

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

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

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26

27 **Abbreviations**

28 ACTH: Adrenocorticotropic hormone

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

30 CAT: Catalase

31 COX: Cyclooxygenase

32 CYP11 β : cytochrome 11 β - hydroxylase

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

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

35 GH: Growth Hormone

36 GLC: Gas Liquid chromatography

37 GPX: Glutathione peroxidase

38 GR: Glucocorticoid receptor

39 HSP: Heat Shock Protein

40 HPI: Hypothalamic-pituitary-Interrenal

41 IGF: Insuline growth factor

42 LC-PUFA: Long chain polyunsaturated fatty acid

43 MW: Molecular weight

44 PIn: Peroxidation index

45 PGE: Prostaglandin E

46 RT-PCR: Real time polymerase chain reaction

47 SGR: Specific growth rate

48 SOD: Superoxide dismutase

49 StAR: Steroidogenic acute regulatory protein.

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51

52 **Abstract**

53 This study reports for the first time in European sea bass, *Dicentrarchus labrax* (L.), larvae,
54 the effect of different levels of dietary arachidonic acid (ARA; 20:4n-6) on the expression of
55 genes related to the fish stress response. Copies of mRNA from genes related to
56 steroidogenesis (StAR (steroidogenic acute regulatory protein), c-Fos, and CYP11 β (11 β -
57 hydroxylase gene)), glucocorticoid receptor complex (GR (glucocorticoid receptor) and HSP
58 (heat shock proteins) 70 and 90) and antioxidative stress (catalase (CAT), superoxide
59 dismutase (SOD), and glutathione peroxidase (GPX)) were quantified. Eighteen day-old
60 larvae were fed for 14 days with three experimental diets with increasing levels of ARA
61 (0.3, 0.6 and 1.2% d.w.) and similar levels of docosahexaenoic (DHA; 22:6n-3) and
62 eicosapentaenoic (EPA; 20:5n-3) acids (5 and 3%, respectively). The quantification of
63 stress-related genes transcripts was conducted by One-Step TaqMan real time RT-PCR
64 with the standard curve method (absolute quantification). Increase dietary levels of ARA
65 induced a significantly ($p < 0.05$) down-regulation of genes related to cortisol synthesis,
66 such as StAR and CYP11 β and up-regulated genes related to glucocorticoid receptor
67 complex, such as HSP70 and GR. No effects were observed on antioxidant enzymes gene
68 expression. These results revealed the regulatory role of dietary ARA on the expression of
69 stress-related genes in European sea bass larvae.

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77 **Introduction**

78 Stress response has been widely studied in fish within the last years [1,2,3,4,5,6]. The
79 rapid elevation of plasma corticosteroid levels in response to stressor challenges is a
80 constant within the different vertebrates. The stress response in vertebrates is initiated by
81 the activation of the hypothalamic–pituitary–interrenal (HPI) axis, leading to the increase of
82 plasma levels of cortisol, which is the main corticosteroid in teleost [1]. Fish interrenal cells
83 located at the head kidney synthesize cortisol from cholesterol through a series of
84 isomerizations and hydroxylations, including a final step catalyzed by the P450 11 β -
85 hydroxylase enzyme (11 β). This enzyme belongs to the cytochrome P450 (CYP) family
86 proteins, and is encoded by the CYP11 β gene [7]. Another protein, the steroidogenic acute
87 regulatory protein (StAR), is involved in the transport of cholesterol through the
88 mitochondrial membrane of the steroidogenic cells to be used as substrate for steroids
89 synthesis [8].

90 Following the stimulation by the adrenocorticotrophic hormone (ACTH), cortisol is
91 released into the blood and enters into the cells by passive diffusion. Cortisol effects in the
92 cells are mediated by the intracellular glucocorticoid receptors (GR), which are members
93 of the nuclear receptor superfamily and act as ligand-dependent transcription factors to
94 control and regulate gene expression [4,9]. Glucocorticoid receptor is part of a multiprotein
95 heterocomplex, which includes several heat shock proteins (HSP) such as HSP70 and
96 HSP90, whose functions are the assembly, functionality and transport of GR [10]. HSP90
97 is associated to the GR until a hormone signal induces a conformation with lower affinity
98 for HSP90. This action enables the GR to target sites of transcriptional activation [10].
99 HSP90 and HSP70 play a fundamental role on the folding and assembling of other cellular
100 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

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

128 Like in mammals, there are some reported evidences on the role of ARA on the
129 expression of stress-related genes in fish [27]. Indeed, ARA has been reported to have an
130 effect on the expression of StAR gene in Senegalese sole whole post-larvae [27], but a
131 correlation between dietary levels of ARA and the expression of this gene was not found.
132 In another study, Montero and co-authors [21] found no effect of ARA in the expression of
133 the steroidogenic gene StAR, in European sea bass head kidney but demonstrated a
134 direct effect of ARA on the *in vitro* mRNA levels of another steroidogenic gene, the
135 CYP11 β .

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

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

146

147 **Materials and methods**

148 All the experimental conditions and sampling protocols have been approved by the
149 Animal welfare and bioethical committee of the University of Las Palmas de Gran Canaria
150 (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 \pm SD), mean dry body weight 350
164 ± 21 µg), 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 °C. 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 O₂ 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: $SGR = (\ln \text{ final body weight}) - (\ln \text{ initial body weight}) \times$
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 -80°C 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°Cmin⁻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 (PI_n) was used to

201 estimate the susceptibility of lipids to oxidation and was calculated by the formula: $PI_n =$
202 $0.025 \times (\text{percentage of monoenoics}) + 1 \times (\text{percentage of dienoics}) + 2 \times (\text{percentage of}$
203 $\text{trienoics}) + 4 \times (\text{percentage of tetraenoics}) + 6 \times (\text{percentage of pentaenoics}) + 8 \times$
204 $(\text{percentage of hexaenoics})$ [35].

205

206 Quantitative real-time RT-PCR

207 *Preparation of total RNA*

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

211 The quantity of the extracted RNA was calculated using the absorbance at 260 nm,
212 whereas the integrity of RNA was assessed by agarose gel electrophoresis. Crisp 18S and
213 28S bands, detected by ethidium bromide staining were indicator of the intact RNA.

214

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

216 The approach used for the real-time quantification of our target genes expression relied on
217 the standard curve method for target mRNA quantification. The target genes were c-Fos,
218 StAR, CYP11 β , HSP70 and HSP90, SOD, CAT, and GPX. Following this method, the
219 number of each gene transcript copies could be quantified by comparing them with a
220 standard graph constructed using the known copy number of mRNAs of each target gene.

221 The first step in this direction is the generation of standards of mRNAs by *in vitro*-
222 transcription. As an example, in the case of c-Fos, a forward and a reverse primer were
223 designed based on the mRNA sequences of *D. labrax* c-Fos we have previously identified
224 [36] (Genebank accession n°. [DQ838581](https://www.ncbi.nlm.nih.gov/nuccore/DQ838581)). This primer pair was used to create templates
225 for the *in vitro* transcription of mRNAs for c-Fos: the forward primer was engineered to

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

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

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

237 $c\text{-Fos MW} = [129(n^\circ \text{ of A bases}) \times 329.2) + 69 (n^\circ \text{ of U bases}) \times 306.2) + 66(n^\circ \text{ of C}$
238 $\text{bases}) \times 305.2) + 98(n^\circ \text{ of G bases}) \times 345.2)] + 159$. The result was 126182.2.

239 Spectrophotometry at 260 nm gave a concentration of 132.8 ng/μl for c-Fos. Therefore,
240 the concentration of the final working solution was 6.34×10^{11} molecules/μl.

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

244 The MW of the *in vitro*-transcribed RNAs calculated according to the aforementioned
245 formula were 117433.8 for HSP70; 73451.4 for StAR, and 96414.6 for CYP11β.

246 Spectrophotometry at 260 nm gave a concentration of 33.7 ng/μl for HSP70; 201.1 for
247 CYP11β, and 104.0 for StAR. Therefore, the concentration of the final working solutions
248 were 1.73×10^{11} molecules/μl for HSP70, 1.26×10^{12} for CYP11β, and 8.53×10^{11}
249 molecules/μl for StAR.

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

260

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

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

267 TaqMan[®] PCR was performed on a StepOne Real Time PCR System (Life Technologies).
268 To reduce pipetting errors, master mixes were prepared to set up duplicate reactions (2 x
269 30 μ l) for each sample.

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

275

276 *Statistical analysis*

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

284

285 **Results**

286 *Larval culture performance*

287 Fish accepted well all the experimental diets, regardless the ARA content in the diet.
288 Increase in dietary ARA significantly ($R^2=0.9739$, $p= 0.0002$) reduced the PIn in the larvae
289 (Fig.1a), despite similar PIn values were found in the diet. Raising dietary ARA levels also
290 increased ($R^2=0.9963$, $p=0.0001$) incorporation of ARA into larval tissues (Fig.1b), which in
291 turn were also correlated ($R^2=0.9905$, $p=0.1619$) with slight increase in larval survival
292 (Fig.1c). Nevertheless, final survival rate was not significantly different among larvae fed
293 the different affected ARA levels. Larval growth in terms of SGR was also significantly
294 ($p<0.05$) increased by dietary ARA levels (4.03 ± 0.38 , 4.71 ± 0.62 and 5.26 ± 0.24 for 0.3, 0.6
295 and 1.2 %ARA respectively), and was highly correlated ($R^2=0.9908$, $p=0.0096$) with the
296 ARA content in larval whole body (Fig. 1d). Whole body lipid content of the larvae was not
297 significantly affected by the experimental diets (data not showed) and fatty acid
298 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, CYP11 β , 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 CYP11 β 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

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

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

331

332 **Discussion**

333 As it occurs in other species [17,37,38,39,40], in the present study dietary ARA levels
334 included in microdiets significantly improved European sea bass growth and were
335 positively correlated to final larval survival ($R^2 = 0.9997$), suggesting the importance of
336 ARA to promote European sea bass performance along larval development. Accordingly,
337 higher Insuline Growth Factor-1 (IGF-1) and growth hormone (GH) gene expressions were
338 found in larvae fed higher levels of ARA (data not shown) denoting the role of this fatty
339 acid as a growth promoter. Fatty acids seem to increase IGF-1 gene expression through
340 the effect of arachidonic acid- cyclooxygenase (COX)-derived prostaglandin E (PGE) [41,
341 42]. These growth factors are also related to stress and stress-mediated cortisol release,
342 being IGF-I expression reduced by cortisol in sea bass juveniles [43] and in silver sea
343 bream (*Sparus sarba* Forsskål) hepatocytes [44]. In the present study, fish fed higher
344 dietary ARA levels showed higher growth-related genes expression and low expression of
345 CYP11 β , suggesting a combined effect of dietary ARA on growth and resistance to stress
346 in European sea bass larvae.

347 The mRNA levels of genes related to cortisol synthesis (StAR and CYP11 β) were
348 reduced in larvae fed the highest ARA level, in agreement with studies conducted in

349 gilthead sea bream larvae [40]. Since increased abundance of CYP11 β mRNA has been
350 related to cortisol synthesis, this result suggests lower basal cortisol levels when ARA
351 increases in diet [45]. Furthermore, ARA is a preferred substrate for COX to produce
352 prostaglandins [46] that mediate a rapid ACTH-induced release of corticosteroid stored in
353 microvesicles [47] and regulate steroidogenesis, inhibiting StAR gene expression and
354 decreasing progesterone production in a dose-dependent manner [47]. Indeed, the effect
355 of ARA levels on *in vitro* ACTH-induced release of cortisol from gilthead sea bream
356 interrenal cells depends, at least in part, on COX activity [26]. Moreover, there is a dose-
357 dependent effect of fatty acids on the *in vitro* ACTH-induced release of cortisol by
358 interrenal cells in fish [26]. Accordingly, in the present study, increased dietary ARA would
359 enhance COX activity and prostanoids synthesis down-regulating StAR gene expression
360 that would reduce basal cortisol release.

361 Dietary ARA did not seem to have an effect on the other steroidogenic gene
362 studied, c-Fos, which is a member of the activation protein one (AP-1) response elements
363 [49]. The role of the AP-1 response elements on the steroidogenesis has been described
364 in mammals [50]. In fish, we have previously found an effect of DHA but not of ARA on the
365 mRNA levels of c-Fos [21] that could explain the lack of an effect of dietary ARA levels on
366 c-Fos mRNA levels in the present study, since DHA levels were equal in both diets and
367 whole larvae. On the other hand, it could also be feasible that c-Fos regulation occurs at
368 protein level, as opposed to transcriptional level of mRNA expression.

369 A positive relationship between dietary ARA and larval GR mRNA copies was found
370 in the present study, whereas a negative relationship was found in the case of CYP11 β ,
371 which is involved in cortisol synthesis. These results are in agreement with the high
372 correlation between basal GR expression levels and ARA content in Senegalese sole
373 larvae [28]. Besides, the type of dietary oils and, hence, dietary essential fatty acids

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

392 In this way, a dose-dependent relationship has been also described between ARA
393 and the transcription levels of chaperone proteins associated to GR, the heat shock
394 proteins, via acquisition of DNA-binding activity and phosphorylation of heat shock factor
395 [55]. In the present study, larvae fed with highest ARA level showed the highest HSP70
396 basal expression, together with the highest GR basal expression, pointing out the
397 important function of HSP70 in GR assembly and maintenance. Besides, larvae with
398 highest expression of HSP70 had the lowest transcript levels of CYP11 β , 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

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

437 In conclusion, 1.2% of ARA in the diet seems to have beneficial effects on
438 European sea bass larvae, when EPA and DHA requirements are fulfilled, by optimizing
439 the basal levels of stress-related genes. By decreasing cortisol-synthesis related CYP11 β
440 gene expression, basal (unstressed) circulating cortisol concentration is expected to be
441 lower, avoiding the negative effects of chronic high levels of glucocorticoids in the blood.
442 Besides, by increasing glucocorticoid receptor complex-related genes (GR and HSP70)
443 basal expression, tissues are expected to be better prepared to cope with a stress-related
444 increase of circulating glucocorticoids, optimizing thus the ability of these animals to cope
445 with a stressful situation and enhancing larvae welfare.

446

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450

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658

659 **List of Figures**

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

665 Fig. 2.- Absolute mRNA levels of genes related with steroidogenesis: a) StAR, b) c-Fos
666 and c) CYP11 β in whole body European sea bass larvae fed 3 different dietary levels of
667 ARA. n = 3 (20 larvae in each tank). Different letters indicate significant ($p < 0.05$)
668 differences among fish fed different diets

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

673 Fig. 4.- Absolute mRNA levels of genes related with oxidative stress enzymes: a) SOD, b)
674 CAT and c) GPX in whole body European sea bass larvae fed 3 different dietary levels of
675 ARA and d) their relation with the peroxidation index. n = 3 (20 larvae in each tank).
676 Different letters indicate significant ($p < 0.05$) differences among fish fed different diet
677

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.)

	<i>Diets</i>		
	<i>0.3ARA</i>	<i>0.6ARA</i>	<i>1.2ARA</i>
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 (PI_n)	75.18	75.18	75.25

680

681 ¹ Riber and Son, Bergen, Norway. ²Polaris, Pleuven, France. ³Merck, Darmstadt, Germany. ⁴Vitamin 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; α-tocopherol acetate, 150.000. ⁵Mineral 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 O, 14.837; CuSO₄ ·5H₂ O, 1.247; MnSO₄ ·H₂ O, 2.998; KI, 0.742; CoSO₄ ·7H₂
 688 O, 10.706.

689

690

691

	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
E Saturated	2.63	2.77	3.13
E Monoenoic	5.16	5.05	4.93
En-3	10.07	10.14	9.88
En-6	1.89	2.21	3.01
En-9	4.34	3.79	3.14
En-3 HUFA	9.22	9.02	8.79
HPA/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: <i>gtaatacgaactactataggg</i> ACTCAGCACCCGAAAATGC R: ACTTTGCCAACCCACCTCAG
Cytochrome P450 11 β	CYP11 β	AF449173	F: <i>gtaatacgaactactataggg</i> CTCAAGAACGGTGAGGAGTGG R: CTTCTCCTCATCTCCCTCCA
Glucocorticoid receptor	GR	AY549305	F: <i>gtaatacgaactactataggg</i> GCCTTTTGGCATGTACTCAAACC R: GAACAGGTATGGAGAGTCGTCCC
Heat shock protein 90	HSP90	AY395632	F: <i>gtaatacgaactactataggg</i> CAAACGACTGGGAGGATCAC R: GAGTTCCGGGCCCTGC
Heat shock protein 70	HSP70	AY423555	F: <i>caattaaccctcactaaaggg</i> CCATCCTGACCATCGAAGAC R: TTGTCCATCTTGGCGTCAC
Superoxide dismutase	SOD	FJ860004	F: <i>gtaatacgaactactataggg</i> GTTGGAGACCTGGGAGATGT R: GAAAAGGAGGCAATGAGGAG
Catalase	CAT	FJ860003	F: <i>gtaatacgaactactataggg</i> ATGGTGTGGGACTTCTGGAG R: CGTTTCTACTGCAAGTTCCA
Glutathione peroxidase	GPX	FM013606	F: <i>gtaatacgaactactataggg</i> AGTTAATCCGGAATTCGTGAGA R: CAACAACCAGGGACTACACTCA

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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 Rv: TGGACTTCTCATCTCTAGCTGATC Taqman Probe: GAGCTTACAGACACTCTG	R ² = 0.995 Efficiency = 89.45%
Steroidogenesis acute regulatory protein	StAR	Fw: AGCGGAGAATGGACCTACCT Rv: GAAGACCCAAATAAGACCAAGTTCAC Taqman Probe: ATAGTCATGAAGCCCTGTG	R ² = 0.982 Efficiency = 90.379%
Cytochrome P450 11β	CYP11 β	Fw: CTTGCGCAGTAAAGTGCTTTCTAC Rv: GGATTTCTGTCTGAATGCTGCG Taqman Probe: GCTTGATGAGGTGGCGA	R ² = 0.993 Efficiency = 82.47%
Heat shock protein 70	HSP70	Fw: GGACATCAGCCAGAACAAGAGA Rv: GAGAACCCTGTCCCTCCAGC Taqman Probe: GCTTGTGAGAGGGCCAA	R ² = 0.996 Efficiency = 99.276%
Glucocorticoid receptor	GR	Fw: GCCTTTTGGCATGTACTIONAAACC Rv: GAACAGGTATGGAGAGTCGTCC Taqman Probe: GTGGTTGGGGAGAGCTG	R ² = 0.997 Efficiency = 94.417%
Heat shock protein 90	HSP90	Fw: CCAACGACTGGGAGGATCAC Rv: GAGTTCCGGGCCCTGC Taqman Probe: CTGTCAAGCACTTCTCG	R ² = 0.998 Efficiency = 86.23%
Superoxide dismutase	SOD	Fw: TGGAGACCTGGGAGATGTAACCTG Rv: CAAGATAGACATCACGGACAAGA Taqman Probe: CAGGAGGAGATAACATTG	R ² = 0.994 Efficiency = 96.23%
Catalase	CAT	Fw: ATGGTGTGGGACTTCTGGAG Rv: CATCAGGTGTCTTTCTTGTTTCAGC Taqman Probe: TGAGGCCTGAGTGTCTG	R ² = 0.996 Efficiency = 97.23%
Glutathione peroxidase	GPX	Fw: AGTTAATCCGGAATTCGTGAG Rv: GTTTTACGACCTGACAGCTAAGCT Taqman Probe: AATGGCTGGAAACGTG	R ² = 0.998 Efficiency = 93.23%

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704 **Table 5. Fatty acid composition of total lipids from European sea bass larvae after**705 **14 days of feeding different levels of dietary ARA.**

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	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	0.14±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 ($P \leq 0.05$) for each tissue. Values expressed in mean \pm SD. (n = 3 tanks/diet).

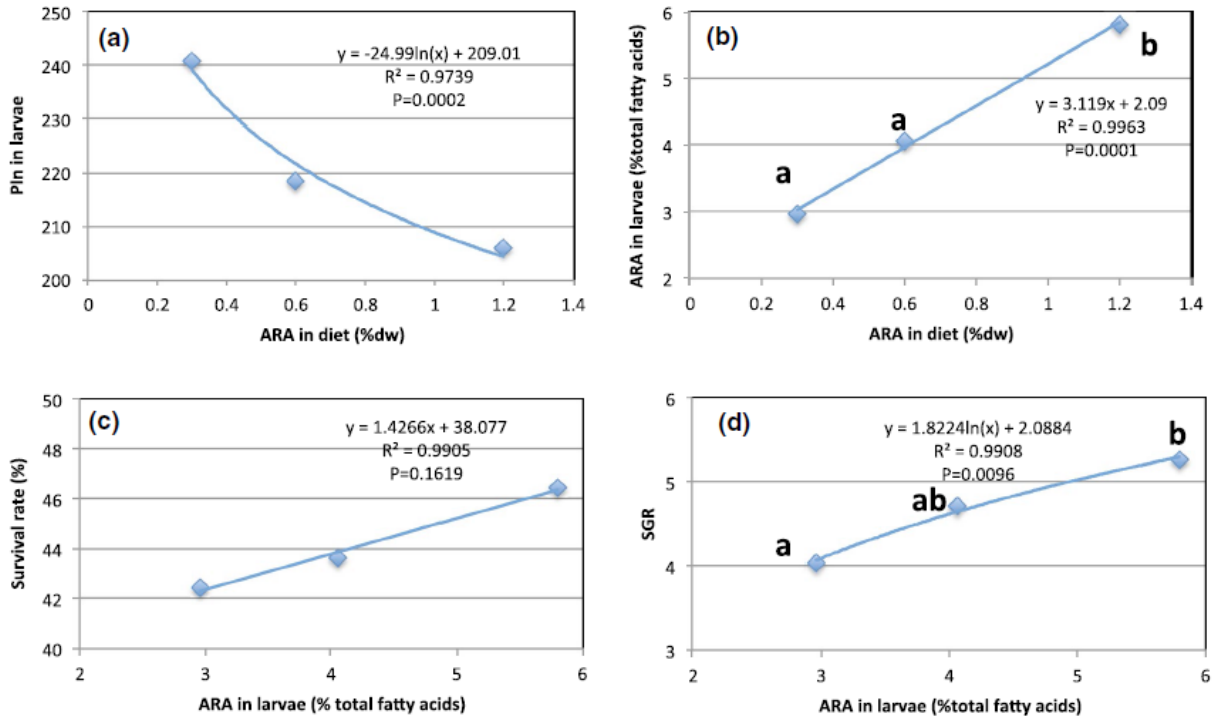
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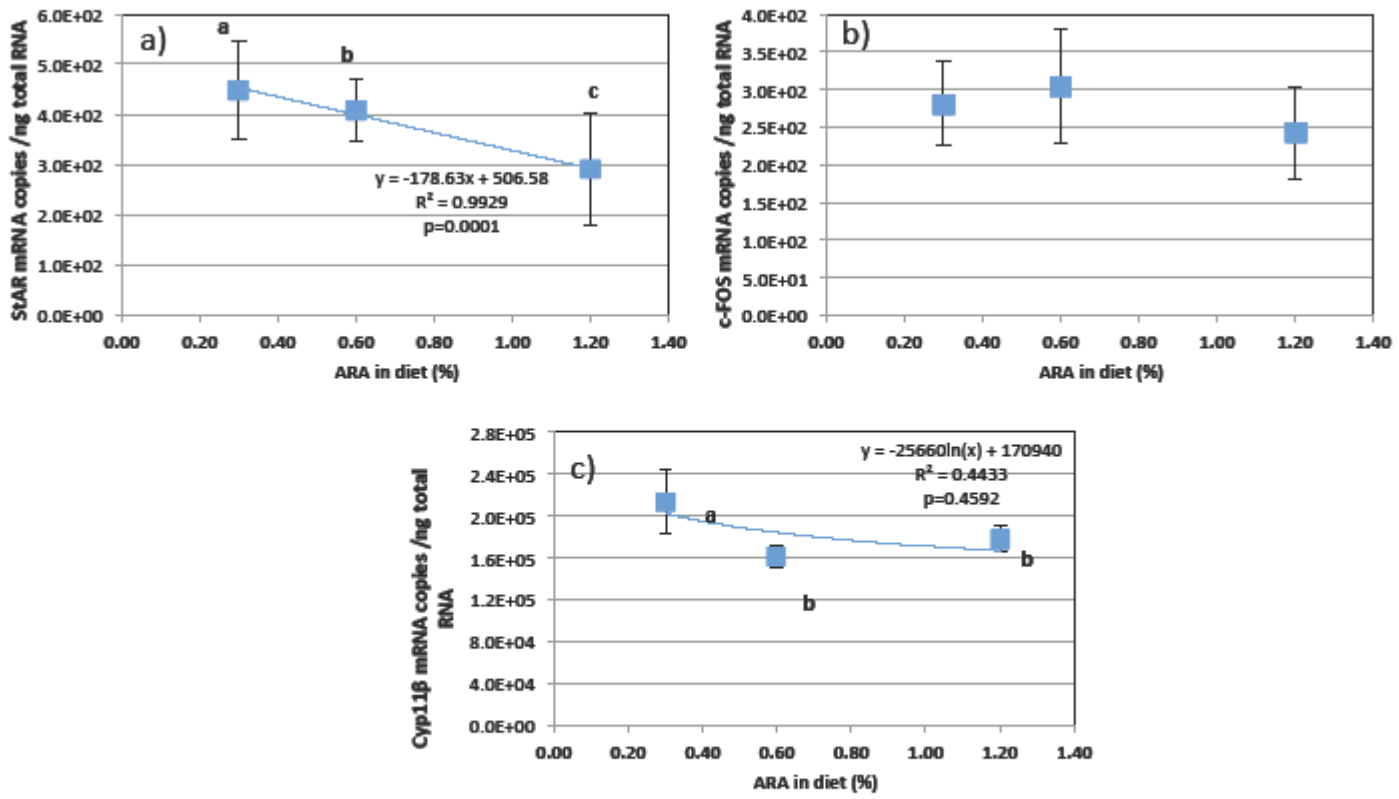
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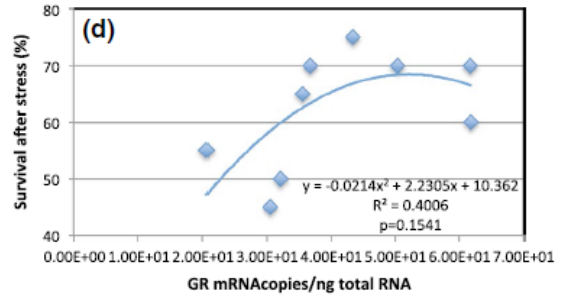
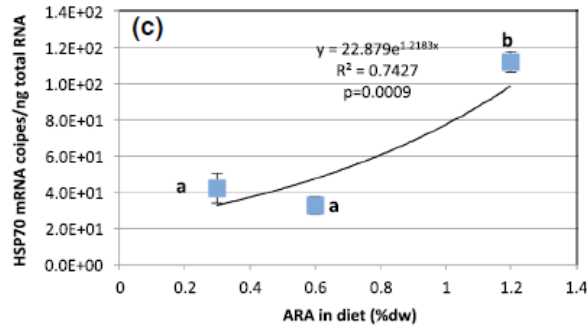
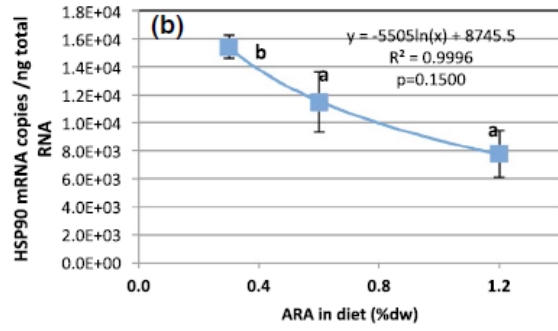
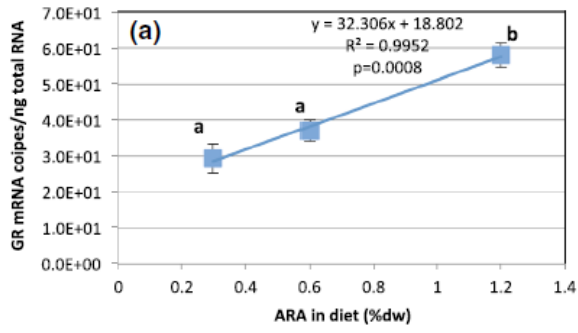
714 Figure 1

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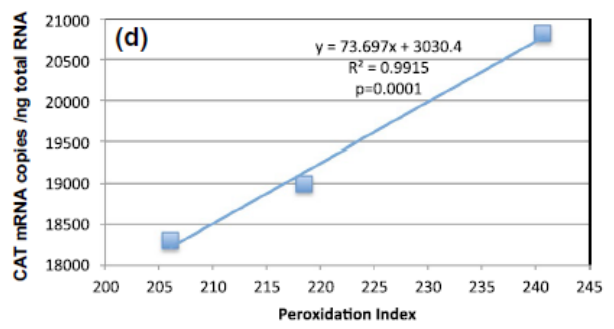
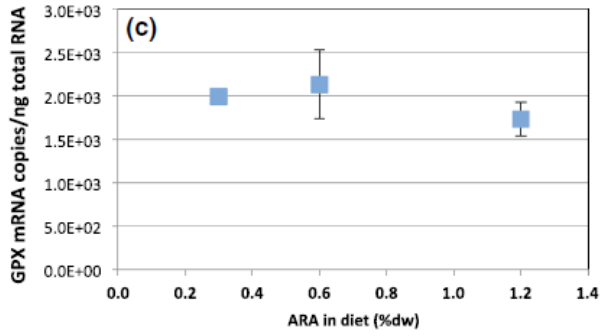
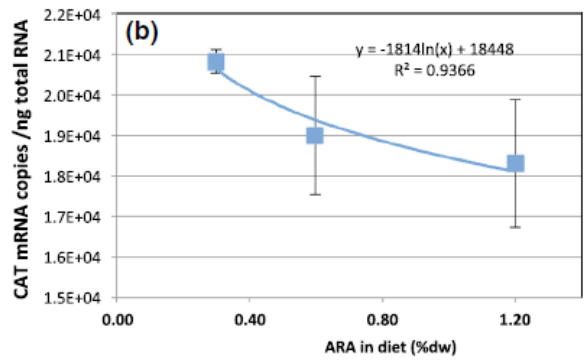
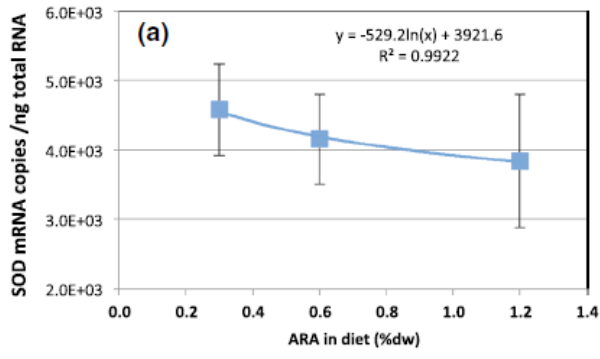
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Figure 4

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