Cortisol response to air exposure in *Solea senegalensis* post-larvae is affected by dietary arachidonic acid to eicosapentaenoic acid ratio

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Abstract

An experiment was conducted in order to evaluate the effects of feeding frozen *Artemia* diets differing in arachidonic acid to eicosapentaenoic acid ratios (ARA/EPA) on growth, survival, and stress coping ability of Senegalese sole post-larvae (19-31 days after hatch). Two experimental diets presenting high (“High”; 3.0) or low (“Low”; 0.7) ARA/EPA ratios were tested under two rearing conditions: undisturbed (C) and stressed by a 2 min air exposure every two days (S). Growth, survival, and basal cortisol levels were similar between groups indicating that independently of dietary ARA/EPA ratios fish were able to cope with the repeated stress imposed. Also, cortisol levels at 3 h past air exposure were determined in all groups at the end of the experiment. Among fish fed the “Low” diet, C groups seemed to present a quicker recovery from the acute stress (basal-like levels) than S groups. Repeated stress effects were not apparent in fish fed the “High” diet and, relative to basal levels, two-fold higher cortisol concentrations were detected at 3 h, in both C and S groups. This study suggests the importance of ARA in steroidogenesis regulation and the modulatory role of EPA in this process. Despite the tolerance to a wide range of dietary ARA/EPA as indicated by growth and survival results, acute stress coping response may be more efficient in Senegalese sole post-larvae fed low ARA/EPA ratios and, under these particular conditions, a faster recovery of cortisol to basal values could be indicative of rearing conditions (undisturbed *versus* repeatedly stressed).

Keywords: *Solea senegalensis*, cortisol, stress, fatty acids, arachidonic acid, eicosapentaenoic acid

Abbreviations: ALA, alpha-linolenic acid; ARA, arachidonic acid; DAH, days after hatch; DHA, docosahexaenoic acid; DPAn-3, docosapentaenoic acid; EPA, eicosapentaenoic acid; LC-PUFA, long-chain polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; OLA, oleic acid; RGR, relative growth rate; SAFA, saturated fatty acids.

Introduction

Senegalese sole (*Solea senegalensis*) is a species of high commercial value in southern European countries like Portugal and Spain. Larval rearing depends greatly on live feed supply and various studies during these life stages have focused on weaning strategies (Engrola et al. 2009a,b, Mai et al. 2009, Engrola et al. 2010). In hatcheries, during metamorphosis, it is common practice to gradually replace live feed with frozen *Artemia* metanauplii, generally enriched in lipids. However, lipid nutrition studies in this species have warned against excessive lipid supply in juveniles (Borges et al. 2009, Campos et al. 2010) as well as in larvae, especially in the triacylglyceride form, which can affect dietary fatty acid utilization (Morais et al. 2007). Research on long-chain polyunsaturated fatty acid (LC-PUFA) requirements suggests that, up to 35-40 days after hatch (DAH), Senegalese sole have a low need for their dietary provision as growth was not affected in various dose-response studies, under optimized rearing conditions (Villalta et al. 2005a,b, 2008b); however, specific LC-PUFA, namely arachidonic acid (ARA; 20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3), have commanded some attention due to their involvement in skin pigmentation development (Villalta et al. 2005a, 2008a,b). Despite low requirements for LC-PUFA, several of the above mentioned studies show these are in general selectively retained in sole tissues. Their physiological importance is well recognized in fish nutrition regarding cell membrane structure and functions, growth, and development, particularly of the neural and visual systems (Tocher 2003).

The ability to cope with stress and maintain homeostatic balance is presently recognized as a valuable parameter for the evaluation of the physiological condition in fish, along with survival and growth performance. Long chain-polyunsaturates, mainly ARA, are often investigated for their implication in stress response, most commonly attributed to their role as eicosanoid precursors (Bessonart et al. 1999, Koven et al. 2003, Van Anholt et al. 2004a). Significant ARA effects seem to occur when its dietary intake exceeds the requirement, leading to a higher abundance of the free intracellular form which can potentially directly or indirectly modulate various signalling pathways (Van Anholt et al. 2004b). However, these effects also depend on the abundance of n-3 LC-PUFA, EPA and docosahexaenoic acid (DHA; 22:6n-3), which can inhibit ARA metabolism and alter the profile of regulatory eicosanoids (Calder 2009). These relations are indeed thought to be a main cause for the previously mentioned effects on skin pigmentation in Senegalese sole. Furthermore, EPA oxidized derivatives differ in their biological activities compared to those produced from ARA which generally (not always) present higher potency (Calder 2009). Hence, ratios between LC-PUFA are extremely important and, considering their low requirements in Senegalese sole, it is plausible that unbalanced ratios or excessive supply in the diet of such fatty acids could reflect in altered stress coping ability.

Fatty acids and their derivatives can act as transcription factors that regulate protein synthesis, as ligands in signal transduction, and as membrane components that regulate the fluidity, permeability, and dynamics of cell membranes (Tocher 2003, Clarke 2004). In terms of the fish cortisol response to stress, effects of LC-PUFA supply have been observed *in vitro* in gilthead seabream interrenal cells (Ganga et al. 2006, Ganga et al. 2010) and *in vivo* in the same species (Van Anholt et al. 2004a). However, various factors such as stress type, developmental stage, diet (Koven et al. 2003), species, early life history and individual differences, can contribute to ambivalent results in stress resistance and cortisol production in fish (Van Weerd and Komen 1998).

Understanding the lipid requirements of a fish species is essential for the development and optimisation of diets, and stress studies can help narrowing the range at which essential fatty acids should be provided. This has become of major importance for the aquaculture sector within which continuous attempts at decreasing the dependence of the aquafeed industry on fish oils and meals have been made in order to promote more sustainable growth of the sector. Commercial marine fish microdiets generally present very low ARA/EPA ratios and minimum n-3 LC-PUFA levels of 2% (Yildiz 2009).

This experiment was designed as a first approach to understand the potential effects of different dietary ARA/EPA ratios on the acute stress coping ability of Senegalese sole post-larvae. Additionally, the outcome of regular air exposure during the experimental period was assessed. As such, data on growth, survival, and whole-body cortisol concentration were collected and analysed in light of the fish fatty acid profiles.

Materials and Methods

Larval rearing

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

This experiment was conducted at the Centre of Marine Sciences (University of Algarve, Faro, Portugal). Senegalese sole eggs were obtained from INRB/IPIMAR EPPO facility (Olhão, Portugal). The larvae were kept in 100 L cylindro-conical tanks with an initial density of 100 larvae L⁻¹, in a recirculation system supplied with seawater (salinity about 35 ppt, dissolved oxygen around 6 mg L⁻¹ and 18 – 19 °C temperature) with constant aeration and under 12 h light: 12 h dark conditions. Tank water renewal was 4 times daily until 3 DAH and increased to 8 times per day until the transfer of the fish into the experimental tanks at the end of metamorphosis (18 DAH). During this period, the green water technique was applied to the rearing tanks with the addition of *Tetraselmis chuii*. The larvae were fed rotifers (*Brachionus rotundiformis*) until 3 DAH and *Artemia* nauplii (INVE Aquaculture NV) thereafter, 3 times daily, until 9 DAH. From 9 to 18 DAH, enriched *Artemia* metanauplii (Selco, INVE Aquaculture NV) were fed to the larvae, according to standard rearing procedures (Conceição et al. 2007). *Artemia* enrichments were performed in a single dose at densities of about 250 nauplii mL⁻¹, with 0.6 g enrichment L⁻¹, at 28 °C, during 16 h. During metamorphosis (14 to 18 DAH) live *Artemia* was gradually replaced by frozen metanauplii. Monitoring of water quality, tank maintenance and removal of mortalities were performed daily.

Experimental diets

Two lipid emulsions were formulated (table 1) to enrich *Artemia* nauplii in a single batch per dietary treatment. These formulations were designed to confer distinct ARA/EPA ratios (“High” and “Low”) to the *Artemia*, which were kept frozen until required during the experiment. Enrichments were conducted as described above and *Artemia* samples were analysed for lipid content and fatty acid profile (table 2).

Experimental design and sampling procedures

At 18 DAH Senegalese sole post-larvae were distributed into a recirculation system of 12 rectangular tanks (4 L) at a density of 3000 post-larvae m⁻² (220 larvae per tank). During the experimental period, 19 to 31 DAH, fish were kept at around 20 °C, 36 ppt salinity, and under a 14 h light: 10 h dark photoperiod (100 lux). Water quality monitoring and tank maintenance were performed daily, in the morning. Post-larvae were hand-fed the previously enriched and frozen *Artemia* metanauplii, four times daily, in slight excess (based on daily visual inspection and adjustment). Feeding rates ranged from 20-30 % body weight day⁻¹ (dry matter basis). This experiment included the testing of two diets, termed “High” and “Low” in relation with their ARA/EPA ratios, combined with either control (C) or repeated stress (S) conditions. The repeatedly stressed groups were submitted to a 2 minute air exposure every two days, by gentle siphoning of the total volume of tank water (water level below 1 mm), before the first daily meal. As such, four experimental treatments were defined, each tested in triplicate: HighC, HighS, LowC, and LowS.

Initial average dry weight and total length of the post-larvae were determined from a 20 fish sample stored at -20 °C until measurements could be conducted. At the end of the experiment, samples were taken for the analyses of effects of *Artemia* lipid enrichment and repeated air exposure on growth performance (20 fish per tank for dry weight, 10 per tank for total length), and whole-body fatty acid composition (50 post-larvae per tank). Whole-body cortisol levels were also determined at the end of the experiment in all experimental groups, under basal conditions and 3 h after a 2 min air exposure (pools of 7 to 10 post-larvae per tank, per sampling time), as cortisol response to stress usually develops from 1 to 3 h after air exposure in Senegalese sole post-larvae (personal observation; Ruane et al. 2005). Samples collected for both lipid and cortisol analyses were stored in eppendorf tubes at -80 °C. Survival was determined by total counting of individuals in each tank at the end of the experiment (31 DAH). All post-larvae sampled were previously anaesthetised with an overdose of 2-phenoxyethanol and washed with distilled water before storage or measurements.

Analytical methods

Whole-body dry weight was determined individually in a Sartorius M5P scale (0.001 mg precision; Sartorius micro, Göttingen, Germany) after freeze-drying the samples for 48 h in a Savant SS31 (Savant Instruments Inc., Hokbrook, NY, USA). Total length was measured on individual photographs of each post-larva with an image processing and analysis program (UTHSCSA Image Tool, version 3.0, C.D. Wilcox, S.B. Dove, W.D. McDavid, and D.B. Greer, University of Texas Health Science Centre, Texas, USA).

In order to evaluate the lipid composition of *Artemia* and post-larvae, total lipids were extracted using an adaptation of the Bligh and Dyer method (1959) in which a mixture of solvents - namely dichloromethane (replacing chloroform in the original method for lower toxicity purposes) and methanol - was used to extract the lipid fraction from fresh tissues. Hexane was also used to improve the recovery of the non-polar lipids (e.g. triacylglycerols or cholesterol esters). The combined organic layers were added to a saline solution to improve the partition of the mixture into aqueous and organic phases and the solvents evaporated for the gravimetric determination of total lipid content. The fatty acid profile of each sample was analysed using an adaptation of the Lepage and Roy method (1986) to the amount isolated in each fraction, in a Varian Star 3800 CP (Walnut Creek, CA) gas chromatograph equipped with an auto-sampler and fitted with a flame ionisation detector at 250 °C. The separation was performed in a polyethylene glycol capillary column DB-WAX with 30m-length 0.25 mm i.d. and 0.25 mm film thickness from J&W Scientific (Folsom, CA, USA). The column was subjected to a temperature program starting at 180 °C for 5 min, heating at 4 °C min⁻¹ for 10 min, and held up at 220 °C for 25 min. The injector (split ratio 100:1) and detector temperatures were kept constant at 250 °C during the 40 min analysis. Fatty acid peaks were identified by directly comparing retention times with those of a known standard ("PUFA 3"; SIGMA-ALDRICH®, USA) and quantified by means of the response factor to an internal standard (21:0) which was used at 5 ml mg⁻¹ of sample.

A commercial cortisol enzyme-linked immunosorbent assay kit (ELISA; Neogen Corporation, Lexington, KY, USA) was used to assess whole-body cortisol levels in pooled post-larvae samples of about 80 mg per tank (wet weight). This was preceded by sample homogenization in a phosphate buffered saline solution and extraction with diethyl ether. The latter was performed following recommendations by

Sink et al. (2007) which involve the addition of 100 µL of a food-grade vegetable oil per gram of body weight analysed. Olive oil was used for this end and previously assayed for cortisol to ensure no cross contamination would occur from animal fats. After extraction and solvent evaporation under a stream of nitrogen, lipid extracts containing cortisol were reconstituted in the extraction buffer of the kit, diluted to an appropriate factor and analysed according to the manufacturer's instructions. All samples and standards were run in duplicate. Intra-assay variation was checked and a coefficient of variation (% CV) of $\leq 20.0\%$ was set as acceptable. Parallelism and linearity (passing limit $r^2 > 0.90$) were tested using serial dilutions of sample extracts.

Statistical analysis

Senegalese sole growth was determined at the end of the experiment for all treatment groups and is expressed as relative growth rate (RGR) using the formula: $(e^g - 1) \times 100$, with $g = [(\ln \text{final weight} - \ln \text{initial weight}) / \text{time}]$ (Ricker 1958). For the assessment of differences between experimental groups regarding growth performance, whole-body fatty acid composition and basal cortisol levels a two-way ANOVA was used with dietary and repeated stress treatments as independent variables. Differences were considered statistically significant when $P < 0.05$, except for data regarding whole-body cortisol concentrations for which the alpha level was set at 0.10 due to combined low sample number (3 tanks per treatment) and high biological variation for cortisol typical in Senegalese sole (Aragão et al. 2008, Costas et al. 2008, Silva et al. 2010). Furthermore, the effect size (partial eta squared) was considered as an indicator of the magnitude of the differences between means i.e. the amount of total variance in the dependent variable that is predictable from knowledge of the levels of the independent variable. Also, within each dietary treatment, cortisol data were analysed by a 2 X 2 mixed-design ANOVA to assess differences in whole-body levels between groups exposed or not to repeated stress (between-subjects variable), and at two time periods (within-subjects variable), before and 3 h after an acute stress which all groups were submitted to at the end of the experiment. The relationships between fatty acid concentrations in diet and in fish can differ among fatty acids. Hence, the differences (Δ values) between the percentages of selected fatty acids in larval lipids and in dietary lipids were calculated (% total fatty acids). All statistical analyses were conducted using the SPSS 16.0 software package.

Results

Fatty acid composition analyses of the *Artemia* showed ARA/EPA ratios of 3.0 (High diet) and 0.7 (Low diet) were obtained after enrichment with the formulated emulsions (table 2). This difference was due mostly to variation in EPA content (2.5 mg g⁻¹ sample vs. 11.0 mg g⁻¹ sample, respectively), as ARA remained at 7.3 - 7.5 mg g⁻¹ diet in both treatments. Also, a relatively higher oleic acid (OLA; 18:1n-9) level was present in the High diet whereas DHA content was more elevated in the Low *Artemia*. At the start of the experiment the dry weight of the post-larvae was 1.14±0.32 mg and total length was 10.3±1.3 mm.

No significant interaction effects were detected between diet and repeated stress regarding results of the growth parameters analysed and survival ($P > 0.05$). Also, no statistically significant effects for either diet or repeated stress were found ($P > 0.05$). Senegalese sole more than doubled their body weight during the course of the experiment and grew at a relative growth rate of 6.8 to 7.9 % daily (table 3). Final dry weight was 2.7-3.1 mg per post-larva and total length averaged from 14.2 to 14.8 mm. Survival was above 90 % in all groups.

Whole-body fatty acid composition of the post-larvae generally mirrored dietary profiles and no interaction effects between dietary treatment and repeated stress were found. Effects detected for dietary treatment are identified in table 4, whereas no significant effects for repeated air exposure on fish fatty acid composition were found, at the end of the experiment. As such, fish fed the Low diet showed lower monounsaturated fatty acid levels, particularly OLA ($P < 0.05$). Also, ARA levels were similar between experimental groups, whereas EPA was more abundant in fish fed the Low diet. These differences resulted in ARA/EPA ratios of 4.5 and 1.6 for fish fed diets High and Low, respectively. DHA was significantly more deposited in groups fed the Low diet ($P < 0.05$), which also reflected dietary content. Nonetheless, despite similar DHA/EPA ratios in the diets, these differed between experimental groups as the difference in EPA levels in the post-larvae between groups fed different diets was more pronounced than that of DHA.

Selective utilization of C18 fatty acids, particularly linolenic acid (ALA; 18:3n-3), was evident in all experimental groups since their concentration in larval lipids was lower than that provided through dietary lipids, as shown in table 5. OLA was also metabolized by the larvae, especially when in higher abundance in the diet (High diet fed groups). Conversely, ARA and DHA appeared to be preferentially retained in the larvae in all groups, whereas Δ values for EPA pointed to a preferential utilization of this fatty acid for metabolic purposes when supplied in higher dietary doses (Low diet fed larvae).

Whole-body cortisol measurements were performed before and at 3 h past air exposure in all experimental groups, at the end of the experiment. Survival at the end of this challenge was 100 % in all groups. Intra-assay coefficient of variation for the ELISA was 11.1 %. Displacement curves for cortisol standards and sample serial dilutions confirmed parallelism and linearity ($r^2 = 0.9997$). These results are summarised in figures 1 and 2. Basal cortisol concentration analysis revealed the interaction effect between diet and repeated stress treatment was not statistically significant ($P = 0.68$), neither were the main effects for diet ($P = 0.34$) nor repeated stress ($P = 0.93$). Overall, mean basal cortisol values ranged between 83.3 and 141.4 ng g⁻¹ of post-larvae sample. Results of the mixed ANOVA within each of the

dietary groups revealed no significant interactions between repeated stress treatment and sampling time ($P = 0.74$ in groups fed High diet; $P = 0.24$ in groups fed Low diet). However, regarding repeated stress (between-subjects factor), soles fed the two diets showed different trends in cortisol levels. As such, analysis of the groups fed the High diet showed repeated stress did not affect cortisol levels ($P = 0.48$), whereas in Low diet fed fish repeated stress affected whole-body cortisol ($P = 0.06$) which was further corroborated by the partial eta squared statistical value (62 %). Indeed, overall cortisol concentrations were higher in LowS than in LowC groups, which was particularly noticeable for determinations at 3 h after stress (two-fold higher than basal levels in LowS groups). On the other hand, P values obtained for sampling time (within-subjects factor) were not statistically significant (0.14 and 0.20 for High and Low diet fed groups, respectively). At 3 h after stress, both HighC and HighS groups presented average cortisol levels of 245.8 and 183.6 ng g⁻¹ post-larvae, respectively, meaning a two-fold increase relative to basal values which can be considered biologically significant. Besides, partial eta squared value indicates that 46 % of the total variance found in cortisol in High diet groups was due to sampling time. On the other hand, in Low diet fed fish, 37 % of this variance could be assigned to sampling time and the P value for this factor was slightly higher (0.20). The dispersion in values found at 3 h after stress within this dietary group was likely accountable for the lack of effect of sampling time; that is, whereas LowS groups showed average cortisol levels of 302.8 ng g⁻¹ post-larvae, those maintained under control conditions presented values around 130.0 ng g⁻¹ which were within the range observed for basal cortisol levels in this experiment.

Discussion

Relative growth rates and dry weights obtained at the end of this study were not significantly affected by the experimental treatments and were within values previously reported for this species, at similar developmental stages, when fed frozen *Artemia* diets (Engrola et al. 2007, 2009a). Alongside good survival these results revealed that Senegalese sole post-larvae present high tolerance towards a relatively wide range of dietary ARA/EPA ratios, which could be due to the exceptionally low dietary requirements for LC-PUFA identified for Senegalese sole larvae, relative to most marine fish studied (Villalta et al. 2005a, b, 2008b). Also, growth performance and survival results suggested that the ability to cope with the regularly imposed stress throughout the experimental period was not compromised. Unaffected growth in Senegalese soles regularly exposed to acute handling stress (air exposure) or reared under chronic stress conditions (high stocking density) has been reported at the juvenile stage, also (Aragão et al. 2008, Costas et al. 2008).

The importance of dietary ARA/EPA ratios is generally recognized due to the known competition between these two fatty acids for both inclusion into cellular membrane phospholipids by fatty acyltransferases, and subsequent eicosanoid formation and action (Sargent et al. 1999). Eicosanoids are mostly studied for their inflammatory and immunoactive functions but their role in stress response has been acknowledged in fish (Van Anholt et al. 2003, Ganga et al. 2006, 2010). In principle, an important shift in dietary ARA/EPA ratios could thus compromise the overall physiological condition of the fish including its health status and stress coping capacity. Moreover, in Senegalese sole in particular, high dietary ARA levels were found to be linked to albinism (Villalta et al. 2005a, 2008a), although in this study no incidence of malpigmentation was noted for any of the experimental groups, as this effect may be most predominant during the premetamorphosis period (Lund et al. 2008).

Specific fatty acids are either selectively retained or metabolised, and preferential metabolism often occurs when a particular fatty acid is supplied at high concentrations in the diet (Karalazos et al. 2007). Assuming that lipid digestibility is high in Senegalese sole post-larvae, especially that of LC-PUFA (Conceição et al. 2007, Mai et al. 2009), the identification of the preferential metabolic fate of fatty acids should be possible by comparing fish fatty acid profiles with those presented in the diets. Delta values for C18 fatty acids, like OLA and particularly ALA, provide indication that these were preferentially metabolised, likely for energy production purposes through β -oxidation, as observed in various fish species (Bell et al. 2001, Tocher 2003, Morais et al. 2006). On the other hand, ARA, docosapentaenoic acid (DPA_n-3; 22:5_n-3), and particularly DHA, appeared to be preferentially retained in all experimental groups, as reported by other authors (Morais et al. 2004).

Dietary ARA levels *per se* may not be sufficient to cause significant changes in basal cortisol levels (Van Anholt et al. 2004b); it seems from the present results that in Senegalese sole the same can be concluded regarding dietary ARA/EPA ratios. Basal cortisol levels were relatively high which seems to be a characteristic feature in dark coloured post-larvae of this species (Ruane et al. 2005), such as those reared in this study. High variability found also for this parameter before and after acute stress, as reported by other authors in post-larvae (Ruane et al. 2005) and juveniles (Aragão et al. 2008, Costas et

al. 2008), is a typical trait of the species attributed to individual differences in stress coping styles (Silva et al. 2010). Therefore, it is important to consider that a low sample number and high biological variation for this parameter may contribute to the lack of statistically significant differences detected by variance analyses. In this study, we further explored these results by using an effect size measure (partial eta squared).

A cortisol response classically develops from 1 to 3 h after air exposure in Senegalese sole post-larvae (personal observation). Although a time-course study of the cortisol response was not conducted, data suggested a faster recovery back to basal levels after air exposure in previously undisturbed LowC groups, in contrast with observations in other groups. As such, these results suggest that cortisol stress coping response to air exposure was delayed or more intense in fish fed high ARA/EPA ratios and/or repeatedly stressed animals, whereas feeding low ARA/EPA ratios may facilitate stress coping in Senegalese sole reared under undisturbed conditions. Indeed, it appears that the dietary induced differences in cortisol response were not caused by the absolute amount of ARA in fish tissues, which remained similar between groups, but rather by EPA deposition levels. The availability of either of these fatty acids to enter the eicosanoid synthesis cascade or other cellular signalling pathways, to influence cell membrane fluidity or to regulate the expression of various genes thus affecting numerous metabolic pathways, is generally recognised and could potentially affect cortisol release. Arachidonic acid and/or its metabolites have been linked to enhanced steroidogenesis in mammal (Hirai et al. 1985) and fish studies (Koven et al. 2003), and considered particularly for their involvement in the regulation of the steroidogenic acute regulatory protein (StAR), acknowledged as the key rate-limiting enzyme in steroidogenesis (Wang and Stocco 1999). Thus, it is possible that the abundance of ARA relative to EPA (or their oxidized derivatives) in HighC fish could influence StAR gene or protein expression, increase cortisol production, and ultimately imply higher energy expenditure to cope with stress. In fact, fish fed higher amounts of EPA (4.9-fold increase in Low diet relative to High diet) deposited EPA in the tissues in larger quantity but also directed it for metabolism. Hence, the physiological action of EPA or that of its oxidized derivatives in Low diet groups could likely have counteracted those of ARA (or its metabolites) in steroidogenesis enhancement. In addition, EPA appeared to be partially elongated into DPAn-3 as significantly higher levels of the later were noted in Low diet fed soles, despite the absence of this fatty acid in *Artemia*.

Whereas in LowC groups cortisol concentrations at 3 h post-stress were among the range of values determined for basal cortisol in this experiment, LowS fish showed approximately double the concentrations detected prior to air exposure. Therefore, in Low diet groups, repeated exposure to stress altered the cortisol response to acute stress suggesting that, when reared in a stressful environment, Senegalese soles could require longer periods to regain homeostasis. However, the effect of repeated stress was not detected in groups fed with the High diet. It is possible that the enhancement of cortisol response to acute stress by ARA relative abundance, as previously proposed, could mask the effects of repeated stress between the High diet fed groups.

Repeated stress could have been expected to affect weight gain through decreased feed intake, absorption or utilization, as well as owing to higher metabolic energy expenditure to cope with stress (Van Weerd and Komen 1998). However, it is clear from data regarding growth performance, survival at the end of the experiment, and cortisol before and after stress that, for the duration of the experiment, adaptation to both dietary conditions tested was successful, and that stress coping ability was not compromised. Judging from the survival rates after air exposure (100 % in all groups), the stress response of soles was adaptive, regardless of the cortisol levels determined for the various experimental groups. Nonetheless, cortisol concentrations at 3 h post-stress suggest that this response could be more efficient in Senegalese sole post-larvae fed low dietary ARA/EPA ratios, whereas high ratios and repeated stress exposure seem to promote steroidogenesis. The regulation of enzymes involved in cortisol production, such as StAR, should be envisaged in future studies regarding potential roles for fatty acids on fish stress response. Understanding these mechanisms is an important contribution for the development of optimised diets used in intensive fish farming, particularly during early life stages.

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Fig1 Linearity (a) and parallelism (b) of the ELISA cortisol analysis method. Each point is the mean of duplicate determinations

Fig2 Whole-body cortisol levels prior to air exposure stress (basal) and 3 h post-stress, at the end of the experimental period. Sole post-larvae were fed different ARA/EPA ratios (High and Low) and either submitted (S) or not (C) to repeated stress during the experiment. Cortisol levels of post-larvae fed the “High” diet are shown left of the dashed line and those of soles fed the “Low” diet are shown right of the dashed line. Values are mean $\pm$ SE. Asterisks mean statistical differences due to repeated stress within one dietary treatment (mixed ANOVA; $P < 0.10$). Absence of letters means no statistical differences due to sampling time (mixed ANOVA; $P > 0.10$)