/K+ ATPase mRNA in black sea bass *Centropristis striata*. Aquaculture 319: 111-121.

64. Furuita, H., Takeuchi, T., Toyota, M., Watanabe, T. (1996) EPA and DHA requirements in early juvenile red sea bream using HUFA enriched Artemia nauplii. Fisheries Science, 62, 246–251.

65. Izquierdo, MS, Socorro, J, Arantzamendi, L, and Hernández-Cruz CM (2000) Recent advances in lipid nutrition in fish larvae. Fish Physiol. Biochem. 22: 97–107.

**List of Figures**

Fig. 1.- Composition and performance of European sea bass fed 3 different dietary ARA levels for 14 days: a) Peroxidation index in larval whole body, b) ARA contents in larval whole body, c) survival rate at the end of the trial, d) larval growth during the trial. n = 3 (20 larvae in each tank). Different letters indicate significant ($p<0.05$) differences among fish fed different diets

Fig. 2.- Absolute mRNA levels of genes related with steroidogenesis: a) StAR, b) c-Fos and c) CYP11β in whole body European sea bass larvae fed 3 different dietary levels of ARA. n = 3 (20 larvae in each tank). Different letters indicate significant ($p<0.05$) differences among fish fed different diets

Fig. 3.- Absolute mRNA levels of genes related with glucocorticoid receptor complex: a) GR, b) HSP90 and c) HSP70 in whole body European sea bass larvae fed 3 different dietary levels of ARA, and their relation to stress resistance. n = 3 (20 larvae in each tank). Different letters indicate significant ($p<0.05$) differences among fish fed different diets

Fig. 4.- Absolute mRNA levels of genes related with oxidative stress enzymes: a) SOD, b) CAT and c) GPX in whole body European sea bass larvae fed 3 different dietary levels of ARA and d) their relation with the peroxidation index. n = 3 (20 larvae in each tank). Different letters indicate significant ($p<0.05$) differences among fish fed different diet

678 Table 1. Main lipid ingredient composition and analyzed lipid, protein, moisture contents

679 and peroxidation index of the experimental diets (g/100g diet d.w.)

| | ***Diets*** | | |
|---|---|---|---|
| | ***0.3ARA*** | ***0.6ARA*** | ***1.2ARA*** |
| **Defatted squid powder [1]** | 69.00 | 68.85 | 68.60 |
| **DHA45[2]** | 8.00 | 8.00 | 7.90 |
| **EPA45[2]** | 4.50 | 4.50 | 4.30 |
| **ARA44[2]** | 0.00 | 0.70 | 2.00 |
| **Oleic acid[3]** | 2.40 | 1.70 | 0.70 |
| **Vitamin premix[4]** | 6.00 | 6.00 | 6.00 |
| **Mineral premix[5]** | 2.50 | 2.50 | 2.50 |
| **Lipid content (d.w.)** | 20.72 | 20.46 | 20.50 |
| **Protein content (d.w.)** | 70.38 | 70.27 | 70.17 |
| **Moisture content (%)** | 9.2 | 8.91 | 8.93 |
| **ARA (% dw)** | 0.30 | 0.62 | 1.25 |
| **Peroxidation index (PIn)** | 75.18 | 75.18 | 75.25 |

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681 [1] Riber and Son, Bergen, Norway. [2]Polaris, Pleuven, France.[3]Merck, Darmstadt, Germany. [4]Vitamin premix
682 supplied per 100g diet: Cyanocobalamine, 0.030; Astaxanthin, 5.00; folic acid, 5.44; pyridoxine-HCl, 17.28;
683 thiamine, 21.77; riboflavin, 72.53; Ca-pantothenate, 101.59; paminobenzoic acid, 145.00; nicotinic acid,
684 290.16; myo -inositol, 1450.90; retinol acetate, 0.180; $\alpha$-tocopherol acetate, 150.000. [5]Mineral premix
685 supplied g per 100g diet: NaCl, 215.133; MgSO4 ·7H2 O, 677.545; NaH2 PO4 ·H2 O, 381.453; K2 HPO4 ,
686 758.949; Ca(H2 PO4 )·2H2 O, 671.610; FeC6 H5 O7 , 146.884; C3 H5 O3 ·1/2Ca,1617.210; Al2 (SO4 )3
687 ·6H2 O, 0.693; ZnSO4 ·7H2 0, 14.837; CuSO4 ·5H2 O, 1.247; MnSO4 ·H2 O, 2.998; KI, 0.742; CoSO4 ·7H2
688 O, 10.706.

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| | 0.3 ARA | 0.6 ARA | 1.2 ARA |
|---|---|---|---|
| 14:0 | 0.23 | 0.22 | 0.21 |
| 14:1n-7 | n.d. | 0.02 | 0.01 |
| 14:1n-5 | 0.05 | 0.01 | 0.01 |
| 15:0 | 0.04 | 0.04 | 0.03 |
| 15:1n-5 | n.d. | 0.01 | 0.01 |
| 16:0ISO | n.d. | 0.01 | 0.01 |
| 16:0 | 1.45 | 1.53 | 1.77 |
| 16:1n-7 | 0.43 | 0.38 | 0.32 |
| 16:1n-5 | n.d. | 0.02 | 0.02 |
| 16:2n-6 | 0.02 | 0.03 | 0.02 |
| 16:2n-4 | 0.03 | 0.05 | 0.05 |
| 17:0 | 0.04 | 0.06 | 0.07 |
| 16:3n-4 | 0.06 | 0.10 | 0.08 |
| 16:3n-3 | 0.11 | n.d. | n.d. |
| 16:3n-1 | 0.02 | 0.02 | 0.02 |
| 16:4n-3 | 0.02 | 0.02 | n.d. |
| 16:4n-1 | 0.05 | 0.05 | 0.05 |
| 18:0 | 0.69 | 0.78 | 0.91 |
| 18:1n-9 | 3.76 | 3.18 | 2.49 |
| 18:1n-7 | 0.60 | 0.54 | 0.45 |
| 18:1n-5 | 0.03 | 0.02 | 0.02 |
| 18:2n-6 | 1.33 | 1.32 | 1.45 |
| 18:3n-6 | 0.05 | 0.08 | 0.13 |
| 18:3n-4 | 0.06 | 0.02 | 0.02 |
| 18:3n-3 | 0.22 | 0.21 | 0.22 |
| 18:4n-3 | n.d. | 0.28 | 0.27 |
| 18:4n-1 | 0.28 | 0.03 | 0.03 |
| 20:0 | 0.08 | 0.11 | 0.12 |
| 20:1n-9 | 0.49 | 0.48 | 0.45 |
| 20:1n-7 | 0.04 | 0.04 | 0.04 |
| 20:2n-9 | 0.04 | 0.05 | 0.06 |
| 20:3n-9 | 00.05 | 0.08 | 0.14 |
| 20:4n-6 | 0.30 | 0.62 | 1.25 |
| 20:4n-3 | 0.05 | 0.05 | 0.04 |
| 20:5n-3 | 4.06 | 3.97 | 3.87 |
| 22:1n-11 | 0.19 | 0.19 | 0.19 |
| 22:4n-6 | 0.04 | 0.16 | 0.16 |
| 22:5n-6 | 0.16 | n.d. | n.d. |
| 22:5n-3 | 0.44 | 0.43 | 0.42 |
| 22:6n-3 | 5.16 | 5.05 | 4.93 |
| ΣSaturated | 2.63 | 2.77 | 3.13 |
| ΣMonoenoics | 5.16 | 5.05 | 4.93 |
| Σn-3 | 10.07 | 10.14 | 9.88 |
| Σn-6 | 1.89 | 2.21 | 3.01 |
| Σn-9 | 4.34 | 3.79 | 3.14 |
| Σn-3 HUFA | 9.22 | 9.02 | 8.79 |
| EPA/DHA | 0.79 | 0.79 | 0.79 |
| ARA/EPA | 0.07 | 0.16 | 0.32 |
| n-3/n-6 | 5.32 | 4.60 | 3.29 |

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**Table 3** Sequences of primers used to synthesize *in vitro* standard mRNA

| Gene | Symbol | Acc. nr. | Primer sequence (5′–3′) |
|---|---|---|---|
| Steroidogenesis acute regulatory protein | StAR | EF409994 | F: *gtaatacgactcactatagggACTCAGCACCCGAAAATGC*<br>R: ACTTTGCCAACCACCTCAG |
| Cytochrome P450 11β | CYP11 β | AF449173 | F: *gtaatacgactcactatagggCTCAAGAACGGTGAGGAGTGG*<br>R: CTTCTCCTCATCTCCCTCCA |
| Glucocorticoid receptor | GR | AY549305 | F: *gtaatacgactcactatagggGCCTTTTGGCATGTACTCAAACC*<br>R: GAACAGGTATGGAGAGTCGTCCC |
| Heat shock protein 90 | HSP90 | AY395632 | F: *gtaatacgactcactatagggCCAACGACTGGGAGGATCAC*<br>R: GAGTTCCGGGCCCTGC |
| Heat shock protein 70 | HSP70 | AY423555 | F: caattaaccctcactaaagggCCATCCTGACCATCGAAGAC<br>R: TTGTCCATCTTGGCGTCAC |
| Superoxide dismutase | SOD | FJ860004 | F: gtaatacgactcactatagggGTTGGAGACCTGGGAGATGT<br>R: GAAAAGGAGGCAATGAGGAG |
| Catalase | CAT | FJ860003 | F: gtaatacgactcactatagggATGGTGTGGGACTTCTGGAG<br>R: CGTTTCTACTGCAAGTTCCACT |
| Glutathione peroxidase | GPX | FM013606 | F: gtaatacgactcactatagggAGTTAATCCGGAATTCGTGAGA<br>R: CAACAACCAGGGACTACACTCA |

698 **Table 4**. Primers and probes used for One Step quantitative real-time RT-PCR.

| Gene | Symbol | Nucleotide sequence (5'-3') | One step Taqman real time standard curve quality |
|---|---|---|---|
| **c-Fos** | **c-Fos** | **Fw**: CAGCAAAATGCCGCAACAG<br>**Rv**: TGGACTTCTCATCCTCTAGCTGATC<br>**Taqman Probe**: GAGCTTACAGACACTCTG | $R^2$ = 0.995<br>Efficiency = 89.45% |
| **Steroidogenesis acute regulatory protein** | **StAR** | **Fw**: AGCGGAGAATGGACCTACCT<br>**Rv**: GAAGACCCAAATAAGACCAAGTTCAC<br>**Taqman Probe**:<br>ATAGTCATGAAGCCCTGTG | $R^2$ = 0.982<br>Efficiency = 90.379% |
| **Cytochrome P450 11β** | **CYP11 β** | **Fw**: CTTCGGCAGTAAAGTGCTTTCTAC<br>**Rv**: GGATTTCTGTCGAATGCTGCG<br>**Taqman Probe**: GCTTGATGAGGTGGCGA | $R^2$ = 0.993<br>Efficiency = 82.47% |
| **Heat shock protein 70** | **HSP70** | **Fw**: GGACATCAGCCAGAACAAGAGA<br>**Rv**: GAGAACCCTGTCCTCCAGC<br>**Taqman Probe**: GCTTGTGAGAGGGCCAA | $R^2$ = 0.996<br>Efficiency = 99.276% |
| **Glucocorticoid receptor** | **GR** | **Fw**: GCCTTTTGGCATGTACTCAAACC<br>**Rv**: GAACAGGTATGGAGAGTCGTCC<br>**Taqman Probe**: GTGGTTGGGGAGAGCTG | $R^2$ = 0.997<br>Efficiency = 94.417% |
| **Heat shock protein 90** | **HSP90** | **Fw**: CCAACGACTGGGAGGATCAC<br>**Rv**: GAGTTCCGGGCCCTGC<br>**Taqman Probe**: CTGTCAAGCACTTCTCG | $R^2$ = 0.998<br>Efficiency = 86.23% |
| **Superoxide dismutase** | **SOD** | **Fw**: TGGAGACCTGGGAGATGTAACTG<br>**Rv**: CAAGATAGACATCACGGACAAGA<br>**Taqman Probe**: CAGGAGGAGATAACATTG | $R^2$ = 0.994<br>Efficiency = 96.23% |
| **Catalase** | **CAT** | **Fw**: ATGGTGTGGGACTTCTGGAG<br>**Rv**: CATCAGGTGTCTTTCTTGTTCAGC<br>**Taqman Probe**: TGAGGCCTGAGTGTCTG | $R^2$ = 0.996<br>Efficiency = 97.23% |
| **Glutathione peroxidase** | **GPX** | **Fw**: AGTTAATCCGGAATTCGTGAG<br>**Rv**: GTTTTACGACCTGACAGCTAAGCT<br>**Taqman Probe**: AATGGCTGGAAACGTG | $R^2$ = 0.998<br>Efficiency = 93.23% |

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**Table 5. Fatty acid composition of total lipids from European sea bass larvae after 14 days of feeding different levels of dietary ARA.**

| | 0.3ARA | 0.6ARA | 1.2ARA |
|---|---|---|---|
| **14:0** | 0.64±0.01 | 0.97±0.56 | 0.60±0.04 |
| **14:1n-7** | 0.09±0.02 | 0.10±0.01 | 0.07±0.0 |
| **14:1n-5** | 0.08±0.03 | n.d. | 0.09±0.01 |
| **15:0** | 0.32±0.03 | 0.34±0.04 | 0.30±0.03 |
| **15:1n-5** | 0.13±0.01 | 0.13±0.00 | 0.11±0.00 |
| **16:0ISO** | 0.23±0.02 | 0.22±0.03 | 0.24±0.06 |
| **16:0** | 15.84±1.73 | 15.20±0.90 | 19.74±4.48 |
| **16:1n-9** | 0.79±0.35 | 1.27±1.20 | 19.74±4.48 |
| **16:1n-7** | 6.07$^{a}$±0.25 | 6.65$^{b}$±0.07 | 0.65±0.21 |
| **16:1n-5** | 0.14±0.09 | 0.18±0.14 | 0.15±0.12 |
| **16:2n-6** | 0.28±0.01 | 0.29±0.04 | 0.24±0.04 |
| **16:2n-4** | 0.34±0.01 | 0.41±0.17 | 0.29±0.01 |
| **17:0** | 0.66±0.09 | 0.62±0.07 | 0.85±0.26 |
| **16:3n-4** | 0.44±0.03$^{a}$ | 0.45±0.10$^{a}$ | 0.31±0.04$^{b}$ |
| **16:4n-3** | 0.47±0.30 | 0.68±0.10 | 0.70±0.12 |
| **16:4n-1** | 0.53±0.15 | 0.40±0.06 | 0.36±0.11 |
| **18:0** | 7.51±6.67 | 8.70±1.02 | 14.05±6.49 |
| **18:1n-9** | 11.84±2.38 | 12.76±01.08 | 10.51±2.12 |
| **18:1n-7** | 6.85±6.42 | 3.18±0.13 | 2.74±0.51 |
| **18:1n-5** | 1.25±1.91 | 0.17±0.02 | 0.17±0.01 |
| **18:2n-6** | 2.32±1.89 | 3.52±0.27 | 3.18±0.61 |
| **18:3n-6** | 0.29±0.1$^{a}$ | 0.35±0.01$^{ab}$ | 0.50±0.09 $^{b}$ |
| **18:3n-3** | 1.02±0.05 | 1.08±0.02 | 0.94±0.21 |
| **18:4n-3** | 0.53±0.02 | 0.84±0.54 | 0.45±0.04 |
| **20:0** | 0.45±0.15 | 0.34±0.05 | 0.58±0.35 |
| **20:1n-9** | 1.79±0.05$^{a}$ | 1.77±0.08$^{a}$ | 1.46±0.19 $^{b}$ |
| **20:1n-7** | 014±0.04 | 0.10±0.01 | 0.09±0.02 |
| **20:2n-9** | 0.73±0.01 | 0.70±0.05 | 0.69±0.09 |
| **20:3n-9** | 0.11±0.05 | 0.19±0.00 | 0.22±0.08 |
| **20:4n-6** | 2.96±0.15$^{a}$ | 4.06±0.38$^{a}$ | 5.80±1.22 $^{b}$ |
| **20:4n-3** | 0.36±0.01 | 0.42±0.12 | 0.29±0.02 |
| **20:5n-3** | 10.18±0.64 $^{ab}$ | 11.32±2.21$^{a}$ | 7.92±1.17$^{b}$ |
| **22:1n-11** | 0.22±0.04 | 0.34±0.19 | 0.21±0.04 |
| **22:4n-6** | 0.72±0.05 | 0.69±0.05 | 0.61±0.11 |
| **22:5n-3** | 1.13±0.11 | 1.27±0.29 | 1.04±0.11 |
| **22:6n-3** | 26.26±2.31 | 25.95±0.90 | 23.09±4.48 |
| **ΣSaturated** | 25.23±8.10 | 25.95±1.52 | 35.94±11.57 |
| **ΣMonoenoics** | 24.10±6.24 | 20.70±0.39 | 17.22±3.17 |
| **Σn-3** | 39.93±3.02 | 38.74±1.79 | 32.34±5.75 |
| **Σn-6** | 6.57±1.87$^{a}$ | 8.91±0.66$^{ab}$ | 10.32±2.25$^{b}$ |
| **Σn-9** | 15.27±2.5 | 16.69±0.18 | 13.53±2.25 |
| **Σn-3 HUFA** | 37.93±3.02 | 38.74±1.79 | 32.34±5.75 |
| **EPA/DHA** | 0.39±0.01 | 0.44±0.10 | 0.34±0.02 |
| **ARA/EPA** | 0.29±0.01$^{a}$ | 0.36±0.09$^{a}$ | 0.73±0.07$^{b}$ |
| **n-3/n-6** | 6.08±2.24$^{a}$ | 4.64±0.61$^{ab}$ | 3.34±0.03$^{b}$ |

n.d.: not detected. Different letters within a line denote significant differences (P≤0.05) for each tissue. Values expressed in mean ± SD. (n = 3 tanks/diet).

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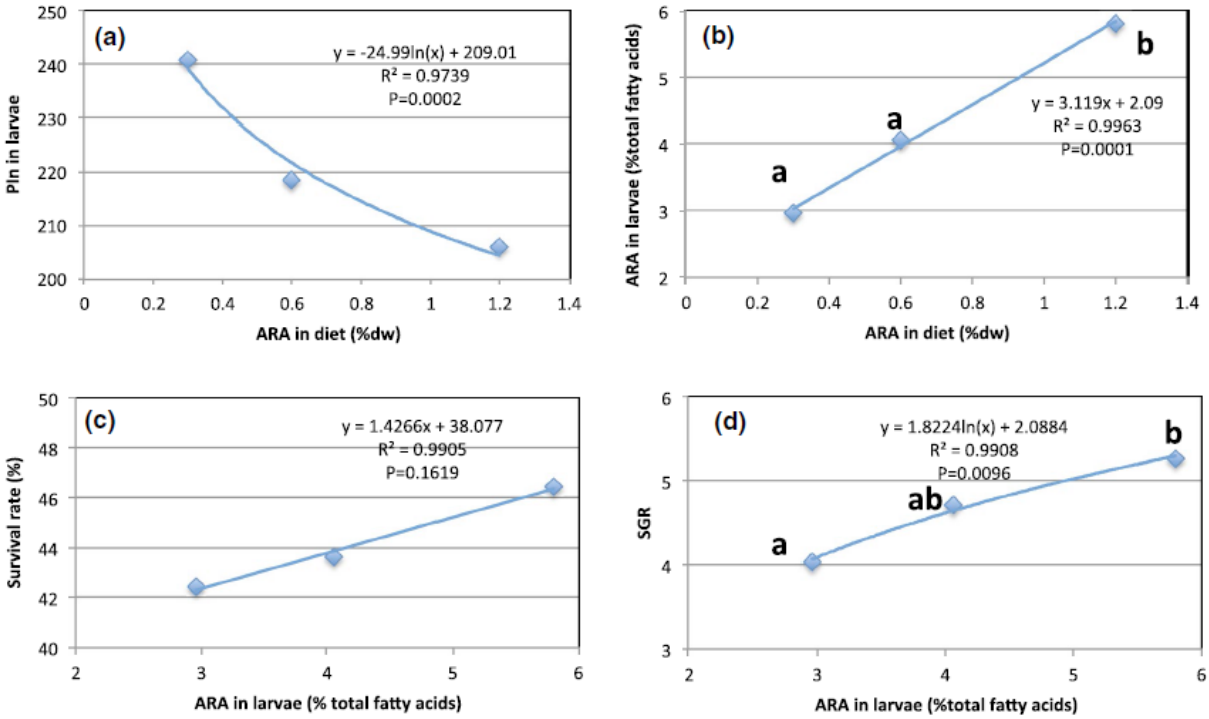


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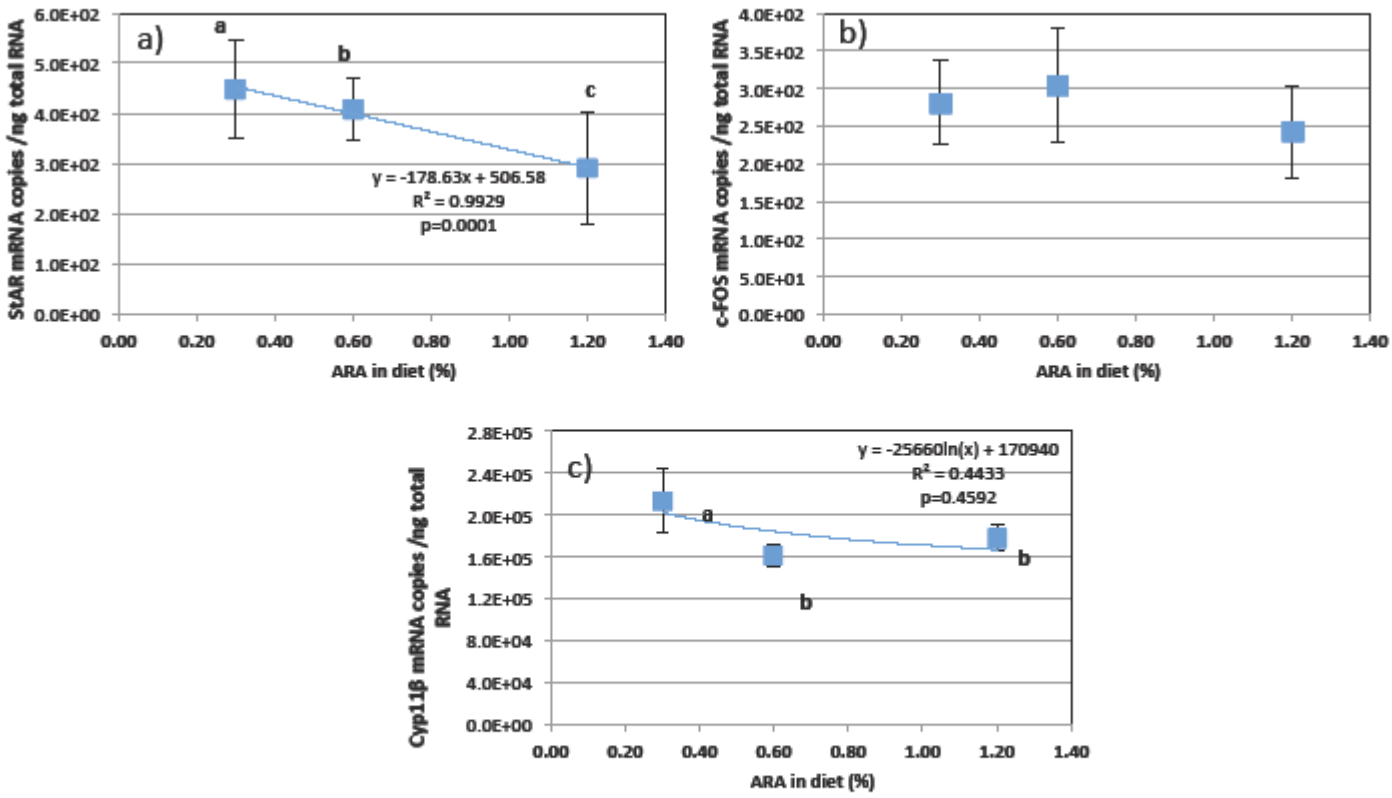

a)
b)
c)
StAR mRNA copies /ng total RNA
c-FOS mRNA copies /ng total RNA
Cyp11β mRNA copies /ng total RNA
ARA in diet (%)
y = -178.63x + 506.58
R² = 0.9929
p=0.0001
y = -25660ln(x) + 170940
R² = 0.4433
p=0.4592

Figure 2

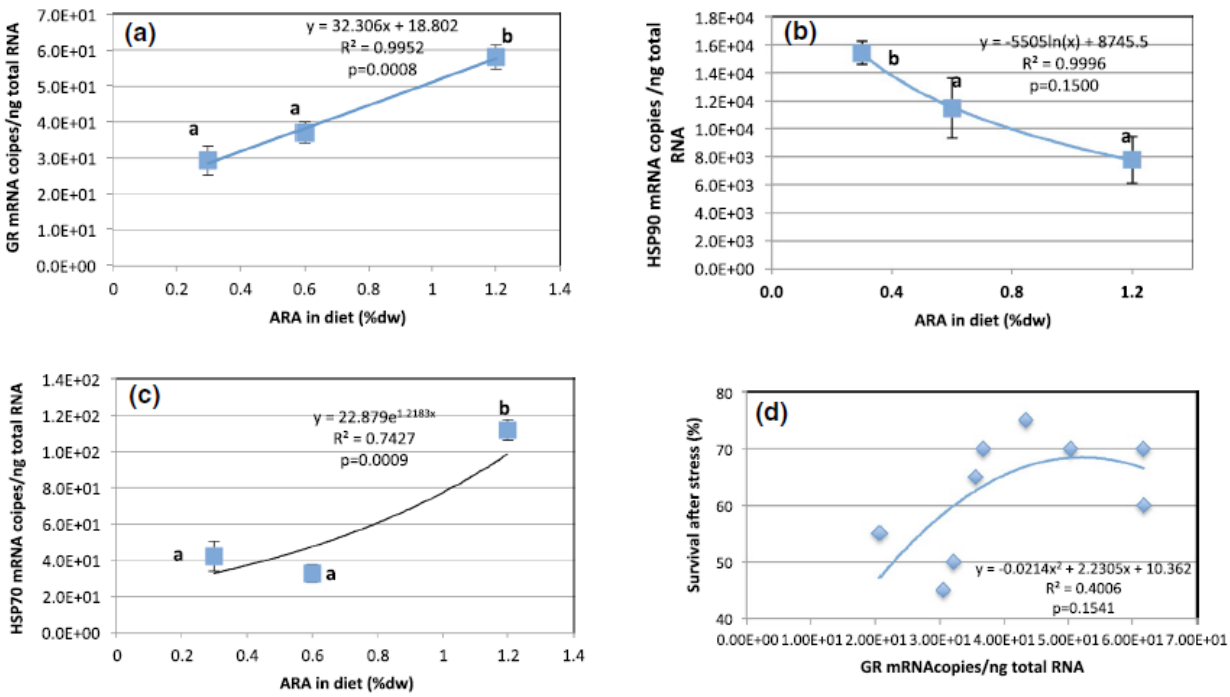
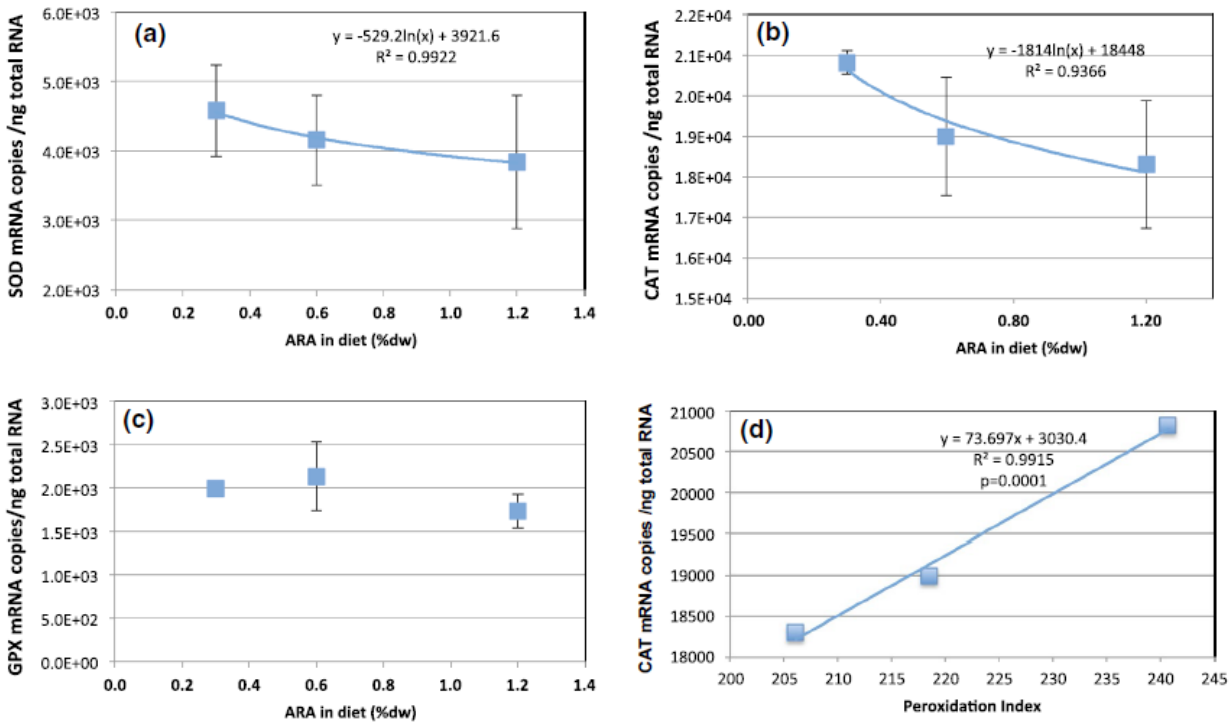

(a)
y = 32.306x + 18.802
R² = 0.9952
p=0.0008
GR mRNA coipes/ng total RNA
ARA in diet (%dw)
(b)
y = -5505ln(x) + 8745.5
R² = 0.9996
p=0.1500
HSP90 mRNA copies /ng total RNA
ARA in diet (%dw)
(c)
y = 22.879e^1.2183x
R² = 0.7427
p=0.0009
HSP70 mRNA coipes/ng total RNA
ARA in diet (%dw)
(d)
y = -0.0214x² + 2.2305x + 10.362
R² = 0.4006
p=0.1541
Survival after stress (%)
GR mRNAcopies/ng total RNA

Figure 3

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732 Figure 4
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