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The effect of dietary n-3 LC-PUFA on the responses to acute and prolonged stress of meagre (*Argyrosomus regius*, Asso 1801) juveniles

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Abstract

The replacement of fish oils in aquafeeds by vegetable oil sources is known to cause a depletion in dietary n-3 LC-PUFA, particularly in DHA and EPA. This decrease may influence several performance indicators in fish, including health status and stress resistance and response. The present study aimed to evaluate the effect of dietary n-3 LC-PUFA levels (0.8, 1.4 and 2.6 %), below and above the requirement estimated for growth, in stress response of meagre juveniles exposed to an acute and prolonged stress. Fish were submitted to an acute stress by chasing for 15 seconds, and a prolonged stress by cage confinement for 7 days. The lowest n-3 LC-PUFA levels (0.8 and 1.4 %) led to higher post-stress plasma cortisol levels than fish fed 2.6 %. Besides, acute stress led to higher levels of post-stress plasma lactate, as well as a strong neuronal serotonergic activity in fish fed the lowest n-3 LC-PUFA diets, while prolonged confinement resulted in the highest relative *catalase* mRNA levels in fish fed 1.4 % n-3 LC-PUFA. Therefore, the dietary changes in n-3 LC-PUFA, essential for marine fish, induce a change in the stress response in meagre, significantly increasing glucocorticoid and serotonergic response in fish fed low n-3 LC-PUFA contents. Furthermore, low n-3 LC-PUFA diets generates greater alterations in relation to basal levels of oxidative stress-related genes and, possibly increasing the oxidative stress

damage. However, meagre denotes a good adaptation to both acute and prolonged stress, even when fed with low n-3 LC-PUFA diets, highlighting the high stress resistance of this species.

Keywords: n-3 long-chain polyunsaturated fatty acids; essential fatty acids; meagre *Argyrosomus regius*; stress response

Abbreviations: ALA, linolenic acid; ARA, arachidonic acid; cat, catalase; DHA, docosahexaenoic acid; DM, dry matter; EFA, essential fatty acid; EPA, eicosapentaenoic acid; gpx, glutathione peroxidase; h, hour(s); LA, linoleic acid; LC-PUFA, long-chain polyunsaturated fatty acids; VO, vegetable oil(s); 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin

1. Introduction

In the past decades, an intensive search for alternative sources to fish meal (FM) and fish oil (FO) has been accomplished, and, nowadays, the aquaculture industry can incorporate considerable amounts of vegetable meals and oils in aquafeeds without compromise growth rates of several target species, including meagre. However, the replacement of FM and FO by vegetable sources is known to cause a depletion in dietary n-3 LC-PUFA, which are recognized to, not only play important roles in fish cellular metabolism and structure, but also to modulate immune system and stress response and resistance (Izquierdo, 1996; Montero and Izquierdo, 2011; Tocher, 2015). Therefore, the alteration of the dietary fatty acid profile, for instance through the inclusion of vegetable oils (VO) in aquafeeds, may modify the contents and proportions between different essential fatty acids (EFA) and, consequently, affect fish susceptibility and response to stress (Montero et al., 2003).

The response to a stressful situation comprises endocrine, neural, haematological, metabolic, transcriptomic, antioxidant and immune alterations (Barton and Iwama, 1991; Tort, 2011). In a primary stage, this response is initiated by neural

alterations, with a stimulus of fish hypothalamus–pituitary–inter-renal cells axis (HPI), and the subsequent release of cortisol to the blood stream. Upon stress exposure, an increased release of serotonin (5-hydroxytryptamine, 5-HT) at the brain, including in telencephalon region, seems to be involved in the downstream activation of the HPI axis (Gesto et al., 2013; Medeiros et al., 2014). Indeed, this brain region is believed to have a key role when integrating sensorial information about stressors and in the organization of a response (Vindas et al., 2017). Furthermore, stress is also recognised to stimulate the production of the reactive oxygen species (ROS), resulting from animal normal aerobic processes, and which are known to be deleterious for fish. To counterbalance ROS and protect against oxidation damage, fish have an antioxidant defence system, which is composed by vitamins (α -tocopherol) and enzymes, including, among others, the catalase (CAT) and the glutathione peroxidase (GPX) (Halliwell, 1996). However, when the production of ROS surpasses organism detoxification power or the antioxidant defence system is inefficient, cell membrane oxidation may lead to tissue damage that can affects genes transcription levels (Olsvik et al., 2011). Moreover, despite fish having a specific requirement for n-3 LC-PUFA, they are also more susceptible to oxidation (Halliwell and Chirico, 1993). Several studies reported an effect of dietary nutrients in fish oxidative stress, particularly lipids and PUFA (Mourente et al., 1999; 2000; 2002). However, fewer studies have evaluated the effect of dietary n-3 LC-PUFA on antioxidant defence system under stressful conditions (Pérez-Sánchez et al., 2013; Welker and Congleton, 2003). Finally, if all the former responses are insufficient to promote fish adaptation to stress and the recovery of homeostasis, changes in the whole-animal occur, including immunosuppression (Tort, 2011). Besides, more energy is deviated to deal with stress and, consequently, less energy is available for animal vital functions and growth, which, in extreme cases, could lead to death and generating important economic losses in aquaculture farms (Tort, 2011; Barton and Iwama, 1991; McCormick et al., 1998).

Meagre (*Argyrosomus regius*) is a carnivorous species that has been most recently incorporated into large-scale aquaculture production in Europe, mainly due to its high growth rate and good feed conversion ratios (FAO, 2017). Meagre lipid requirement was established at 17 % wet weight of the diet (Chatzifotis et al., 2010), while, at least, 2.0 % of n-3 LC-PUFA in dry weight of feed is necessary for maximizing growth performance and feed conversion of fingerlings (Carvalho et al., 2018). However, for a precise determination of nutritional requirements, other parameters besides growth, such as the ability of the fish to cope with physiological or oxidative stress, should be considered. Moreover, the requirements for fish growth may differ from the requirements under stress conditions (Montero and Izquierdo, 2011). Since the stress response is known to be species-specific (Fanouraki et al., 2011), the present study aimed to evaluate the effect of increasing dietary n-3 LC-PUFA on the response to an acute and a prolonged stress in meagre juveniles.

2. Materials and Methods

This experiment was conducted according to the European Union Directive (2010/63/EU) and Spanish legislation (RD 53/2013) on the protection of animals for scientific purposes at ECOAQUA Institute of University of Las Palmas de Gran Canaria (Canary Islands, Spain).

2.1 Experimental diets, fish and conditions

Previous to stress challenges, a feeding trial was performed with meagre fingerlings (initial body weight of ~3 g), in which five increasing n-3 LC-PUFA levels from 0.8 to 3.6 % in dry weight of feed were tested to check growth and feed utilization (Carvalho et al., 2018). In the same previous study, the requirement estimated for maximum growth was determined, at least, 2.0 % DM n-3 LC-PUFA. After the growth trial, two stress challenges were conducted, in the 66th and 90th day of the feeding period, for

experiment I and II, respectively, in order to assess the effect of the dietary n-3 LC-PUFA on stress response of meagre juveniles. For that, three of the five diets were selected: two below the requirement estimated for optimal growth (0.8 and 1.4 %), and one above (2.6 %). Experiment I evaluated the effect of 0.8 %, 1.4 % and 2.6 % dietary n-3 LC-PUFA on meagre stress plasmatic parameters and telencephalic serotonergic activity in response to a short acute stress. Experiment II evaluated the effect of 1.4 and 2.6 % n-3 LC-PUFA diets, on meagre oxidative imbalance generated by a prolonged stress exposure, using the expression of antioxidant defence-related genes as an indirect measure of potential oxidative stress. Treatment 0.8% was not included in Experiment II because there was insufficient number of animals to perform the trial with the adequate level of replication. Diets were manufactured by Skretting ARC (Stavanger, Norway) and the composition and respective FA profiles are presented in Table 1.

Table 1. Composition (%), proximate analysis and fatty acid profile of the experimental diets

	Dietary n-3 LC-PUFA level (% DM)		
	0.8	1.4	2.6
<i>Ingredients (%)</i>			
Fish meal, N. Atlantic ¹	15.0	15.0	15.0
Corn gluten ²	10.0	10.0	10.0

Faba beans ¹	10.0	10.0	10.0
Wheat ¹	8.0	8.0	8.0
Wheat gluten ¹	18.4	18.4	18.4
Soy protein concentrate ¹	25.0	25.0	25.0
Fish oil, S. American ¹	0.0	2.7	8.2
Linseed oil ³	1.6	1.2	0.4
Palm oil ³	3.3	2.5	0.8
Rapeseed oil ¹	6.0	4.5	1.5
Premix ⁴	2.8	2.8	2.8
<i>Proximate analysis (% DM)</i>			
Protein	56.5	54.5	56.0
Lipids	16.2	17.0	16.9
Ash	4.9	5.0	5.2
Moisture	8.7	8.5	8.2
<i>Fatty acid composition (% total FA)</i>			
ALA (18:3n-3)	8.3	7.3	3.5
LA (18:2n-6)	18.0	16.6	12.6
ARA (20:4n-6)	0.1	0.2	0.5
EPA (20:5n-3)	2.1	3.4	6.8
DHA (22:6n-3)	2.8	4.0	7.2
EPA/ARA	21	17	13.6
EPA/DHA	0.8	0.9	0.9
Σ n-6 LC-PUFA	0.3	0.5	0.9
Σ n-3 LC-PUFA	5.2	8.0	15.1

1: Skretting, Stavanger, Norway;

2: Cargill Nordic AS, Charlottenlund, Denmark;

3: AAK AB, Karlshamn, Sweden;

4: Trouw Nutrition, Boxmeer, the Netherlands. Proprietary composition Skretting ARC, including vitamins and minerals;

Vitamin and mineral supplementation as estimated to cover requirements according NRC (2011).

2.2 Stress challenges and samples collection

2.2.1 Experiment I: acute stress

Triplicate groups of meagre juveniles (initial body weight ~25 g) fed 0.8, 1.4 % and 2.6 % dietary n-3 LC-PUFA levels were allocated into 9 tanks of 250 L (working volume of 100 L), at a density of 10 fish per tank (2.5 kg m^{-3}). After 4 days of acclimation to the experimental conditions, a short-standardized handling stress protocol, by chasing fish with a net for 15 seconds, was applied. When the stress protocol started, the water volume in the tanks was reduced to 15 L in around 45 s, by opening the drainpipe. The 15 seconds of chasing were applied 15 s after starting to reduce the water level. The first seconds of chasing were used for capturing fish and take samples at control sampling point (time 0), while the remaining 10 s were used for chasing the fish that remained in the tank, when the water volume was minimum (15 L). At this point, stock density was 11.7 kg m^{-3} (7 fish per tank, since control fish were already captured). When the water volume in the tank reached the minimum (15 L), the drainpipe was closed again, and the water inflow was increased to 5 L min^{-1} for a rapid recovery of the usual water level. Samples, including blood and head collection were taken from 3-4 fish per tank at specific sampling points: time 0, 1 hour (h) and 5 h for a total of 10 animals per diet and time. All samples at each specific sampling point were collected in less than 5 min to avoid changes in stress parameters between individuals. Blood was collected from the caudal vein using heparinized syringes and then plasma was obtained by centrifugation at 4°C for 10 minutes, at 2250 g (relative centrifugal force). After that, fish were sacrificed by a cut in the head, which were immediately collected and frozen. All samples were stored at -80°C until the analysis.

2.2.2 Experiment II: prolonged stress

Triplicate groups of meagre juveniles (with an initial body weight of ~70.3 g) fed 1.4 and 2.6 % dietary n-3 LC-PUFA were allocated in 500 L tanks, at a stock density of 6 fish per tank. After 1 week of acclimation to the experimental conditions, fish were

confined in two cages of 10 L, at a density of 3 fish per cage (21 kg m^{-3}), for 7 days. Samples from 3 fish per tank were sampled at control time (pre-confinement), 2 h of confinement (one cage from each tank was sampled), and 7 days of confinement (the remaining cage in the tanks), for a total of 9 animals per diet and time. Blood collection, plasma obtaining, and fish sacrifice followed the conditions described above for Experiment I. After that, livers were collected, conserved in RNA later (Sigma-Aldrich, Madrid, Spain) and stored at -80°C until analysis. Plasma cortisol and the hepatic relative expression of antioxidant-related genes was further determined.

2.3 Biochemical analysis

2.3.1 Plasmatic stress indicators

Plasma cortisol levels were determined using the immunoassay method by ELISA (Access Immunoassays system, Cortisol ref: 33600, Beckman Coulter, Inc., USA) (Moore et al., 1985). Plasma glucose and lactate levels (Experiment I) were assessed using a colourimetric kit (Sigma, MAK013, MAK064, St. Louis, MO, USA).

2.3.2 Brain serotonergic activity

Prior to analysis, brains were individually collected out from the frozen head and immediately processed. The telencephalon was homogenized in 0.3 mL of a perchloric acid solution 4 % and then centrifuged. A diluted aliquot of the supernatant was analysed using high performance liquid chromatography with electrochemical detection (HPLC-EC), according to the conditions described previously by Gesto et al. (2017). The levels of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were quantified by comparing peak areas with proper standards.

2.4 Gene expression analysis

Total RNA extraction and cDNA synthesis were carried out following the same methodology described previously (Carvalho et al., 2018). Gene expression of *catalase*

(*cat*) and *glutathione peroxidase (gpx)* genes were analysed by individual and measured by Real-Time PCR (RT-PCR) in an iQ5 Multicolour Real-Time PCR detection system (Bio-Rad). β -*actin* was used as housekeeping gene, and RT-PCR was run according to the following conditions: a first step of 2 min at 50 °C followed by 10 min at 95 °C, and 35 cycles of 15 s at 95 °C, 30 s at gene annealing temperature (T_m) and 30 s at 72 °C. β -*actin*, *cat* and *gpx* primer sequences used were the same described in Ruíz et al. (2018) and are shown in Table 2, as well their respective annealing temperatures. All PCR reactions were run in a final volume of 20 μ l, with 7.5 μ l of Brilliant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA), 0.6 μ l of each primer (10 mM), 5 μ l of cDNA (1:10 dilution) and 1.3 μ l of MiliQ water. MiliQ water replaced cDNA to use as blank reactions.

Table 2. Primer sequences and respective annealing temperature used for real-time PCR (RT-PCR)

Gene	Primer sequence (5' - 3')		Annealing temperature (T_m , °C)
<i>B-actin</i>	Forward	CCATCGAGCACGGTATTGT	60
	Reverse	CAGCTTCTCCTTGATGTCACG	
<i>cat</i>	Forward	GCTTCCACAACCCAGATTA	59
	Reverse	GGTTCCTGTTTCAGCACCATT	
<i>gpx</i>	Forward	AAGCAGTTTGCCGAGTCCTA	57
	Reverse	GCTGGTCTTTCAGCCACTTC	

Primers were previously described in Ruiz et al. (2018).

2.5 Data treatment and statistical analysis

The obtained gene expression data were transformed using the quantification method of $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen, 2011), which allows the comparison of the C_t (cycle threshold) of the target genes with the C_t of housekeeping gene.

All data were statistically tested with Shapiro-Wilk test for normality and Levene's test for homogeneity of variances. When normal distribution or homogeneity of

variances were not assumed, data transformation was required, using ln or square root. Then, a two-way analysis of variances (ANOVA) was applied to the data, using the dietary n-3 LC-PUFA level and time post-stress as variables. When *P-value* was significant ($P < 0.05$), means and interactions were compared with Holm-Sidak post hoc test. All statistical analysis were carried out using the IBM* SPSS Statistic 20 (New York, USA) and SigmaPlot version 12.0 for Windows.

3. Results

3.1 Experiment I: acute stress

3.1.1 Plasmatic stress indicators

Basal plasma cortisol was unaffected by the different dietary n-3 LC-PUFA levels (Figure 1). However, at 1 h post-stress, an increase of this stress marker was observed, which was followed by a recovery 5 h post-stress ($P < 0.05$; Figure 1). Although no statistical differences were observed in 1 h post-stress plasma cortisol levels between fish fed different n-3 LC-PUFA diets, due to inter-individual variation, a clear tendency to present the highest levels in fish fed 0.8 % n-3 LC-PUFA was noted (Figure 1).

Additionally, plasma glucose levels were not affected by stress neither by dietary n-3 LC-PUFA content. In contrast, an increase in plasma lactate levels at 1 h post-stress was found, which was the highest in fish fed the lowest n-3 LC-PUFA diet (0.8 %) ($P < 0.05$; Figure 2). For this sampling point, a significant interaction between the dietary n-3 LC-PUFA level and time after stress was noted ($P_{\text{int.}} < 0.05$). Even though, at 5 h post-stress, fish showed recovered lactate baseline values, regardless of the dietary n-3 LC-PUFA content ($P < 0.05$; Figure 2).

3.1.2 Brain serotonergic activity

Neuronal 5-HT levels seemed to be unaffected by stress neither by the dietary n-3 LC-PUFA. However, brain 5-HIAA levels, the main metabolite of 5-HT, showed a significant increase 1 h post-stress, irrespective of the dietary treatment, as well as a recovery 5 h post-stress ($P < 0.05$; Figure 3). Interestingly, the 5-HIAA / 5-HT ratio, often used as an indicator of brain serotonergic activity, increased 1 h post-stress in meagre fed the two lowest n-3 LC-PUFA diets (0.8 and 1.4 %), whereas in meagre fed 2.6 % n-3 LC-PUFA this ratio remained unaffected by the stressor ($P < 0.05$; Figure 4). At 5 h after stress, fish fed 0.8 and 1.4 % n-3 LC-PUFA diets showed lower 5-HIAA / 5-HT values than meagre fed 2.6 % ($P < 0.05$; Figure 4). Also, a significant interaction between the dietary n-3 LC-PUFA level and time post-stress was observed ($P_{int.} < 0.05$).

3.2 Experiment II: prolonged stress

3.2.1 Plasmatic stress markers

Plasma cortisol levels showed a rise 2 h after confinement in meagre fed both n-3 LC-PUFA diets, which was the highest for fish fed the 1.4 % n-3 LC-PUFA diet ($P < 0.05$; and Figure 5). However, at 7 days of confinement, the two treatment groups recovered basal cortisol levels ($P < 0.05$). A significant interaction was found between the dietary n-3 LC-PUFA content and time of confinement ($P_{int.} < 0.05$).

3.3.2 Antioxidant-related genes

Meagre fed 1.4 % n-3 LC-PUFA significantly increased *cat* relative expression at 2 h of confinement, which recovered basal values at 7 days of confinement ($P < 0.05$; Figure 6). In contrast, in meagre fed 2.6 % n-3 LC-PUFA, the relative expression of this gene was unaffected by the stressor, remaining constant during the prolonged confinement ($P < 0.05$; Figure 6). Besides, a significant interaction in *cat* relative expression was noted between the dietary n-3 LC-PUFA and time ($P_{int.} < 0.05$), which contrasted with the no interaction found between these two variables in *gpx* relative

expression. For the latter gene, an increase after 2 h of confinement followed by a recovery of basal expression values after 7 days of confinement was observed, regardless the dietary n-3 LC-PUFA ($P < 0.05$; Figure 7).

4. Discussion

Both types of stressors applied, a short acute stress by chasing and a prolonged stress by confinement in cage for 7 days, induced a primary activation of fish neuroendocrine system, reflected by a rapid increase of meagre plasma cortisol levels post-stress in both trials. However, fish submitted to chasing stress presented a maximum of a 4-fold increase in plasma post-stress cortisol, a lower increment when compared to that observed in 2 h post-stress plasma cortisol of meagre exposed to confinement (maximum 7-fold increase). These differences in the primary acute response and intensity of meagre plasma cortisol peaks are reasonable and may be related to various factors: The first one is the acute response sampling point, which in experiment I was at 1 h post-stress to cover the rapid response of serotonergic indicators, while in experiment II was at 2 h post-stress. Indeed, previous studies indicate, that meagre cortisol peaks after stress are achieved between 1-2 h, with maximum levels at 2 h post-stress (Fanouraki et al., 2011; Samaras et al., 2016). Besides, the recovery of control plasma cortisol levels at 5 h and 7 days post-stress in meagre exposed to an acute and a prolonged stress, respectively, suggests a good adaptation capacity of meagre, even to a prolonged confinement in cage, in agreement with previous studies (Fanouraki et al., 2011; Samaras et al., 2016). These results, added to the low maximum plasma cortisol levels (70 ng / mL) in comparison to other fish species, such as carp (*Cyprinus carpio*) (150-500 ng / mL; Pottinger, 1998) or gilthead seabream (*Sparus aurata*) (150 ng/ mL; Rotllant et al., 2001), suggest a high stress resistance of meagre, as already pointed out by Samaras et al., 2016, although

different stressors applied in these studies may also induce differences in the cortisol response.

The intensity of cortisol release is not only mediated by the type and severity of stress, but also by the nutritional status of the fish and, therefore, deficient diets can be also considered a stressor (Montero and Izquierdo, 2011; Tort, 2011). For instance, EFAs modulate cortisol synthesis and release by interrenal cells (Montero et al., 1998; 2003; 2015; Ganga et al., 2006; 2011a,b). In the present study, meagre basal plasma cortisol was unaffected by the replacement of FO by VO, suggesting that 0.8 % of n-3 LC-PUFA was sufficient to avoid causing a basal nutritional stress in meagre. This could be related to the high selective retention of n-3 LC-PUFA showed by meagre fed this lowest n-3 LC-PUFA diet at the end of the growth period, as well as the a higher *elov15* and *fads2* expression, which denotes a certain capacity to synthesize LC-PUFAs (Carvalho et al., 2018). However, when exposed to a stressful situation, in particular, a confinement in cage, meagre fed low n-3 LC-PUFA diet (1.4 %) showed a higher plasma cortisol post-stress response at 2 h, than meagre fed 2.6 % n-3 LC-PUFA. These results are in agreement with the estimation of n-3 LC-PUFA requirements for meagre juveniles at 2 % n-3 LC-PUFA in dry matter for optimal growth (Carvalho et al., 2018) and normal hepatic physiological status (Carvalho et al., unpublished). A similar higher post-stress cortisol response was also found in gilthead seabream fed n-3 LC-PUFA deficient diets and high VO diets (low in n-3 LC-PUFA) when submitted to crowding stress (Montero et al., 2003; Ganga et al., 2011). In contrast, the lack of a dietary effect on cortisol levels in the acute stress by chasing could be related to the different sampling points performed in both trials, as discussed before, or the different type and severity of the stressor applied.

Additionally, plasma glucose concentration after stress is usually recognised as an additional stress marker as a consequence of an increase of catecholamines and corticosteroids in blood (Pottinger, 1998). However, in the present trial, plasma glucose levels were unaffected by stress neither by the dietary n-3 LC-PUFA. In agreement, in

a previous study with meagre exposed to a similar stress, a lack of effect in plasma glucose at 1 h post-stress was noted, whereas this parameter was significantly affected at 0.5 h post-stress (Fanouraki et al., 2011). Therefore, it may be possible that the lack of effect in plasma glucose post-stress levels was due to a short response of glucose and the late sampling point of the present stress challenge.

While cortisol and glucose are considered direct stress markers, lactate is an indirect stress indicator, that gives information about the magnitude of oxygen demand in fish tissues due to severe exercise, which may occur when fish are submitted to an acute stress (Milligan and Girard, 1993). In the present study, fish fed the lowest n-3 LC-PUFA diet (0.8%) presented the highest post-stress lactate levels, suggesting the highest metabolic acidosis resulting from the highest oxygen demand in these fish. Similarly, increased lactate levels in liver of Senegalese sole (*Solea senegalensis*) exposed to an acute stress (Conde-Sieira et al., 2018), and in plasma lactate of sturgeon (*Acipenser naccarii*) submitted to hypoxia (Randall et al., 1992) were found, when both species were fed low n-3 LC-PUFA diets. However, further studies are necessary to better understand the role of specific fatty acids on lactate metabolism under stressful conditions.

Brain monoamine neurotransmitters synthesis and turnover have been reported to participate in controlling of stress response in mammals as well as in fish, by stimulating the HPA and HPI axis, respectively (Øverli et al., 1999; 2001; Hoglund et al., 2000; Gesto et al., 2013). However, studies evaluating the effect of diet composition, in particular of the dietary n-3 LC-PUFA, on brain serotonergic activity under stressful conditions are very scarce. Telencephalon is believed to host structures involved in the emotional reactivity and high-level processing in the fish brain (Vindas et al., 2017), and therefore, has a key role when integrating sensorial information about stressors and in the organization of a response. In fact, this brain macro-area has been consistently demonstrated to show stress-induced effects in its serotonergic outcome in fish. In the present study, telencephalic 5-HT was unaffected by stress or by dietary n-3

LC-PUFA, in agreement with a previous study in rainbow trout (*Oncorhynchus mykiss*) (Gesto et al., 2013). This could be related to the fact that the amount of serotonin released by neurons is low when compared to the serotonin amounts still stored in the neurons, even if serotonergic activity is increased, and may be compensated by new serotonin synthesis from tryptophan. However, the release of serotonin in response to stress is partly transformed to 5-HIAA, and, therefore, despite the effect of this increase in total serotonergic stores is usually minor, an increase in brain 5-HIAA can be noted, as shown in the present study, and in agreement with a previous study (Gesto et al., 2013). Furthermore, the metabolite/monoamine ratio is used as an indirect biochemical indicator of brain serotonergic activity (Winberg and Nilsson, 1993). It has been reported that this ratio increases in stressed animals (Emerson et al., 2000; Gesto et al., 2013) and prolonged elevated brain serotonergic activity seems to be an indicator of chronic stress (Øverli et al., 2005). In the present study, this ratio increased in meagre fed 0.8 and 1.4 % n-3 LC-PUFA at 1 h post-stress, while it remained unaffected in fish fed 2.6 % n-3 LC-PUFA during the whole sampling time (1 h and 5 h). These results suggest that fish fed low n-3 LC-PUFA diets may have a different and, possibly, less controlled perception of the stressor, resulting in a stronger neuronal serotonergic activity. The influence of PUFA on the modulation and activation of HPI axis during stress conditions seems to be related to their electrophysiological effect on hypothalamus cells related to an inhibitory control on the activation of the HPI axis (Conde-Sieira et al., 2018), as described for mammals (Dallman, 1984; Chalon et al., 2001), or a direct effect on the expression of steroidogenesis-related genes (Montero et al., 2015). There was also a lower 5-HIAA/5-HT ratio 5 h after stress, in meagre fed lower levels of n-3 LC-PUFA (0.8 and 1.4 %) when compared to fish fed 2.6 % n-3 LC-PUFA. This agrees well with the reduction in post-stress 5-HIAA/5-HT ratios, in Senegalese sole fed low n-3 LC-PUFA (Conde-Sieira et al., 2018) and also with the lower basal 5-HIAA/5-HT ratios in mice fed n-3 LC-PUFA deficient diets (Tang et al., 2016).

The dietary components, particularly lipid sources and dietary LC-PUFA, were reported also to affect fish oxidative status (Montero and Izquierdo, 2011; Castro et al., 2018). However, to our best knowledge, few studies evaluated these effects on fish under stressful conditions. In a study conducted in European sea bass (*Dicentrarchus labrax*) fed FO vs VO lipid sources and submitted to handling stress, GPX but not CAT enzyme activity was higher in stressed fish fed VO based diets (Castro et al., 2018). These results contrast with those of the present study, where *cat* post-stress relative expression was more influenced by the dietary n-3 LC-PUFA than *gpx* expression. These differences could be related to the fact that enzyme activity does not always directly correlate with gene expression, but could also denote differences in the oxidative stress risk or the balance between pro-oxidant and anti-oxidant factors in both studies, as well as may suggest a species-specific oxidative response. In the present study, fish fed 1.4 % n-3 LC-PUFA showed higher post-stress *cat* expression than fish fed 2.6 % n-3 LC-PUFA. While CAT degrades hydrogen peroxide into molecular oxygen and water (Michiels et al., 1994), GPX reduces both hydrogen peroxides and organic peroxides into water and alcohols, respectively, through a reaction dependent on glutathione reductase (Morales et al., 2004). The highest cortisol level after confinement found in fish fed the lowest n-3 LC-PUFA diet (1.4 %) was well correlated with the highest post-stress *cat* relative expression in these fish, suggesting that stress and its consequent cortisol increase induces an up-regulation of this gene (Aluru and Vijayan, 2009). This may be related to the oxidant by-products resulting from the catabolic processes stimulated by catecholamine and glucocorticoid stress hormones, which can lead to oxidative damage (Liu and Mori, 1999). In contrast, the lack of effect of the stressor on *cat* post-stress expression of fish fed 2.6 % n-3 LC-PUFA diets may be associated with the higher pro-oxidative environment, as described previously for gilthead seabream (Pérez-Sánchez et al., 2013), although basal relative expression of both anti-oxidant related genes was not significantly affected by the dietary n-3 LC-PUFA in the present study. These results agree with previous studies in teleost fish

that reported a time-dependent pro-oxidative stress reflected by a higher basal activity of CAT and/or GPX enzymes, among others, in fish fed high PUFA diets (Mourete et al., 2002; Rueda-Jasso et al., 2004). Therefore, a previous high PUFA environment, which increases the risk of peroxidation, may result in a forced adaptation of fish antioxidant defence system to avoid tissue damage, taking advantage of this when exposed to stressful situations. The slightly higher relative increase of mRNA levels of *cat* compared to *gpx* could be related to the fact that the former is the first active gene in the oxidative response. However, the reason why *cat* relative expression is unaffected under stress conditions in fish fed high n-3 LC-PUFA diets, whereas *gpx* is affected remains unclear, but could be due to the ROS species produced by the stress applied, which can affect more one enzyme than another. Furthermore, the recovery of both anti-oxidant related genes to basal levels after 7 days of confinement, in concordance what was noted for plasma cortisol, indicates that meagre were not suffering chronic stress because of being confined in a 10 L cage but, instead, denotes a good adaptation capacity in response to a long-time confinement, highlighting the high stress resistance of this species, even when fed with low n-3 LC-PUFA diets.

5. Conclusions

As described for other species, the dietary changes in n-3 LC-PUFA, essential for marine fish, induce a change in the stress response in meagre, significantly increasing glucocorticoid or serotonergic response in fish fed low n-3 LC-PUFA contents. Furthermore, high n-3 LC-PUFA diets may enhance fish basal antioxidant defence system-related genes by generating a pro-oxidative environment, and, therefore, affecting less fish homeostatic balance when exposed to a stressful situation. This implies that low n-3 LC-PUFA diets generates greater alterations in relation to basal levels of oxidative stress-related genes and, possibly increasing the oxidative stress damage. However, the results of the present study showed that meagre denotes a

good adaptation to both acute and prolonged stress, even when fed with low n-3 LC-PUFA diets, highlighting the high stress resistance of this species.

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Figure 1. Plasma cortisol levels (ng / mL) of meagre fed different dietary n-3 LC-PUFA (% DM) and submitted to an acute stress; * denote significant differences with time 0 and 5 hours ($P < 0.05$); Values represent means $\pm$ S.E.M for $n = 10$ fish.

Figure 2. Plasma lactate levels (mM) of meagre fed different dietary n-3 LC-PUFA (% DM) and exposed to an acute stress; Different letters denote significant differences between diets for a specific time ($P < 0.05$); * denote significant differences with time 0 and 5 hours ($P < 0.05$); Values represent means $\pm$ S.E.M for $n = 10$ fish.

Figure 3. 5-Hydroxyindoleacetic acid (5-HIAA; ng / g) levels on telencephalon of meagre fed different dietary n-3 LC-PUFA (% DM) and submitted to an acute stress; * denote significant differences with time 0 and 5 hours ($P < 0.05$); Values represent means $\pm$ S.E.M for $n = 10$ fish.

Figure 4. 5-Hydroxyindoleacetic acid/ Serotonin ratio (5-HIAA / 5-HT) levels on telencephalon of meagre fed different dietary n-3 LC-PUFA (% DM) and submitted to an acute stress; Different letters denote significant differences between diets for a specific time ($P < 0.05$); * denotes significant differences with time 0 ($P < 0.05$); $^{\alpha}$ denotes significant differences with time pre-stress (0 hours) ($P < 0.05$); Values represent means $\pm$ S.E.M for $n = 10$ fish.

Figure 5. Plasma cortisol levels (ng/ mL) of meagre fed two different dietary n-3 LC-PUFA (% DM) and submitted to a confinement for 7 days; Different letters denote significant differences between diets for a specific time ($P < 0.05$); * denotes significant differences with time 0 and 7 days ($P < 0.05$); Values represent means $\pm$ S.E.M for $n = 9$ fish.

Figure 6. Catalase (cat) relative gene expression ($2^{-\Delta\Delta Ct}$) of meagre fed two different dietary n-3 LC-PUFA (% DM) and submitted to a confinement for 7 days; Different letters denote significant differences between diets for a specific time ($P < 0.05$); * denotes significant differences with time 0 and 7 days ($P < 0.05$); Values represent means $\pm$ S.E.M for $n = 9$ fish.

Figure 7. Glutathione peroxidase (gpx) relative gene expression ($2^{-\Delta\Delta Ct}$) of meagre fed two different dietary n-3 LC-PUFA (% DM) and submitted to a confinement for 7 days; Different letters denote significant differences between diets for a specific time ($P < 0.05$); * denotes significant differences with time 0 and 7 days ($P < 0.05$); Values represent means $\pm$ S.E.M for $n = 9$ fish.

Highlights

- The dietary reduction in n-3 LC-PUFA increased glucocorticoid response in meagre either exposed to acute and prolonged stress.
- The acute stress exposure altered serotonergic response in fish fed low n-3 LC-PUFA contents (below 2.6 %).
- Low n-3 LC-PUFA diets generates greater alterations in relation to basal levels of oxidative stress-related genes.
- The dietary changes in n-3 LC-PUFA induce alterations in meagre stress response, although a good adaptation capacity to both types of stress was shown by this species.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7