Dietary lecithin source affects growth potential and gene expression in *Sparus aurata* larvae

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Abstract

Soybean lecithin (SBL), used as phospholipid source in larval fish diets, may compromise growth and survival in marine species, and affect gene expression, due to differences in fatty acid composition relative to marine lecithins (ML). The potential of SBL as phospholipid source in gilthead seabream microdiets as compared to ML was evaluated. Two stocking densities were tested in order to exacerbate possible dietary effects: 5 and 20 larvae L⁻¹. Larvae reflected dietary fatty acid profiles: linoleic acid was higher, whereas eicosapentaenoic and arachidonic acids were lower in SBL fed groups than in ML fed larvae. Highest stocking density decreased survival, and led to elevated saturates and lower docosahexaenoic acid levels in polar lipid. Muscle histology observations evidenced hindered growth potential in SBL fed larvae. Despite similar cortisol levels between treatments, higher glucocorticoid receptor (GR), as well as hormone-sensitive lipase (HSL) mRNA levels in SBL fed groups revealed a role for fatty acids in gene regulation. Further analysed genes suggested these effects were independent from the hypothalamus-pituitary-interrenal axis control and the endocannabinoid system. Cyclooxygenase-2 and gluconeogenesis seemed unaffected. For the first time in fish, a link between dietary lecithin nature and HSL gene transcription, perhaps regulated through GR fatty acid-induced activation, is suggested. Enhanced lipolytic activity could partly explain lower growth in marine fish larvae when dietary ML is not provided.

Keywords: dietary lipid, stocking density, gilthead seabream, larvae, fatty acids, stress, gene expression

Introduction

A dietary phospholipid source, particularly of marine origin, is generally recognized to promote growth and survival, as well as skeletal development, and perhaps stress resistance in fish larvae (1; 2). Phospholipids are key cell membrane constituents, and have a relevant role in lipid transportation from the intestine which seems to be of utmost importance in larvae; they are also thought to influence dietary palatability (1). Commercially available lecithins are mixtures of phospholipids obtained from oils of either vegetable or animal nature. Due to its market availability and relatively stable composition, soybean lecithin (SBL) is generally included as a phospholipid providing ingredient in larval diets, although its fatty acid composition differs greatly from that of marine lecithin (ML). Typically, SBL presents elevated linoleic acid levels and does not contain highly unsaturated fatty acids (HUFAs) which characterize ML. Despite the abundance of HUFAs under the triacylglyceride form in larval diets provided mostly through fish oil ingredients, some supply under the phospholipid form seems necessary to sustain growth in marine fish larvae (3; 4). The reasons underlying this requirement are still poorly understood and further research is needed for the optimization of larval diets. It would be plausible to hypothesize that deleterious effects of dietary SBL as compared with ML on growth and development in fish larvae could be due, at least partly, to changes in cell content of certain fatty acids and their metabolites which can play key roles in many cellular events, such as gene expression regulation (5; 6; 7).

Stress coping ability, which requires energy expenditure, has been increasingly recognized as a valuable parameter for the evaluation of physiological condition in fish. Eicosanoid production, their type and relative amounts, is supposedly influenced by the dietary supply of certain fatty acids and believed to play a major role on the success of the stress response in larvae, although seldom explored in fish stress studies (8; 9). Eicosanoids are generated by the activity of phospholipase A2 (PLA2) and other enzymes, such as cyclooxygenase-2 (COX-2), over fatty acids derived from cell membrane phospholipids (10). Other enzymes (lipoxygenase, epoxygenase) also contribute to eicosanoid production which may stimulate ACTH (adrenocorticotrophic hormone) and modify induced cortisol release (11; 8). The ability to recover homeostatic balance in response to stress is determined by a response system in which a variety of transcription factors and nuclear receptors are implied, possibly under direct or indirect influence of fatty acids or their metabolites (12). Besides, the issue of cell membrane composition is also relevant for genomic steroid actions since the ability of a substance to partition into the membrane is critical for its bioavailability to intracellular receptors (13; 14).

This study was designed to test two diets differing in the phospholipid source, in seabream larvae. A relatively high stocking density was used as a chronic stressor with the aim of possibly intensifying effects of the diets on growth, survival, and stress associated parameters.

Exploring effects of dietary lecithin source on aspects related to stress physiology was expected to provide clues for its metabolic regulation by dietary fatty acids. As such, the gene expressions of pro-opiomelanocortin (POMC, a precursor for ACTH), glucocorticoid receptors (GR, cortisol signaling mediators), COX-2, and fructose-1,6-bisphosphatase (FBPase, involved in stress-induced gluconeogenesis) were analysed. Furthermore, the expression of protein kinase C (PKC) gene was studied as a potential intermediary in steroidogenesis, and responsive to cellular arachidonic acid abundance (15; 16). Other genes targeted were those for the rate-limiting enzyme hormone-sensitive lipase (HSL), growth hormone (GH), and cannabinoid 1 receptor (CB1 receptor). CB1 receptors are distributed within the central nervous system (17; 18) and their expression could reflect changes in dietary fatty acid supply, especially arachidonic acid (ARA). Also, histological analyses of the muscle were conducted in order to identify short-term effects of the experimental conditions on the growth potential of the larvae, which is believed to be related with the number of small sized muscle fibres in fish (19; 20; 21).

Experimental procedure

Experimental conditions

A bifactorial experiment was conducted during seven days on gilthead seabream larvae. Eggs were obtained from the “Planta de Cultivos Marinos” of the University of Cádiz (Puerto Real, Spain) and placed in 200 L incubator tanks in a flow-through system, with constant aeration. After hatching, the larvae were transferred into 300 L tanks and fed on rotifers (*Brachionus rotundiformis* and *B. plicatilis*; 5 to 15 per ml of tank water) from 5 to 25 days after hatching (DAH) during which *Nannochloropsis gaditana* was provided, according to the feeding protocols established by Polo *et al.* (22). After 14 DAH, *Artemia* nauplii was also provided at 1-2 nauplii per ml. From 32 DAH onwards, fish were fed exclusively on microencapsulated diets, prepared according to the method described by Yúfera *et al.* (23), and containing either marine (ML) or soybean lecithin (SBL) as phospholipid source ingredients (table 1). Both diets were formulated to present phosphatidylcholine and phosphatidylinositol, thought to be main growth-promoting fractions in lecithin for fish larvae, in a total content of approximately 1% of the diet as suggested by Coutteau *et al.* (24).

During the one week inert feeding period, larvae were kept in a flow-through system consisting of eight cylindro-conical baskets with a plankton net bottom immersed in one tank. Four of the baskets held seabream groups at a low density (“L” groups) of 5 larvae L⁻¹ and other four held groups kept at the higher stocking density (“H” groups) of 20 larvae L⁻¹. The experimental diets were assigned so that each treatment was tested in duplicate (ML-L, ML-H, SBL-L, SBL-H). Feed was supplied in excess, manually during the day and with automatic feeders at night. Constant aeration was provided so as to maintain dissolved oxygen levels around 6 mg L⁻¹, water temperature was kept at 20 ± 0.5 °C and a light : dark photoperiod of 14 h : 10 h was used. Water salinity was 33 g L⁻¹. Tank maintenance, removal of dead fish and uneaten feed were performed daily.

Sampling procedures

Initial fish samples were taken for determination of individual dry weight and total length at 32 DAH (n = 10). At the end of the experiment (39 DAH), fish samples were collected from all treatments for dry weight and total length determination (n = 10), muscle histology (n = 6), gene expression analyses through mRNA quantification (n = 12), whole-body cortisol (n = 14), and lipid quantification (minimum 100 mg body mass per tank). All larvae sampled were anaesthetized with an overdose of ethyl-4-amino-benzoate and washed with distilled water before storage or measurements, with the exception of animals used for gene expression

analysis. These were immediately stored in an RNA stabilizing solution, RNALater™ (Ambion, Austin, TX, USA), at 4 °C overnight, then at -20 °C until analytical work was conducted. Whole larvae samples for histological analyses were fixated in a 0.1 M phosphate buffer containing 3 % glutaraldehyde 25 % EM and stored at 4 °C. Both cortisol determination and fatty acid analysis samples were stored in a -80 °C freezer to avoid material degradation and peroxidation until further analyses.

Analytical methods

Experimental diets were analysed for proximate composition according to the following procedures: dry matter determined gravimetrically by drying in an oven at 105 °C for 24 h; crude ash by incineration in a muffle furnace at 500 °C for 5 h; crude protein (% N x 6.25) determined using an elemental analyzer (Thermoquest Flash 1112, Rodano, Italy) with sulphanimide as a standard; total lipid extracted in chloroform/methanol (2:1, v/v) according to Folch *et al.* (25) and quantified gravimetrically after evaporation of the solvent under a stream of nitrogen followed by overnight vacuum desiccation. Total lipid was stored in chloroform:methanol (2:1, 20 mg ml⁻¹) with 0.01 % butylated hydroxytoluene (BHT) at -20 °C until final analysis. Fatty acid composition of total lipid and polar lipid fraction of the diets was also determined following an acid catalyzed transmethylation (26). Fatty acid methyl esters were extracted twice using isohexane:diethyl ether (1:1, v/v), purified on TLC plates and analysed by gas-liquid chromatography on a Thermo TraceGC (Thermo Fisher Scientific, Waltham, MA, USA) instrument fitted with a BPX70 capillary column (30 m - 0.25 mm id, SGE), using a two-stage thermal gradient from 50 °C (injection temperature) to 150 °C after ramping at 40 °C min⁻¹ and holding at 250 °C after ramping at 2 °C min⁻¹, using helium (1.2 ml min⁻¹ constant flow rate) as the carrier gas, on-column injection, and flame ionization detection. Peaks were identified by comparison with known standards (Supelco, Madrid, Spain) and a well characterized fish oil, and quantified by means of the response factor to the internal standard, 21:0 fatty acid, added prior to transmethylation, using a Chrompack program (Thermo Finnigan, San Jose, CA, USA). To carry out fatty acid composition analysis of the polar lipid fraction, total lipid extract was evaporated to dryness, diluted in chloroform (about 30 mg of lipids in 500 µl of solvent) and injected on a Sep-Pack silica cartridge (Waters S.A., Milford, MA, USA; 27). After adsorption of the sample, 20 ml of chloroform were pushed through the cartridge with a syringe avoiding the formation of air bubbles and the non-polar fraction discarded. The fraction containing the polar lipids was eluted through the cartridge with 30 ml of methanol, collected and subjected to acid catalyzed transmethylation as indicated above.

The total length of the larvae was measured with a micrometer eye piece and whole body dry weight was determined by drying samples at 70 °C for several days until constant weight was attained. At the end of the trial, relative growth rate (RGR; 28) was calculated for all treatment groups, as well as survival. Theoretical biomass was calculated as the product of survival by the mean weight of the larvae.

For muscle histology analyses complete transverse sections were cut from each fish at the level of the anal vent, washed in the fixation buffer, post-fixed in 1 % osmium tetroxide, dehydrated, and embedded in TAAB resin according to Stickland *et al.* (29). Transverse sections of 1 µm thickness were obtained using a Reichert ultramicrotome and stained with 1 % toluidine blue. Slides were examined using a Zeiss image analysis system (KS 300, Kontron, Munich, Germany). White-muscle cross-sectional areas, small (less than 25 µm²), and large (more than 25 µm²) fibre numbers from a quadrant of each transverse section were quantified (as in 29).

Total lipid from larval tissues was extracted (25). Separation of the polar lipid fraction and determination of its fatty acid profile in larvae samples were carried out as previously described.

A commercial cortisol enzyme-linked immunosorbent assay kit (ELISA; Neogen Corporation, Lexington, KY, USA) was used to assess whole-body cortisol levels in pooled larvae samples of about 20 mg (7 fish per sample). This was preceded by sample homogenization in a PBS solution (phosphate buffered saline) and extraction with diethyl ether. The latter was performed following recommendations by Sink *et al.* (30) involving the addition of 100 µL of a food-grade vegetable oil per gram of sample body weight. Olive oil was used 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 kit's extraction buffer, diluted to an appropriate factor and analysed according to the kit manufacturer's instructions. All samples and standards were run in duplicate. Intra- and inter-assay variation values were checked and a % CV of ≤ 20.0 was set as acceptable. Parallelism and linearity (passing limit $r^2 > 0.90$) were tested using serial dilutions of sample extracts.

For molecular analyses 6 larvae sampled from each tank were pooled, homogenized in Tri Reagent (Sigma, Poole, Dorset, UK) with an ultra-turrax (IKA, Werke GmbH & Co. KG, Staufen, Germany), and RNA extracted with chloroform followed by ethanol precipitation. Qiagen RNeasy columns (Qiagen, Crawley, UK) were used for DNase treatment and sample purification. Samples were eluted with Sigma Pure water (Sigma) and RNA concentration measured spectrophotometrically using a Nanodrop N-1000 system (Nanodrop Technologies,

Wilmington, DE, USA). Formaldehyde gel electrophoresis was performed to determine the integrity of total RNA by analyzing bands under UV light. Reverse transcription of sample RNA was performed with Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to fabricant's instructions, using half a microgram of total RNA. The primers used were designed with the support of the Primer-3 Web-Software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and synthesized by Eurofins MWG Operon (Ebersberg, Germany). Primer sequences and accession numbers for the mRNAs analysed are described in table 2. Genes analysed were growth hormone (GH), pro-opiomelanocortin (POMC), cannabinoid CB1 receptor (CB1 receptor), glucocorticoid receptor (GR), hormone-sensitive lipase (HSL), fructose-1,6-bisphosphatase (FBPase), cyclooxygenase 2 (COX-2), and protein kinase C (PKC). Real-time PCR, based on Quantitect Sybr Green detection (Qiagen), was performed on 2 µl cDNA samples, using a Chromo-4 Thermal Cycler (MJ Research Inc., Waltham, MA, USA). The relative concentrations of the target amplicons were calculated by the cycler's software (Bio-Rad, Hercules, CA, USA) from a standard curve created with serial dilutions of standard DNA. The relative concentrations of target sequences in each run were expressed as numbers of copies and normalized to 0.5 µg of total RNA. All PCR products were checked for specificity and purity from a melting curve produced by the thermal cycler software at the end of each run.

Statistical analysis

Data were analyzed by a two-way ANOVA with SPSS 16.0 software package using diet and stocking density as fixed factors. For data not presenting a normal distribution and/or variance homogeneity, log-transformation was applied. Differences were considered significant at an alpha level of 0.10 due to both small sample sizes caused by feasibility constraints, and the exploratory character of this experiment, as recommended by Rubin (31).

Results

Proximate composition of the experimental diets (dry matter basis) showed high protein content (69-70 %) and lipid levels around 17-18 % (table 1). Saturated fatty acids and n-3 series polyunsaturates, particularly docosahexaenoic acid (DHA, 22:6n-3), were higher in the ML diet, whereas the SBL diet contained higher levels of linoleic (18:2n-6) and linolenic (18:3n-3) acids, as shown in table 3. Concerning dietary polar lipid fractions, the levels of palmitic (16:0), eicosenoic (20:1n-9), and eicosapentaenoic (EPA, 20:5n-3) acids, as well as DHA, were more elevated in the ML diet than in the SBL treatment, whereas the latter presented a considerably higher content in linoleic acid than the ML diet.

At 32 DAH, larvae weighed 0.3 ± 0.0 mg and were about 7.9 ± 0.2 mm long. Although classic growth performance analysis was not a main objective of this study due to its short duration, data relative to larval dry weight, total length and relative growth rate (RGR) at the end of the experiment are presented (table 4), along with survival and theoretical biomass results. No significant interaction effects between diet and stocking density were detected for these parameters. Statistical analyses revealed dietary effects on dry weight, total length and RGR, such that values obtained for ML fed larvae were higher than those presented by SBL fed groups. Groups offered the SBL treatment showed actual weight loss during the one-week period whereas animals fed on the ML diet grew at about 3.6-3.8 % day⁻¹. Nonetheless, high variation was observed between replicate tanks with the ML-L treatment. Although the SBL diet seemed to compromise growth, survival was not affected. Mortality occurred in all treatments, especially until around day 5 in “H” groups which resulted in lower stocking densities at the end of the experiment than initially established (fig. 1). Overall, survival was lower in groups kept at the higher stocking density (54.2 – 59.5 %) than in “L” groups (77.5 – 80.8 %). Theoretical biomass decreased with both larval density ($P = 0.072$) and the SBL regime ($P = 0.092$).

Results from muscle histology analysis are shown in table 5 and revealed no significant interaction effects between diet and stocking density for any of the measured parameters. Measurements of the cross-sectional area of a quadrant of fish muscle (fig. 2) did not reveal statistical differences between treatments. Nonetheless, average values were between 37.4 - 42.1 mm² for fish stocked at low density, whereas those at high density showed average areas of 30.8 - 33.6 mm². Histological analysis of white muscle evidenced dietary effects on the total number and density of small fibres but not of large fibres. As such, fish fed the SBL diet showed about 28 % less small fibre number and density compared to those fed the ML diet. Overall,

histological analysis pointed to a higher growth potential in seabream supplied with ML as compared to SBL groups.

Lipid content and total fatty acid composition of the larvae are described in table 6. Total lipid content revealed a significant interaction effect between diet and stocking conditions such that slightly higher levels were found in SBL-H than SBL-L fish, whereas values were very similar between ML diet groups. Deposition of 18:2n-6 was higher in fish fed SBL than in ML fed groups. Also, slightly higher levels of 18:1n-9, 18:1n-7 and 18:3n-3 were detected in SBL fed animals, whereas ML fed fish showed larger accumulation of arachidonic acid (ARA, 20:4n-6) and EPA than SBL groups. Analyses of the polar lipid showed statistically significant interaction effects between diet and stocking density for 18:0, 20:1n-9, and 18:3n-3. Higher stocking density conditions resulted in increased total saturated fatty acid (SAFA) levels, particularly 18:0 in SBL fed groups (table 7), and a reduction of total PUFA, especially DHA. Differences were also found due to dietary treatments. Linoleic and linolenic acids were higher in the polar lipid of SBL than ML fed animals, whereas ARA remained highest in ML fed fish. Opposite to findings in total lipid fatty acid profile, EPA was kept at similar levels in all treatment groups. Besides, unlike the trend observed in dietary composition, DHA deposition in the larvae was apparently unaffected by its dietary content.

Whole-body cortisol analysis did not show a significant interaction effect between diet and stocking density or statistical differences between treatments. Results are presented in figure 3. Average values were about 8.0 ng g^{-1} (larval wet weight) in fish stocked at high density. In ML-L and SBL-L groups, average cortisol levels were 12.4 and 9.0 ng g^{-1} , respectively. Displacement curves for cortisol standards and sample serial dilutions are presented (fig. 4A). Validation tests for the ELISA involving a vegetable oil volume-boosting method showed values of 5.07% for intra-assay coefficient of variation (% CV) and 18.7% for inter-assay CV. Linearity r^2 value was 0.9997 and parallelism was confirmed (Fig. 4B).

Results of the molecular analyses of gene expression are presented in figure 5. No significant interaction effects between diet and stocking density were identified for any of the genes analysed. Quantification of mRNA for GH, POMC, CB1 receptor, FBPase, COX-2, and PKC showed no significant differences under the experimental conditions tested. Nonetheless, the expression of HSL and GR genes was up-regulated in SBL groups compared to ML fed larvae, whereas no statistical differences were found due to stocking density.

Discussion

Early life stages in gilthead seabream are characterized by hyperplastic growth of white myofibres (32; 33). Small muscle fibres are evidence of ongoing fibre hyperplasia in fish muscle. This appears to have been less in fish fed the SBL diet than in those supplied with ML, which indicates hindered growth potential in the former although essential fatty acid requirements were met for the species, according to recommendations by Sargent *et al.* (34) and Castell *et al.* (35). These authors point at n-3 HUFA requirements of about 1.5 % of the diet when the ratio DHA/EPA is about 2, and ARA levels between 0.5 - 1.0 % of total dietary fatty acids, respectively. On the other hand, survival was not affected by dietary lecithin source. Instead, high stocking density increased mortality, which was also related to higher levels of saturated fatty acids, particularly stearic acid (18:0), and lower DHA in the polar lipid fraction than found in "L" groups. Thus, we hypothesize that membrane fluidity might have been affected in "H" groups which could have disturbed the activity of membrane-bound enzymes, ion channels, or receptors and led to higher mortality. The reasons why high density stocking would cause the observed changes in polar lipid fatty acids are unclear but results suggested that seabream larvae might have a higher requirement for DHA under such rearing conditions. In gilthead seabream larvae, 5 % dietary SBL inclusion as provided in the present study, was reported to increase microdiet consumption rates perhaps due to an attractant effect (36). In another study, elevation of dietary polar lipid levels improved microdiet ingestion in seabream, regardless of its soybean or marine origin (37). Although feed consumption was not determined in this experiment, similar survival between dietary treatments and the fact that whole-body fatty acid composition mirrored differences between diets indicated the acceptance of the experimental diets. Therefore, the detrimental effects observed on growth performance could be attributed to dietary lecithin origin. Surprisingly, despite numerous publications reporting the importance of dietary phospholipids for fish larvae, no studies have been published on the viability of SBL use as compared to ML containing diets in marine species. The efficiency of HUFA supplied through diet under the phospholipid form in sustaining growth, as exemplified in this study, is generally recognized (2). However, the reasons for this are yet unclear. Perhaps due to improved digestibility, marine phospholipids tend to be more efficient in supplying essential fatty acids than neutral lipids, although in freshwater fish SBL seems to satisfy larval phospholipid requirements (1).

As expected, dietary fatty acid composition was reflected in fish tissues. In SBL diet fed groups a significant increase in linoleic acid was noted in both total and polar lipids, concurrent with a decrease in arachidonic acid, relative to ML fed larvae. Besides potential effects on cell

membrane lipid composition and function, dietary fat has profound effects on gene expression. Polyunsaturated fatty acids and their metabolites can act in conjunction with nuclear receptors and transcription factors to affect the transcription of a variety of genes (7) leading to changes in metabolism, growth and cell differentiation (6). Thus, diet induced changes in gene expression could provide indications as to mechanisms by which dietary fatty acids could have regulated metabolism in larvae fed distinct lecithins.

Despite the absence of differences between treatments for whole-body cortisol concentrations, which were in accordance with values reported by Szisich *et al.* (38) for seabream, GR mRNA levels were higher in SBL diet fed fish. A similar trend was noted for HSL which has been identified as a glucocorticoid-sensitive gene in mammals (39), containing glucocorticoid responsive elements (GREs) in its promoter (40; 41). This is also likely in fish as enhanced lipolytic activity and increased free fatty acid levels in the plasma are generally associated to the stress response (see review by 12). HSL catalyses the rate-limiting step in fatty acid mobilization from stored triglycerides, determining the supply of energy substrates in the body (42). A relative up-regulation of this enzyme in SBL fed fish could hinder or even impair growth as observed due to energy deviation away from anabolic metabolism. The observed effects on larval growth were not determined by differences in the gene expression of GH, the main regulator of postnatal somatic growth stimulating cell division, skeletal growth, and protein synthesis. GH can also enhance lipolysis in starved fish, as seen in seabream adipocytes (43); however, feeding conditions did not differ among experimental groups and HSL results did not appear to be influenced by GH as its mRNA levels indicated. Although GR mRNA and protein expression are not always correlated, as seen in rainbow trout, (44) no such information is currently available for gilthead seabream. Present results suggested an effect of dietary fatty acid supply on the regulation of GR gene expression regardless of values detected by whole-body total cortisol analyses. Indeed, cortisol plasma concentrations may not always be correlated to cytosolic GRs (45), and increased intracellular concentrations of glucocorticoids can result from overexpression of 11- β hydroxysteroid dehydrogenase type 1 (11 β HSD1; 46) which converts cortisone to cortisol. Thus, both protein levels of this enzyme and GRs can determine the physiological activity of glucocorticoids. Several possible pathways for the modulation of GR gene expression by fatty acids may exist. Nuclear factor-kappa B (NF- κ B), a GR transcription factor (47), can be activated in the presence of linoleic and oleic acids, as seen in rats (48), and may have contributed for the enhancement of GR expression in larvae fed the SBL diet. Peroxisome proliferator-activated receptors (PPARs), probably the best known factors in fatty acid regulation of gene transcription, also

interact with NF- κ B and AP-1 (49), both transcription factors for GRs (reviewed by 50). Indeed, linoleic acid was reported to be a potent PPAR activator relative to other fatty acids (51). Another hypothesis for GRs fatty acid modulation is that arachidonic acid could bind these receptors at sites different from those bound by steroids, hence inhibiting their activity (52). Dose-dependent suppression of GR binding has been reported for various unsaturated fatty acids likely as part of a negative feedback mechanism (53). Nonetheless, in this study, seabream larvae showing lower GR and HSL transcripts presented only slightly higher arachidonic acid levels in body lipid.

The gene expression of POMC which encodes for ACTH (adrenocorticotrophic hormone) in gnathostomes was analysed due to the central role of ACTH on cortisol production. POMC gene expression results corroborated those obtained from cortisol measurements, further suggesting that the differential expression of the GR gene was not determined by fatty acids at the HPI (hypothalamus-pituitary-interrenal) axis level.

PKC has important roles in various biological processes (e.g. signal transduction, cell proliferation) including the tonic inhibitor control of steroidogenesis as reported in mammal cells stimulated by intracellular arachidonic acid (15; 16; 54). In this study, results do not indicate that PKC was involved in maintaining similar cortisol levels between groups. In fact, PKC gene expression was so identical among samples and experimental conditions that it could potentially be used as a control gene in future studies with seabream, as suggested in humans (55).

An acute stress challenge could be required for the release of ARA in the cells and further evidencing effects of dietary fatty acid supply on the expression of stress response related genes. This could be underlying the lack of differences regarding PKC as well as COX-2 gene expression between treatments which did not appear to relate to the observed results regarding both GR and HSL genes. In fish, the endocannabinoid system is thought to be involved in the modulation of adaptive responses to environmental conditions (56). For example, under non-stressful conditions, production of 2-AG (2-arachidonoylglycerol) is higher than in stressed animals, leading to CB1 receptor up-regulation, which depresses HPA (hypothalamus-pituitary-adrenal) axis activity in mammals (57). Several lines of evidence indicate that, through CB1 receptor activation, endocannabinoids can also regulate NF- κ B and AP-1 activities (58; 59), thus potentially interfering with GR activity and the stress response. The lack of differences between experimental groups was in accordance with results obtained for other analysed genes, like COX-2 and PKC further suggesting that the influence of dietary lecithin on GR and HSL genes was exerted, in non-acutely stressed larvae, at the level of GR activation through factors independent from the classic HPI axis regulation.

Gluconeogenesis, in which FBPase (fructose-1,6-bisphosphatase) is a key enzyme, is responsible for glucose production in response to cortisol (12). It is assumed that glucocorticoids stimulate gluconeogenesis by increasing PEPCK (phosphoenolpyruvate carboxykinase) gene transcription which contains a GRE, or by stimulating the supply of hepatic precursors (reviewed by 12). Thus, both higher GR expression and lipolytic activity could have contributed to enhance gluconeogenesis; instead, statistically similar FBPase mRNA levels found between groups suggested a more dramatic increase in GR activity could be required to affect gluconeogenesis enzyme activities, as this is usually associated with a rise in plasma cortisol levels.

In summary, changes induced in body fatty acid phenotype, through dietary SBL supply, exerted a lipolytic effect, compared to ML fed fish, possibly mediated by GR activity, which could have contributed importantly for the detrimental effects observed on growth with the SBL diet. Furthermore, the proposed regulation of GR activity by fatty acids seems to have been independent from the action of the HPI axis, the endocannabinoid system, cyclooxygenase-2 activity, and did not appear to affect gluconeogenesis. On the other hand, no effects of rearing density were found in terms of the expression of genes analysed in this study, although rearing conditions are generally known to interfere with neuroendocrine homeostasis in fish.

The importance of dietary fatty acids on stress coping ability has been repeatedly demonstrated in fish larvae although with limited understanding of the physiological mechanisms involved. Studies regarding fatty acid control over fish GRs are scarce (60) despite their major role in linking and integrating signaling pathways and regulatory processes in cells, tissues, and whole organisms (50). This aspect is not only relevant in marine fish larval nutrition but should be considered in dietary fish oil replacement research, a recognized priority for the sustainable production of farmed fish.

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Table 1. Formulation and proximate composition of microencapsulated diets prepared by internal gelation (g kg^{-1}), for gilthead seabream larvae

<i>Ingredients</i>	ML	SBL
Fish meal ^a	50	50
Fish hydrolysate ^b	100	100
Cuttlefish meal ^c	450	450
Casein ^d	50	50
Sodium alginate ^e	70	70
Baker yeast ^f	30	30
Dextrine ^g	40	40
Fish oil ^h	73.5	80
Soybean lecithin ⁱ	-	50
Marine lecithin ^j	56.5	-
Vitamin premix ^k	20	20
Vitamin C ^l	30	30
Vitamin E ^m	10	10
Mineral premix ⁿ	20	20
<i>Proximate composition</i>		
Moisture (%)	7.1	8.2
Protein (% DM)	69.9	69.1
Lipid (% DM)	16.9	18.5
Carbohydrates ^o (% DM)	8.7	7.8
Ash (% DM)	4.6	4.7

^a AGLONORSE MICROFEED, Norsildmel Innovation AS, Bergen, Norway.

^b CPSP-90, Soprepêche, France.

^c SQUID POWDER 0278, Rieber & Søn ASA, Bergen, Norway.

^d VWR International, Fontenay sous Bois, France.

^e ICN 154724.

^f Commercial bread yeast.

^g Commercial grade type I, MP Biomedicals, LLC, Illkirch, France.

^h Cod liver oil, José M. Vaz Pereira, S.A., Sintra, Portugal.

ⁱ Lecithin Soy Refined, MP Biomedicals, LLC, Illkirch, France.

^j Marine natural lecithin LC 40, PhosphoTech Laboratories, Saint-Herblain, France.

^k Vitamin premix supplied the following (per kg of diet): vitamin A/D₃ 500/100, 1000 mg; vitamin D₃ 500, 40 mg; vitamin E 50, alfa-tocopherol acetate, 3000 mg; vitamin K₃ 23 %, 220 mg; thiamine HCl, 50 mg; riboflavin 80, 250 mg; d-Ca pantothenate, 1100 mg; niacin, 500 mg; pyridoxine, 150 mg; folic acid, 50 mg; vitamin B₁₂ 0.1, 500 mg; biotin 20, 38 mg; ascorbic acid poliphosphate 35 %, 57.2 g; choline chloride 60 %, 100 g; inositol, 15 g; antioxidants, 1.25 %.

^l Sodium, calcium ascorbyl-2-phosphate, ROVIMIX STAY-C 35, DSM Nutritional Products, Inc.

^m DL-alfa-tocopherol acetate, MP Biomedicals, LLC, Eschwege, Germany.

ⁿ Mineral premix supplied the following (per kg of diet): monocalcium phosphate, 35.2 %; calcium carbonate, 11.5 %; sodium chloride, 20.0 %; potassium chloride, 26.0 %; copper sulphate, 0.024 %; magnesium sulphate, 5.0 %; ferrous sulphate, 0.6 %; manganous sulphate, 0.81 %; zinc sulphate, 0.17 %; potassium iodide, 0.0031 %; sodium selenite, 0.6 %.

$$^{\circ} \text{Carbohydrates} = 100 - (\text{protein} + \text{lipid} + \text{ash})$$

Table 2. Primers in the 5'-to 3' direction used for the real time PCR analysis

Primer	Forward	Reverse	Product size (bp)	Accession number
GH	GCTCAGTGTTGAAGCTGCTG	TGGGTGAAATCTGGTTCCTC	108	S54890
POMC	GAGGAGTCAGCCGAGGTCTT	GCCTTCTCCTCCTCCTCCT	101	AY714372
CB1 receptor	AGCGTGCTGGTTCTTTTCAT	ACAAGGCTCTTCTGGGAGGT	104	EF051620
COX-2	TGATCGAGGACTACGTGCAG	AGTGGGTGCCAGTGGTAAAG	139	AB292357
PKC	GACAGTTTGCCCTGTCTGGT	GAGAAAGCCACGCTCAAAAC	118	DQ111989
GR	CGGTCACTGCTACGTCTTCA	CCTCCCAGCACACAGGTAAT	170	DQ486890
HSL	GTTGCAGCCAGCTCTTCTT	TCGGTTTCAGTGATGTTCCA	134	EU781499
FBPase	CTCTGCAGCCTGGAAGAAAC	TCCAGCATGAAGCAGTTGAC	106	AF427867

Table 3. Fatty acid composition (% total fatty acids, TFA) of the experimental diets

	ML		SBL	
Total fatty acids (mg g ⁻¹ lipids)	500.6		534.5	
	Total lipid	Polar fraction	Total lipid	Polar fraction
<i>Fatty acid (% TFA)</i>				
16:0	18.7	17.5	15.9	12.5
18:0	3.4	5.8	3.2	7.6
Σ SAFA	27.1	24.1	23.2	20.6
16:1n-7	4.7	0.5	4.6	0.5
18:1*	20.1	20.7	19.6	22.1
20:1n-9	6.5	5.2	5.5	2.3
Σ MUFA	31.3	26.4	29.8	24.8
18:2n-6	2.2	1.6	11.0	18.6
20:4n-6	0.9	0.7	0.6	0.4
Σ n-6 PUFA	3.5	2.5	12.2	19.1
18:3n-3	0.9	0.3	1.9	2.6
20:5n-3	12.0	15.4	10.3	11.2
22:5n-3	0.7	0.4	0.8	0.4
22:6n-3	21.8	29.8	17.6	20.7
Σ n-3 PUFA	38.0	46.5	34.9	35.3
Σ PUFA	41.5	49.0	51.0	54.4

* Sum of 18:1n-7 and 18:1n-9.

Table 4. Growth performance of seabream larvae fed different phospholipid sources and stocked under different densities

	Experimental treatment				Significance level		
	ML-L	ML-H	SBL-L	SBL-H	Diet	Density	Diet * Density
Dry weight (mg per larva)	0.5±0.1	0.4±0.0	0.3±0.0	0.3±0.0	*	NS	NS
Total length (mm)	8.5±0.7	8.8±0.1	8.1±0.3	7.8±0.2	*	NS	NS
RGR (% day ⁻¹)	3.8±3.8	3.6±0.6	-0.4±2.3	-0.2±0.8	*	NS	NS
Survival (%)	77.5±1.8	59.5±2.8	80.8±6.6	54.2±5.6	NS	*	NS
Theoretical biomass (mg)	35.4±9.8	26.4±2.4	27.3±2.1	18.5±0.8	*	*	NS

Values are mean ± SD. Significant effects determined by two-way analysis of variance indicated by

*P<0.10 or NS not significant.

Table 5. Histological analysis of a muscle quadrant area, fibre number, large : small fibre ratio and fibre density (per mm²) in gilthead seabream larvae fed the experimental diets and stocked under two different densities

	Experimental treatment				Significance level		
	ML-L	ML-H	SBL-L	SBL-H	Diet	Density	Diet * Density
<i>Total</i>							
Area (mm ²)	42.1±5.9	30.8±5.4	37.4±4.7	33.6±5.0	NS	NS	NS
Small fibres	255.3±18.7	239.0±24.4	196.3±19.2	159.8±16.8	*	NS	NS
Large fibres	315.7±29.6	272.5±28.6	298.2±27.1	257.2±25.3	NS	NS	NS
Small:Large fibres	0.8±0.0	0.9±0.1	0.7±0.1	0.6±0.0	*	NS	NS
<i>Density</i>							
Small fibres	6.3±0.8	8.4±1.0	5.5±0.5	5.0±0.5	*	NS	NS
Large fibres	7.6±0.5	9.3±0.7	8.2±0.4	8.1±0.8	NS	NS	NS

Values are mean ± SE. Significant effects determined by two-way analysis of variance indicated by

*P<0.10 or NS not significant.

Table 6. Total lipid content and fatty acid composition (% TFA) of whole-body gilthead seabream larvae fed the experimental diets and stocked under two different densities

	Experimental treatment				Significance level		
	ML-L	ML-H	SBL-L	SBL-H	Diet	Density	Diet * Density
Total lipids (mg g ⁻¹ DW)	20.7±2.3	19.8±1.0	16.2±2.3	21.8±0.5	NS	*	*
Total fatty acids (mg g ⁻¹ lipids)	433.8±15.4	420.4±50.8	449.1±15.6	427.4±38.3	NS	NS	NS
<i>Fatty acid (% TFA)</i>							
16:0	23.0±1.2	20.9±2.7	22.1±0.6	20.5±0.9	NS	NS	NS
18:0	9.1±0.0	9.2±1.5	9.3±0.4	8.2±0.8	NS	NS	NS
Σ SAFA	36.5±2.3	31.5±4.3	33.2±0.8	31.2±2.1	NS	NS	NS
16:1n-7	3.2±0.2	3.4±0.7	3.5±0.8	4.0±1.0	NS	NS	NS
18:1n-9	13.1±0.1	12.7±0.9	13.9±0.5	13.8±0.1	*	NS	NS
18:1n-7	1.6±0.2	1.8±0.4	2.2±0.0	2.1±0.3	*	NS	NS
20:1n-9	1.7±0.4	1.6±0.6	1.7±0.1	0.7±0.9	NS	NS	NS
Σ MUFA	19.9±0.7	19.8±2.5	21.6±0.2	20.8±0.3	NS	NS	NS
18:2n-6	1.6±0.0	1.7±0.3	6.1±0.3	6.6±0.0	*	NS	NS
20:4n-6	4.0±0.1	4.3±0.5	3.6±0.1	3.8±0.2	*	NS	NS
Σ n-6 PUFA	6.4±0.2	7.0±1.2	10.2±0.3	11.1±0.2	*	NS	NS
18:3n-3	0.3±0.1	0.3±0.0	0.5±0.0	0.6±0.0	*	*	NS
20:5n-3	9.2±0.2	10.5±1.2	8.2±0.4	8.8±0.5	*	NS	NS
22:5n-3	5.6±0.2	6.7±1.0	5.9±0.0	6.0±0.5	NS	NS	NS
22:6n-3	21.8±2.0	23.6±3.6	20.0±0.3	21.0±1.1	NS	NS	NS
Σ n-3 PUFA	37.2±1.9	41.7±5.6	35.0±0.7	36.8±2.2	NS	NS	NS
Σ PUFA	43.6±1.6	48.7±6.8	45.3±1.0	47.9±2.4	NS	NS	NS

Values are mean ± SD. Significant effects determined by two-way analysis of variance indicated by

*P<0.10 or NS not significant.

Table 7. Fatty acid composition of the polar lipid fraction (% TFA) of whole-body gilthead seabream larvae fed the experimental diets and stocked under two different densities

	Experimental treatment				Significance level		
	ML-L	ML-H	SBL-L	SBL-H	Diet	Density	Diet * Density
Total PL (mg g ⁻¹ DW)	570.0±155.6	495.0±49.5	665.0±91.9	360.0±84.9	NS	NS	NS
Total fatty acids (mg g ⁻¹ polar lipids)	481.7±103.3	540.9±114.2	505.1±48.8	558.1 (898.1±48 0.8)	NS	NS	NS
<i>Fatty acid (% TFA)</i>							
16:0	16.2±0.9	17.4±1.6	15.3±2.4	16.2±3.6	NS	NS	NS
18:0	11.3±0.4	12.2±1.3	10.7±0.2	15.3±0.8	*	*	*
Σ SAFA	28.8±1.7	30.5±0.2	26.2±2.1	32.0±2.4	NS	*	NS
16:1n-7	0.9±0.2	0.8±0.1	0.8±0.2	0.6±0.2	NS	NS	NS
18:1	10.7±0.6	11.0±0.2	11.2±0.9	11.1±0.2	NS	NS	NS
20:1n-9	2.4±0.3	2.2±0.2	1.7±0.1	2.4±0.2	NS	NS	*
Σ MUFA	13.9±0.7	14.0±0.3	13.7±1.2	14.0±0.2	NS	NS	NS
18:2n-6	1.7±0.6	1.5±0.1	4.6±0.6	4.3±0.4	*	NS	NS
20:4n-6	5.3±0.5	5.7±0.3	4.7±0.3	4.3±0.0	*	NS	NS
Σ n-6 PUFA	7.2±1.3	7.4±0.2	9.5±1.0	8.9±0.2	*	NS	NS
18:3n-3	0.2±0.0	0.2±0.0	0.3±0.1	0.5±0.1	*	NS	*
20:5n-3	11.0±0.3	11.0±0.7	10.7±0.9	10.0±0.2	NS	NS	NS
22:5n-3	7.0±0.3	7.4±0.6	7.2±0.3	6.8±0.5	NS	NS	NS
22:6n-3	30.4±2.4	28.0±0.3	31.2±3.2	26.8±1.1	NS	*	NS
Σ n-3 PUFA	49.1±2.3	47.1±0.3	49.9±4.1	44.5±1.7	NS	NS	NS
Σ PUFA	56.3±1.0	54.4±0.5	59.4±3.1	53.4±1.9	NS	*	NS

Values are mean ± SD. Significant effects determined by two-way analysis of variance indicated by

*P<0.10 or NS not significant.

Figure 1. Stocking density changes estimated during the experimental period in all treatment groups.

Figure 2. Transverse sections through gilthead seabream larvae from ML-L (a) and SBL-L (b) experimental groups stained with 1 % toluidine blue (20x).

Figure 3. Whole-body basal cortisol levels in seabream larvae fed different phospholipid sources and stocked under different densities. Values are mean $\pm$ SE. Absence of letters means no statistical differences ($p > 0.10$).

Figure 4a. Displacement curves for cortisol standards (black circles) and serial dilutions of a gilthead seabream sample (black squares). B = absorbance reading of the sample or standard. B₀ = absorbance reading of blank. Each point is the mean of duplicate determinations.

Figure 4b. Linearity and parallelism of the ELISA cortisol analysis method. Each point is the mean of duplicate determinations.

Figure 5. Expression of genes using real-time PCR and SyBR green detection in gilthead seabream larvae submitted to the experimental conditions. The mRNA levels relative to ML-L treatment were arbitrarily defined as 1 unit. Data are expressed as mean $\pm$ SE. Different letters mean significant differences ($P < 0.10$). GH, growth hormone; POMC, pro-opiomelanocortin; CB1 receptor, cannabinoid CB1 receptor; GR,

glucocorticoid receptor; HSL, hormone-sensitive lipase; FBPase, fructose-1,6-bisphosphatase; COX-2, cyclooxygenase-2; PKC, protein kinase C.