

Effects of soybean meal on digestive enzymes activity, expression of inflammation-related genes, and chromatin modifications in marine fish (*Sparus aurata* L.) larvae

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Abstract The effects of soybean meal (SBM) in early diet of *Sparus aurata* larvae at two developmental windows were assessed. Prolonged (beyond 14 days post-hatch, dph) feeding with SBM decreased the activity of pancreatic enzymes of larvae. In the absence of SBM these larvae later resumed enzyme activities, but exhibited a significant delay in development. Larvae response to SBM involved up-regulation of extracellular matrix remodeling enzymes and pro-inflammatory cytokines, coupled with a drop in putative intestinal enzymes. Larvae receiving SBM at first feeding appear later to have lower expression of inflammation-related genes, especially those fed SBM until 14 dph. Multivariate analysis confirmed that the duration of the SBM early feeding period drives the physiology of larvae in different directions. Feeding larvae with SBM increased

global histone H3 acetylation, whereas upon removal of SBM the process was reverted. A more in depth analysis revealed a dynamic interplay among several reversible histone modifications such as H3K14ac and H3K27m3. Finally, we showed that SBM feeding of larvae results in global hypomethylation that persist after SBM removal. This study is the first demonstrating an effect of diet on marine fish epigenetics. It is concluded that there are limitations for extending SBM feeding of *S. aurata* larvae beyond 14 dph even under co-feeding with live feed, affecting key physiological processes and normal growth. However, up to 14 dph, SBM does not affect normal development, and produces apparently lasting effects on some key enzymes, genes, and chromatin modifications.

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Keywords Gilthead seabream · Fish larvae · Soybean meal · Digestion · Inflammation-related genes · Chromatin modifications

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Introduction

The use of plant proteins (PP) as alternatives to fish meal (FM) in aquafeeds have been thoroughly studied during the last decades but it remains a major issue in aquaculture research and fish physiology (Hardy 2010; Naylor et al. 2000). FM is expensive and it is expected to be limited available in the future, thus unable to sustain the expected increase in aquaculture production (Hardy 2010). Among several PP, soybean meal (SBM) is considered a suitable alternative to FM, due to its high

availability, relatively low cost, and high nutritional value (Drew et al. 2007). However, there are species-specific limits for the inclusion of SBM in diets for different fish species. In salmonids, inclusion of SBM in diets produces alterations in the normal functioning of the digestive tract such as inflammation and failure to regulate mucosal integrity (Krogdahl et al. 2010), increased cell turnover and sloughing (Chikwati et al. 2013a), reduction in the activity of brush border enzymes and nutrient transport (Venold et al. 2013), increased activity of trypsin (Lilleeng et al. 2007) and other pancreatic proteases in the distal intestine (Chikwati et al. 2013b) and in feces (Krogdahl et al. 2003), and reduced cholesterol and bile salt reabsorption (Kortner et al. 2013). SBM contains different anti-nutritional factors (e.g. lectins, trypsin inhibitors, soy antigens, isoflavones), but soybean saponins have been suggested as the most likely contributing agent of these disorders (Krogdahl et al. 2010, 2015). Whereas salmonids are particularly sensitive to SBM and similar responses have been recently reported in turbot (Gu et al. 2016), higher SBM tolerance occurs in other fish species. For instance, *S. aurata* distal intestine *lamina propria* is moderately expanded due to an increase in mononuclear cells infiltration after feeding 30 % SBM diets, but this does not affect growth and feed conversion rates (Bonaldo et al. 2008). In this species, high level of FM substitution has been attained by using mixtures of vegetal protein sources (Dias et al. 2009) but SBM inclusion level has been regularly maintained well below 30 %. A higher inclusion level such as 40 % decreased feed conversion and plasma cholesterol (Venou et al. 2006). Advance to a higher use of SBM in feeds for this species would require an increased tolerance to SBM alone or in combination with others PPs.

Some studies in fish have provided evidence of nutritional programming (Geurden et al. 2014; Fang et al. 2014; Vagner et al. 2009; Koven et al. 2003; Geurden et al. 2013), including few recent reports on conditioning to SBM (Kemski et al. 2015; Perera and Yúfera 2016). The first step for conducting this kind of studies is the evaluation of the acceptance of the nutritional stimulus (or challenge) during early life without compromising further development. While SBM (~5–10 % inclusion)-induced inflammatory response (i.e., enteropathy) in the distal intestine has been extensively documented in salmonids, now there are evidences that young salmon are relatively tolerant to SBM. SBM

inclusion of 12 % induces cell proliferation in the distal intestine of salmon parr, but it produces no significant histological changes (Sanden et al. 2005). In line with these findings, a further study showed that a higher inclusion of SBM (i.e., 16.7 %) in diet did not produce intestinal inflammation, nor did it negatively affect growth, survival, enzyme activities, bile salt concentration or gene expression of various key functional markers in start feeding salmon fry (and up to three months following start-feeding) (Sahlmann et al. 2015). These studies showed that SBM can be used in diets for these life stages in salmon, but there is no information for other cultured marine species. The gilthead seabream, *S. aurata*, is one of the most important fish species cultured in Europe (Basurco et al. 2011) and it is known to be more tolerant to SBM (Bonaldo et al. 2008; Dias et al. 2009) than salmonids. While it is tempting to hypothesize that *S. aurata* larvae would also tolerate SBM in diet, this species has a prolonged larval period and the maturation of the digestive tract progress slower than in other fish (Moyano et al. 1996; Elbal et al. 2004).

Thus, this study accesses the possibility to include SBM in early diets of *S. aurata*. In addition, given that some components of SBM have both histone deacetylase and DNA methyl transferase inhibitory properties (Link et al. 2010), we hypothesized that SBM effects on fish larvae may include epigenetic modifications. Epigenetics refers to mechanisms for silencing or activating tissue specific gene expression independent of the DNA sequence, and mainly encompass changes in histone signatures and DNA methylation profiles that act in concert to induce transient and long-term changes in gene expression (Lillycrop and Burdge 2012). While several investigations on fish nutrition and digestion physiology have integrated ‘omic’ methodologies and there is now extensive information on the transcriptional responses of fish to different dietary components or nutritional conditions, knowledge on the molecular mechanisms regulating these responses is still limited. To our knowledge, there is no information on the effect of diet on marine fish epigenetics.

Results of this work revealed the limited capacity of *S. aurata* larvae to deal with SBM at first feeding. However, we determined a narrow window of time where SBM produces reversible and lasting effects on some key physiological variables without compromising further development. Also, this study shed some

light on the putative role of different chromatin modifications on the regulation of the transcriptional response of larvae to SBM.

Material and methods

All experiments were conducted in compliance with Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 1201/2005 and law 32/2007) for the use of laboratory animals. Experimental procedures used in this study received the approval of the CSIC Bioethics Subcommittee (ref. number 326,245/2013).

Experimental diets

Microdiets were prepared at the laboratory by the method of calcium internal gelation (Yúfera et al. 2005) and were formulated to have, on a dry weight basis, 50 % crude protein, 25 % lipid, 16 to 20 % carbohydrate (Table 1). One microdiet contained a combination of different animal protein sources including fish meal (FM) and was referred as the FM-diet. The other microdiet contained soybean meal (SBM) instead of FM, whereas keeping almost constant all other ingredients, and was referred as the SBM-diet.

Culture conditions, feeding protocol and sampling procedure

Two experiments were performed to evaluate the effects of early feeding of Fish- and SBM-diet on *S. aurata* larvae. Eggs were obtained from broodstock maintained at the Instituto de Ciencias Marinas de Andalucía, Cadiz, Spain, and incubated in 200 L incubators at 19 °C and 35 g L⁻¹. Hatched larvae remained in incubators for 24 h with flow-through seawater and aeration, and then they were randomly sorted to two different experimental groups in triplicated 100 L cylindrical fiberglass tanks at an initial stocking density of 100 larvae L⁻¹. Larvae were cultured at 19.2 ± 0.06 °C (first experiment) and 19.6 ± 0.08 °C (second experiment), 35 g Kg⁻¹ salinity, pH 8.3, and 12 h light: 12 h darkness. Constant but slight aeration was provided and oxygen level was maintained around 6.0 mg L⁻¹. Rotifers, *Brachionus rotundiformis* and *B. plicatilis* (5–10 individuals mL⁻¹), and microalgae *Nannochloropsis gaditana* (0.5 × 10⁶ to 1 × 10⁶ cells mL⁻¹) were supplied

Table 1 Formulation (%) and proximate composition of the experimental diets

Ingredient	FM-diet	SBM-diet
Fish meal ^a	20	-
SBM ^b	-	30
Cuttlefish meal ^c	29	29
Soluble fish protein concentrate ^d	9	9
Taurine ^e	1	1
Methionine ^e	-	0.4
Lysine ^e	-	2
Threonine ^e	-	0.1
Dicalcium phosphate ^e	-	1.5
Fish oil ^f	10	10
Dextrin ^g	4	-
Soy lecithin ^g	8	8
Vitamin Premix ^h	2.8	2.8
Vit C ⁱ	1.6	1.6
Vit E ^g	1.6	1.6
CMC ^e	4	-
Na-alginate ^g	9	9
Total	100	100
<i>Proximate composition</i>		
Crude protein (% DM)*	53	51.6
Crude lipid (% DM)**	25.1	23.3
Carbohydrate (% DM) [†]	16.6	22.5
Ash (% DM)**	5.4	5.2
Gross energy (MJ/kg DM) ^{††}	24.5	24.1

Microdiets prepared by internal gelation; Diets contained 4 % of water; SBM: soybean meal. DM: dry matter; ^a AgloNorse Microfeed, Norsildmel Innovation AS; ^b Cargill; ^c Squid Powder 0278, Rieber & Søn ASA; ^d CPSP-90, Sopropêche, France; ^e Sigma-Aldrich; ^f La Lorientaise, France; ^g MP Biomedicals

^h Vitamin premix supplied the following (per kg of diet): retinol-cholecalciferol 500:100, 1000 mg; cholecalciferol 500, 40 mg; α -tocopherol acetate, 3000 mg; menadione 23 %, 220 mg; thiamin HCl, 50 mg; riboflavin 80, 250 mg; D-calcium pantothenic acid, 1100 mg; nicotinamide, 500 mg; pyridoxine, 150 mg; pteroylglutamic acid, 50 mg; cyanocobalamin 0.1, 500 mg; biotin 20, 38 mg; ascorbic acid polyphosphate 35 %, 57.2 g; choline chloride 60 %, 100 g; myo-inositol, 15 g; antioxidants, 1.25 %

ⁱ Rovimix STAY-C 35, DSM Nutritional Products, Inc.; * Calculated as N × 6.25 after CNH analyses

** Calculated from proximate composition of ingredients; [†] Calculated as 100 - (protein + lipid + ash + water), ^{††} Calculated as total carbohydrate × 17.2 kJ/g; fat × 39.5 kJ/g; protein × 23.5 kJ/g.

from day 4 post-hatch (dph) following the temporal sequence described by Polo et al. (1992). From 18 dph to 25 dph, the larvae were provided with *Artemia sp.* at

0.5 nauplii mL⁻¹ in addition to rotifers. From 25 dph to 30 dph rotifers were substituted by 1 metanauplii mL⁻¹, and from 30 dph to 36 dph live feeding only consisted on 10 metanauplii mL⁻¹. The two experimental groups were fed two times a day (15:00 and 17:00 h), in addition to live feed as above, with the FM- and SBM-diet since first feeding until 18 dph (14 d of feeding) or 14 dph (10 d of feeding) in the first and second experiment, respectively. Then, all groups were fed the FM-diet up to 36 dph. Microdiet ingestion since mouth opening was checked by the observation of larvae under a microscope. Samples (entire larvae) were taken at 4, 14 or 18, and 36 dph, rinsed with distilled water, and immediately frozen at -80 °C for protein/enzyme/DNA assays or stored on RNAlater for gene expression analysis. Pools of 100 larvae (4 dph), 50 larvae (14 and 18 dph) and 20 larvae (36 dph) were collected in duplicate from each tank for protein/enzyme/DNA assays, whereas duplicated pools of 5 larvae per tank for expression analysis. Total length was determined each sampling day by measuring 10 larvae per tank ($n = 30$ per treatment) under a stereomicroscope.

Digestive enzyme activities

Trypsin, chymotrypsin, amylase, lipase, and leucine aminopeptidase activities were assayed in supernatants of homogenates of entire *S. aurata* larvae in ice-cold 200 mM Tris-HCl, 20 mM NaCl, 20 mM CaCl₂, pH 7.5, after centrifugation at 10,000 × *g*, 4 °C. Brush border anchored enzymes such as alkaline phosphatase and matrix metallo-protease activities were measured after extraction with the same buffer containing 0.2 % Triton X-100.

Trypsin activity was measured using 1.25 mM N-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and chymotrypsin activity with 0.1 mM Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAPNA), both in 200 mM Tris-HCl, 20 mM NaCl, 20 mM CaCl₂, pH 7.5 (referred henceforth as the assay buffer). Esterase activity was assessed by the hydrolysis of 0.3 mM *p*-nitrophenyl butyrate (*p*NPB) in the assay buffer. Lipase activity was measured using 1 mM 4-nitrophenyl palmitate (4NPP) in assay buffer containing 0.3 % Triton X-100. Leucine aminopeptidase activity was assessed by the hydrolysis of 0.5 mM L-Leucine-*p*-nitroanilide (LeupNA) in assay buffer. Substrate stock solutions were prepared in DMSO (BAPNA, SAPNA), acetone (*p*NPB), 1:4 v:v acetonitrile/isopropanol (4NPP), and methanol

(LeupNA). The liberation of *p*-nitroaniline and *p*-nitrophenol were kinetically followed at 405 nm and extinction coefficients of 6800 M⁻¹·cm⁻¹ and 18,000 M⁻¹·cm⁻¹ were used for *p*-nitroaniline and *p*-nitrophenol respectively. Amylase activity was determined using the Amylase Activity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA), and alkaline phosphatase and matrix metallo-protease activities were measured using SensoLyte *p*NPP Alkaline Phosphatase Assay Kit and SensoLyte Generic MMP Assay Kit, respectively, from AnaSpec, Inc. (Fremont, CA, USA). Commercial kits were used according to manufacturer instructions. All assays were performed in a microplate reader BioTek ELx808 (BioTek Instrument, Inc). One unit of enzyme activity (U) was defined as the amount of enzyme that produces one μmol of product per minute (but 1 nmol min⁻¹ for amylase) at 37 °C. All enzyme activities were expressed as specific activity (U μg⁻¹ of protein for all enzymes, but per mg of protein for MMP).

Expression rate of target genes

Expression was assessed by real-time qPCR for genes involved in inflammation/immunity (proinflammatory cytokines interleukin 1β [*il1b*], tumor necrosis factor α [*tnfa*], anti-inflammatory cytokine interleukin 10 [*il10*]), the breakdown of extracellular matrix and tissue repair/remodeling (matrix metalloproteases 9 and 13 [*mmp9*, *mmp13*]), luminal digestion (trypsin [*try*]), and intestinal nutrient absorption/trafficking (intestinal alkaline phosphatase [*alpi*], solute carrier family 15, oligopeptide transporter, member 1b [*slc15a1b*] - here referred as proton/oligopeptides transporter PepT1, fatty acid binding protein 2, intestinal [*fabp2*], and fatty acid binding protein 6, ileal [*fabp6*]).

Total RNA was isolated using an Ultra-Turrax T25 (IKA-Werke) and the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol, including an on-column RNase-free DNase digestion step. Concentration of total RNA was measured with the Qubit 2.0 Fluorometer and the corresponding RNA quantification assay (Invitrogen, Life Technologies), and its quality was determined in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using the Agilent RNA 6000 Nano Kit. Only samples with a RNA Integrity Number (RIN) higher than 8.5 were used. Total RNA (500 ng) was reverse-transcribed in a 20 μL reaction using the qScript DNA synthesis kit (Quanta

BioSciences) (5 min at 22 °C, 30 min at 42 °C and 5 min at 85 °C). Primers used (Online Resource 1) were synthesized by IDT (Integrated DNA Technologies, Leuven, Belgium). Various amounts of cDNA were used in triplicate (6 points of serial 1/5 dilutions from 10 ng to 3.2 pg per reaction) as templates to check the assay linearity (R^2) and the amplification efficiency (E) of primers. Each qPCR reaction mixture (10 μ L) contained 0.5 μ L at 400 nM of each specific forward and reverse primers, 5 μ L of PerfeCTa SYBR Green FastMix (Quanta Biosciences) and 4 μ L of template (10 ng cDNA) in white wells twin.tec real-time PCR plates 96 (Eppendorf). Control reactions with DEPC water and RNA instead of cDNA were included to ensure the absence of contamination or genomic DNA. qPCR was performed with the Eppendorf Mastercycler *eprealplex*² S (Eppendorf, Hamburg, Germany). Cycling conditions were 95 °C, 10 min; [95 °C, 20 s; 60 °C, 35 s] X 40 cycles; melting curve [60 °C to 95 °C, 20 min], 95 °C, 15 s). The melting curve was used to ensure that a single product was amplified and confirm the absence of primer-dimer artifacts. Relative gene quantification was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Data were normalized to both elongation factor 1 α (*ef1a*) and ribosomal 18S, which were previously confirmed to express stably among all samples.

Global DNA methylation and histone (H3, H4) acetylation

Genomic DNA was extracted from whole larvae by the salting-out method (Martínez et al. 1998). DNA concentration was determined using the Invitrogen Qubit 2.0 Fluorometer (Life Technologies, ThermoFisher Scientific). Global methylation status was assessed using the colorimetric ELISA kit (EpiSeeker methylated DNA Quantification Kit, abcam) following manufacturer instructions, with 100 ng of DNA per well as the sample. Total histones were extracted and global acetylation of histones H3 and H4 measured with the EpiSeeker Histone H3 Acetylation Assay Kit (abcam) and the EpiSeeker Histone H4 Acetylation Assay Kit (abcam), respectively, following manufacturer instructions, with 2 μ g of histone per well as the sample. Total histones were quantified by their OD_{230nm} at a BioPhotometer Plus (Eppendorf). Methylated DNA and acetylated H3/H4 were quantified at 450 nm in an ELx808 BioTek (BioTek Instrument, Inc.) microplate reader.

Methylation status (% of 5-methylcytosine, 5-mC%) was expressed relative to the positive control provided in the kit, whereas acetylation expressed as OD_{450nm}.

Global histone (H3, H4) modifications

Acetylation at specific lysine residues of histones H3 and H4, as well as some other key post-translational modifications (i.e., mono-, di-, and tri-methylation at specific lysine residues) of these histones, were quantified at 450 nm in an ELx808 BioTek (BioTek Instrument, Inc.) microplate reader, using the Histone H3 Modification Multiplex Assay Kit (abcam) and the Histone H4 Modification Multiplex Assay Kit (abcam), following manufacturer instructions, with 150 ng of total histone (extracted and quantified as above) per well as the sample.

Statistical analysis

Results are presented as mean values plus standard error of means (SEM). Data were checked for normality and homogeneity of variance using Shapiro-Wilk and Levene's tests, respectively. Data were analyzed by Student's t-test. In few cases, due to heterogeneous variances, we used unequal variance t-test with the Welch correction. Differences with *P* values <0.05 were considered as significant. Also, we performed a forward stepwise discriminant analysis using the expression of studied genes to understand the combination of variables that can best explain the differences between old larvae with different early nutritional experiences. For the analysis, it was considered *F* to enter as 0.01, *F* to remove at 0.0, and tolerance of 0.01. After significant functions development, the relative importance of the original variables was gauged by standardized values. The software package Statistica 7.0 (StatSoft Inc.) was used for all tests performed.

Results

SBM feeding beyond 14 dph produces reversible effects on pancreatic enzymes activities and reduces larval growth

The specific activity of trypsin, chymotrypsin, and amylase (Fig. 1a, c, e) were significantly reduced in larvae fed with the SBM-diet for the first 14 d of feeding (at 18

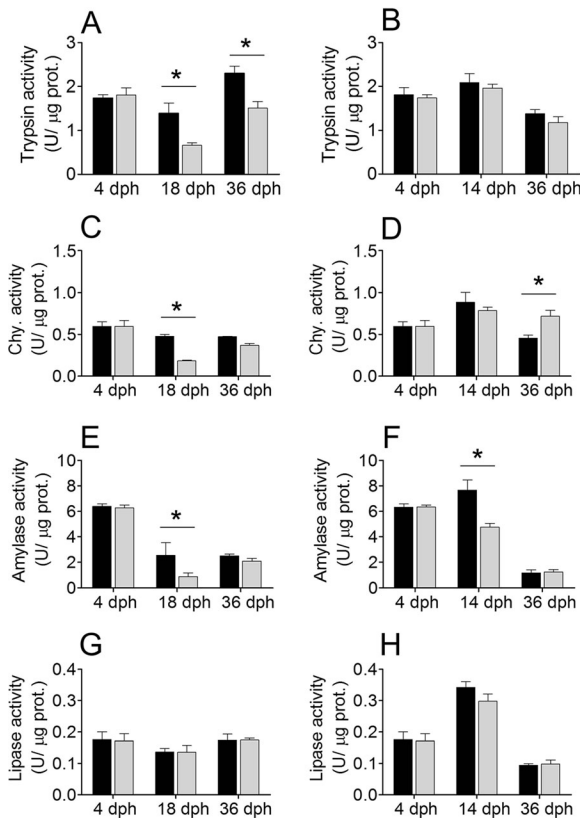


Fig. 1 Effect of feeding gilthead seabream with fish meal (FM)- and soybean meal (SBM)-microdiet (black and gray columns, respectively) from mouth opening to 18 dph or 14 dph (equivalent to 14 and 10 d of feeding, respectively) under a co-feeding regimen with live feed, on specific activity of trypsin (A, B), chymotrypsin (Chy) (C, D), amylase (E, F), and lipase (G, H). After the initial feeding period (up to 18 or 14 dph), all fish were fed with the FM based diet and live feed up to 36 dph. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzed the release of 1 μmol of product per minute ($1 \text{ nmol} \cdot \text{min}^{-1}$ for amylase), and activities were expressed as activity per μg of protein ($\text{U} \mu\text{g}^{-1}$ of protein). Values are means + SEM, * $P < 0.05$

dph), but size of larvae was not affected (Fig. 2a). The exception was lipase, for which no reduction in specific activity was found (Fig. 1g). When SBM was removed from the diet of these larvae, pancreatic enzymes attained at 36 dph similar levels of specific activity to those in fish fed the FM-diet (Fig. 1a, c, e), except for trypsin (Fig. 1a), and exhibited a significant delay in larval growth (Fig. 2a). Reduction of the SBM feeding to 10 d (up to 14 dph) lead to no differences in specific activity of pancreatic enzymes studied (Fig. 1b, d, h) but amylase (Fig. 1f), and following effects at 36 dph only for chymotrypsin (Fig. 1d). The size of these larvae did not differ between the experimental groups at 36 dph (Fig. 2b).

Early SBM feeding produces both reversible and lasting effects on MMP, AP and LAP activities

The specific activity of matrix metalloproteinases (MMP) increased in larvae receiving the SBM-diet for 14 d (Fig. 3a), whereas this effect was not observed when the SBM feeding period was shorter (Fig. 3b). Change from the SBM-diet to live food resulted in a significantly decrease in MMPs activity at 36 dph irrespective of the early SBM feeding period (Fig. 3c). The specific activity of AP was significantly lower in *S. aurata* larvae feeding SBM for 14 d than in larvae fed the FM-diet (Fig. 3c). Later, feeding these larvae with live food up to 36 dph did not allow larvae to exhibit mean activities similar to those of larvae fed with the FM-diet since first feeding (Fig. 3c). Conversely, 10 d of feeding had no effect on AP activity either during or after SBM feeding (Fig. 3d). Moreover, we found that LAP specific activity significantly decreased after 14 d of SBM feeding, a change that was reverted after SBM removal from diet (Fig. 3e). The reduction of

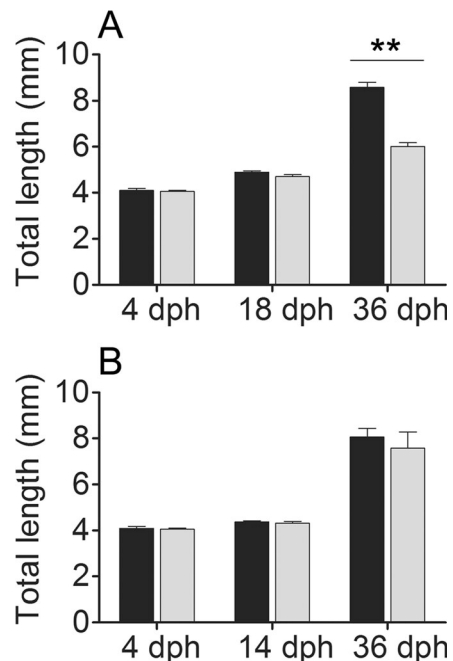


Fig. 2 Growth of gilthead seabream larvae fed with fish meal (FM)- and soybean meal (SBM)-microdiet during two different periods. Gilthead seabream larvae were fed with a FM-microdiet (black columns) or a SBM-microdiet (gray columns) from mouth opening to 18 dph (A) or 14 dph (B) (equivalent to 14 and 10 d of feeding, respectively) under a co-feeding regimen with live feed, and then fed with the FM-diet and live feed up to 36 dph. Values are means + SEM, ** $P < 0.01$

SBM period to 10 d leads to no differences in specific LAP activities or final size of larvae (Fig. 2b), but to an increase in activity in the absence of SBM at 36 dph (Fig. 3f).

Early SBM feeding produces both reversible and lasting effects on gene expression of different markers of inflammation and tissue remodeling

Feeding *S. aurata* with SBM for 14 d (up to 18 dph) up-regulated the inflammatory cytokine *il1b* (Fig. 4a) and the remodeling enzyme *mmp13* (Fig. 4e) gene expression. We also observed a non significant trend for *mmp9* expression to increase at this time (Fig. 4d). At 36 dph and in the absence of SBM from diet, these larvae exhibited lower expression of *il1b* (Fig. 4a) and both *mmp9* (Fig. 4d) and *mmp13* (Fig. 4e) than larvae fed with the FM-diet since first feeding. The analysis of expression

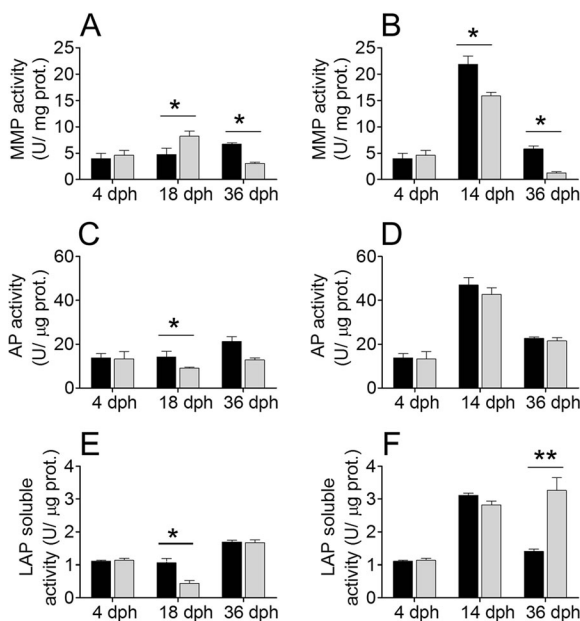


Fig. 3 Effect of feeding gilthead seabream with fish meal (FM)- and soybean meal (SBM)-microdiet (black and gray columns, respectively) from mouth opening to 18 dph (left side graphs) or 14 dph (right side graphs) (equivalent to 14 and 10 d of feeding, respectively) under a co-feeding regimen with live feed, on the specific activities of matrix metalloproteases (MMP) (A, B), alkaline phosphatase (AP) (C, D), and leucine aminopeptidase (LAP) (E, F). After the initial feeding period (up to 18 or 14 dph), all fish were fed with the FM-diet and live feed up to 36 dph. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzed the release of 1 μmol of product per minute, and activities were expressed as activity per μg of protein ($\text{U } \mu\text{g}^{-1}$ of protein). Values are means + SEM, * $P < 0.05$, ** $P < 0.01$

data from larvae fed the SBM-diet only during 10 d (until 14 dph) allowed us corroborate the up-regulation of *il1b* (Fig. 5a), *mmp9* (Fig. 5d), and *mmp13* (Fig. 5e), but also to observe other changes that occurs earlier and become buried at 18 dph, such as the increased expression of *tnfa* (Fig. 5c) and *alpi* (Fig. 5g). After the removal of SBM from diet of these larvae, we observed lower expression of *il1b* (Fig. 5a), *mmp9* (Fig. 5d), and *fabp2* (Fig. 5i), and higher expression of the anti-inflammatory interleukin *il10* (Fig. 5b) at 36 dph. Thus, both SBM feeding periods triggered changes in gene expression of some markers of inflammation and tissue remodeling, but these were reverted when SBM was removed from diet. Also, both feeding periods led to older larvae with higher expression of an anti-inflammatory cytokine.

Duration of the SBM feeding period drives the physiology of larvae in different directions

Two significant discriminant functions could be developed from the relative expression of studied genes in 36 dph larvae. These functions collectively accounted for 100 % of the total variance (Table 2). Expression of genes involved in inflammatory/anti-inflammatory processes, tissue remodeling, and nutrient transport were the variables with the highest relevance in the first discriminant function (Table 3), which accounted for 73 % of the total variance (Table 2). In this function, two groups of SBM feeding larvae showed opposed central values depending on early nutrition (Fig. 6, axis x). On the other hand, the second discriminant function better discriminated among SBM and FM diets irrespective of the SBM feeding period (Fig. 6, axis y).

Early SBM feeding results in global hypomethylation

We observed no difference in global methylation level between *S. aurata* larvae fed with SBM up to 14 or 18 dph and larvae fed with the FM-diet (Fig. 7a,b). However, *S. aurata* larvae that faced SBM at first feeding showed a significant global DNA hypomethylation at 36 dph irrespective of the duration of the SBM feeding period (Fig. 7a,b).

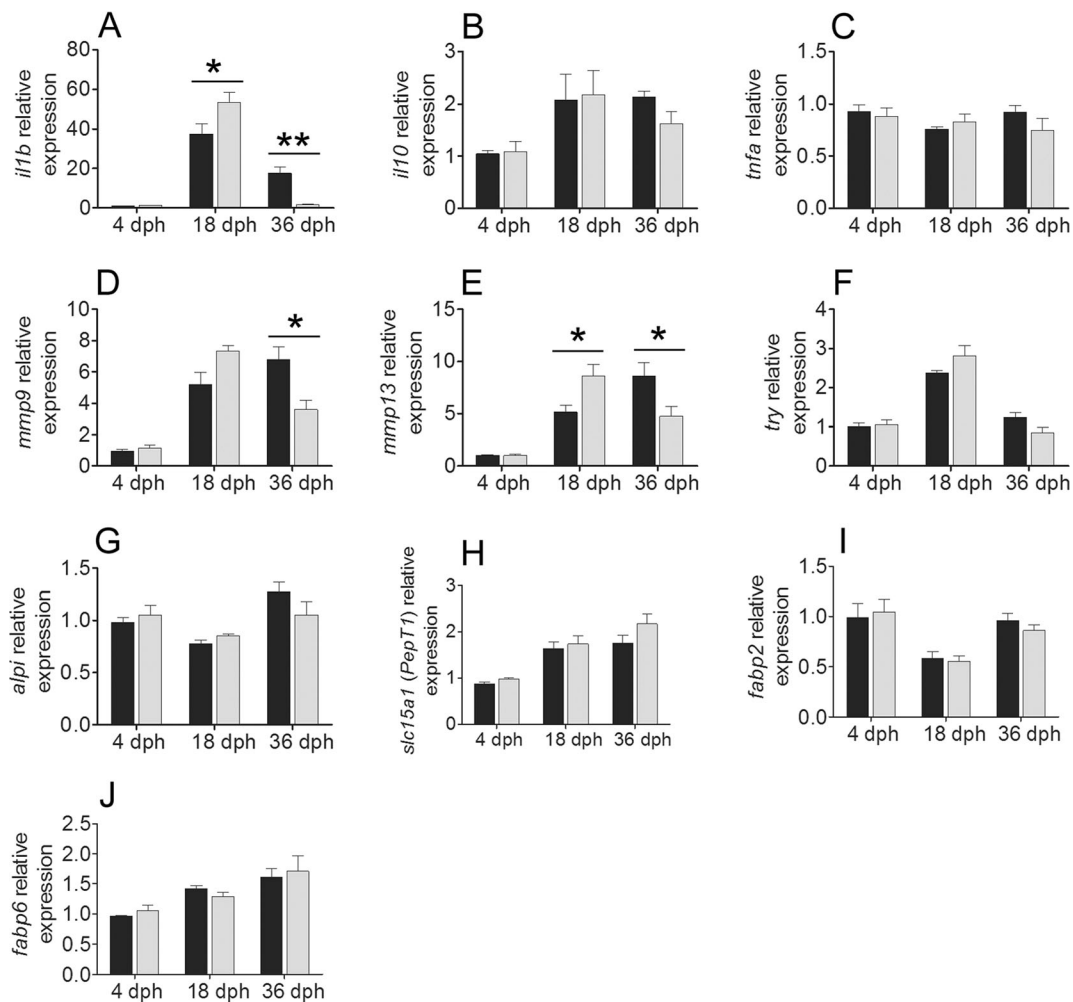


Fig. 4 Effect of feeding gilthead seabream with fish meal (FM)- and soybean meal (SBM)-microdiet (black and gray columns, respectively) from mouth opening to 18 dph (equivalent to 14 d of feeding) under a co-feeding regimen with live feed, on relative expression of selected genes related to inflammation/immunity (interleukin 1 β [A], interleukin 10 [B], tumor necrosis factor α [C]), breakdown of extracellular matrix and remodeling (matrix metalloprotease 9 [D], matrix metalloprotease 13 [E]), luminal digestion (trypsin [F]), and intestinal nutrient absorption/

trafficking (intestinal alkaline phosphatase [G], solute carrier family 15, member 1 (proton/oligopeptide transporter) [H], fatty acid binding protein 2, intestinal [I], fatty acid binding protein 6, ileal (gastrotropin) [J]). After the initial feeding period (up to 18 dph), all fish were fed with the FM based diet and live feed up to 36 dph. Relative expression was assessed by real-time qPCR and normalized to both elongation factor 1 α (*ef1a*) and ribosomal *18S*. Values are means + SEM. * $P < 0.05$, ** $P < 0.01$

H3K14ac and H3K27m3: the most dynamic, but not the only, epigenetic marks responding to SBM

Feeding *S. aurata* with SBM from mouth opening up to 14 dph (10 d of feeding) significantly increased global H3 acetylation (Fig. 7d). This SBM-induced H3 hyperacetylation was transient, as the level of acetylation was restored in later larvae in the presence (at 18dph, Fig. 7c) or absence of SBM (at 36 dph, Fig. 7d). We found a slight hypoacetylation of histone

H4 at 18 dph in larvae fed with the SBM-diet (Fig. 7e), but no such effect if the feeding period was restricted up to 14 dph (Fig. 7f) or if SBM was removed from later diet (Fig. 7e,f).

To gain insight on the type of histone modifications that occurred as response to SBM in *S. aurata* larvae, we further assessed H3 and H4 modifications at 14 and 36 dph in fish fed with the SBM- and the FM-diet during the first 10 d of feeding. SBM feeding resulted in hyperacetylation of histone H3 at lysine 14 (H3K14ac)

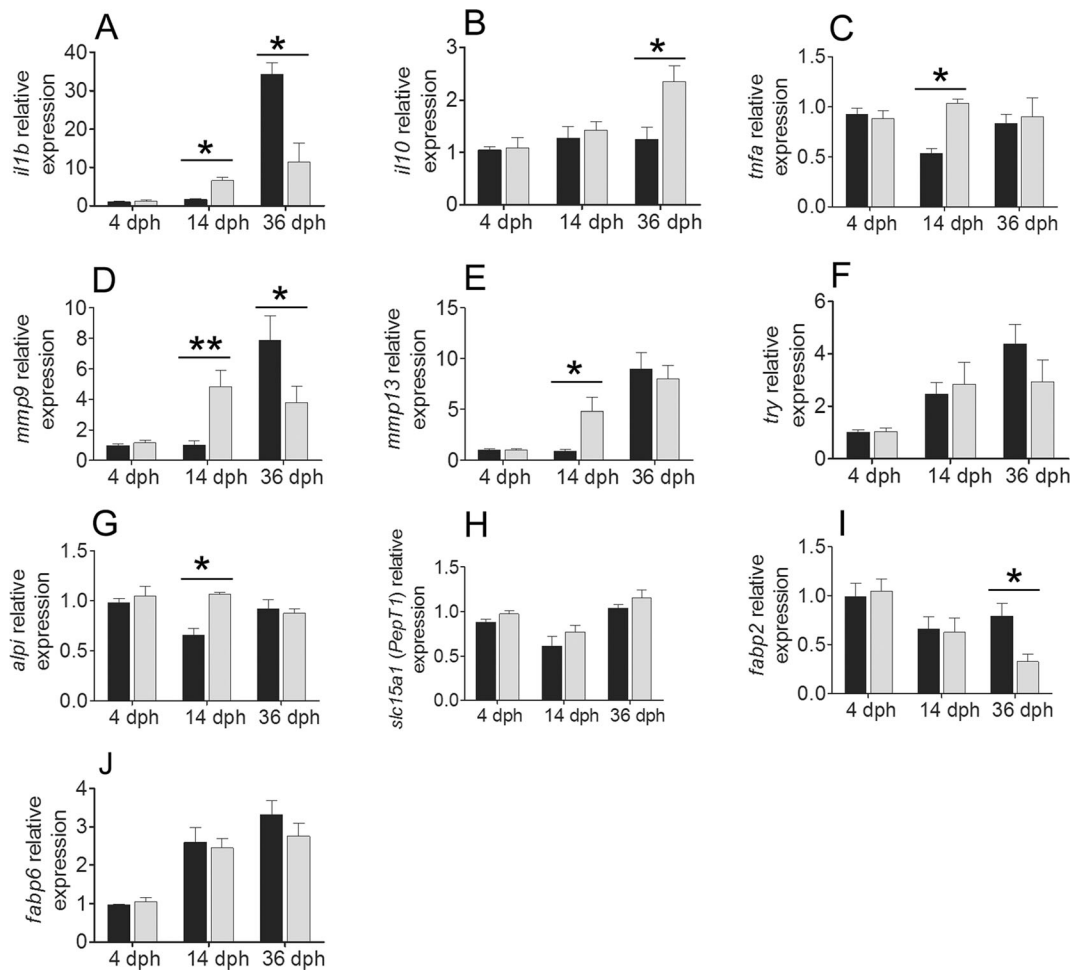


Fig. 5 Effect of feeding gilthead seabream with fish meal (FM) and soybean meal (SBM)-microdiet (black and gray columns, respectively) from mouth opening to 14 dph (equivalent to 10 d of feeding) under a co-feeding regimen with live feed, on relative expression of selected genes related to inflammation/immunity (interleukin 1 β [A], interleukin 10 [B], tumor necrosis factor α [C]), breakdown of extracellular matrix and remodeling (matrix metalloprotease 9 [D], matrix metalloprotease 13 [E]), luminal digestion (trypsin [F]), and intestinal nutrient absorption/

trafficking (intestinal alkaline phosphatase [G], solute carrier family 15, member 1 (proton/oligopeptide transporter) [H], fatty acid binding protein 2, intestinal [I], fatty acid binding protein 6, ileal (gastrotropin) [J]). After the initial feeding period (up to 14 dph), all fish were fed with the FM based diet and live feed up to 36 dph. Relative expression was assessed by real-time qPCR and normalized to both elongation factor 1 α (*ef1a*) and ribosomal *18S*. Values are means + SEM. * $P < 0.05$, ** $P < 0.01$

(Fig. 8a), whereas acetylation level of other H3 lysine residues was not modified or even decreased (H3K18ac) (Fig. 8a). Once SBM was removed from diet, we observed a significant hypoacetylation of the same lysine

residue (H3K14ac) but also of H3K56ac (Fig. 8b). These reversible SBM-induced H3 acetylations occurred along with other histone modification such as the tri-methylation of the same histone at lysine residue

Table 2 Summary of the canonical discrimination analysis

Variable/Function	Eigen value	Percentage of variance	Canonical correlation	Wilks Lambda	Chi-Square	d.f.	P
1	222.6	72.92	0.99	0.0012	70.21	20	<0.001
2	20.09	27.08	0.97	0.0569	30.09	9	<0.001

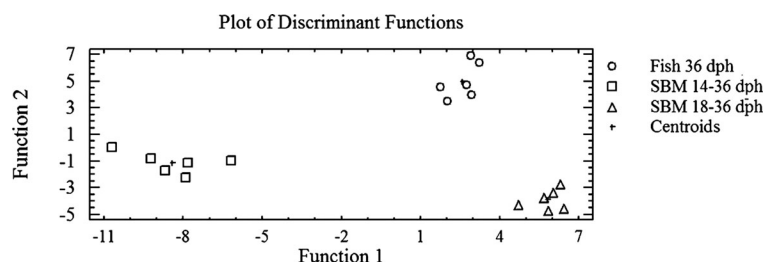
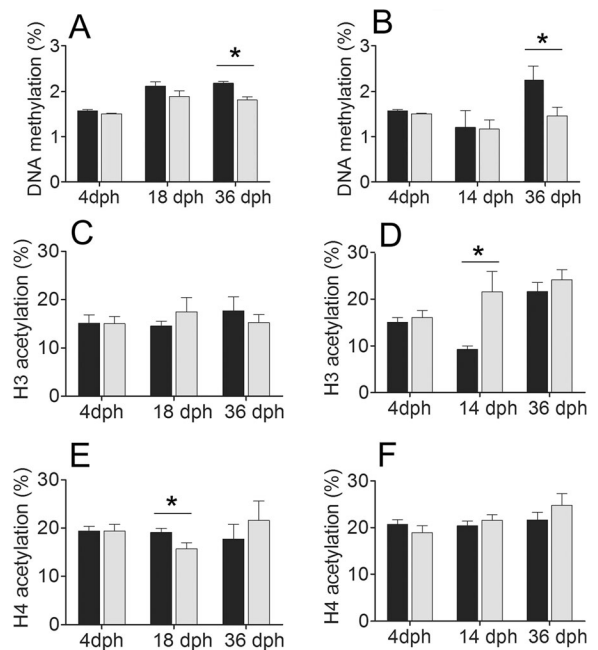
Table 3 Standardized function coefficients for each of the significant discriminant functions

Variable (Gene expression)	Function	
	1	2
<i>illb</i>	1.6666	0.7369
<i>slc15a1</i>	2.2869	-1.3163
<i>alpi</i>	-1.9761	-0.1186
<i>fabp6</i>	-1.1452	0.1294
<i>il10</i>	0.5279	1.0773
<i>fabp2</i>	-3.6642	0.3953
<i>mmp13</i>	0.4298	0.1995
<i>mmp9</i>	-2.0600	0.7941
<i>tnfa</i>	-0.7709	0.6036
<i>try</i>	2.6688	-0.1604

27 (H3K27m3). After 10 d of SBM feeding, we found a significant decrease in H3K27m3 (Fig. 8a), but upon removal of SBM from diet, H3K27m3 significantly increased over the level observed in fish fed the FM-diet (Fig. 8a). We found no difference between dietary treatments on acetylation level of H4 at lysine residues 5, 8, 12, and 16, or in other modifications such as mono- and tri-methylation of lysine 20 (Fig. 8c,d).

Discussion

In this work we examined the effects of SBM in diet on *S. aurata* larvae at first feeding during two different developmental windows. Early SBM feeding of *S. aurata* larvae significantly affects the activity of most pancreatic enzymes in a time-of-exposure dependent

**Fig. 6** Plots of the two axes from the forward stepwise discriminant function analysis of gene expression in gilthead seabream larvae. Larvae were fed, under a co-feeding regimen with live feed, from first feeding up to 14 dph with a FM-based diet or a SBM-based diet, or up to 18 dph with the same diets. Later, all larvae**Fig. 7** Effect of feeding gilthead seabream with fish meal (FM)- and soybean meal (SBM)-microdiet (black and gray columns, respectively) from mouth opening to 18 dph or 14 dph (equivalent to 14 and 10 d of feeding, respectively) under a co-feeding regimen with live feed, on chromatin modifications. Global DNA methylation (A, B), histone H3 (C, D) and H4 (E, F) acetylation of entire larvae. After the initial feeding period (up to 18 or 14 dph), all fish were fed with the FM- based diet and live feed up to 36 dph. Values are means + SEM. * $P < 0.05$

form. More than 10 d of SBM feeding (i.e., beyond 14 dph) delayed the normal development of *S. aurata* larvae digestive capacities as the activities of all trypsin, chymotrypsin, and amylase were significantly reduced. This is opposed to the typical response of juvenile and adult fish to SBM. Protease inhibitors present in SBM can partially abrogate the activity of trypsin and chymotrypsin in the proximal intestine (Robaina et al. 1995),

were fed with the FM-diet and live feed up to 36 dph. These older larvae were referred as FM 36 dph when fed on the FM-diet, SBM 14–36 dph if fed with the SBM-diet up to 14 dph and then with the FM-diet, and SBM 18–36 dph if fed with the SBM-diet up to 18 dph and then with the FM-diet

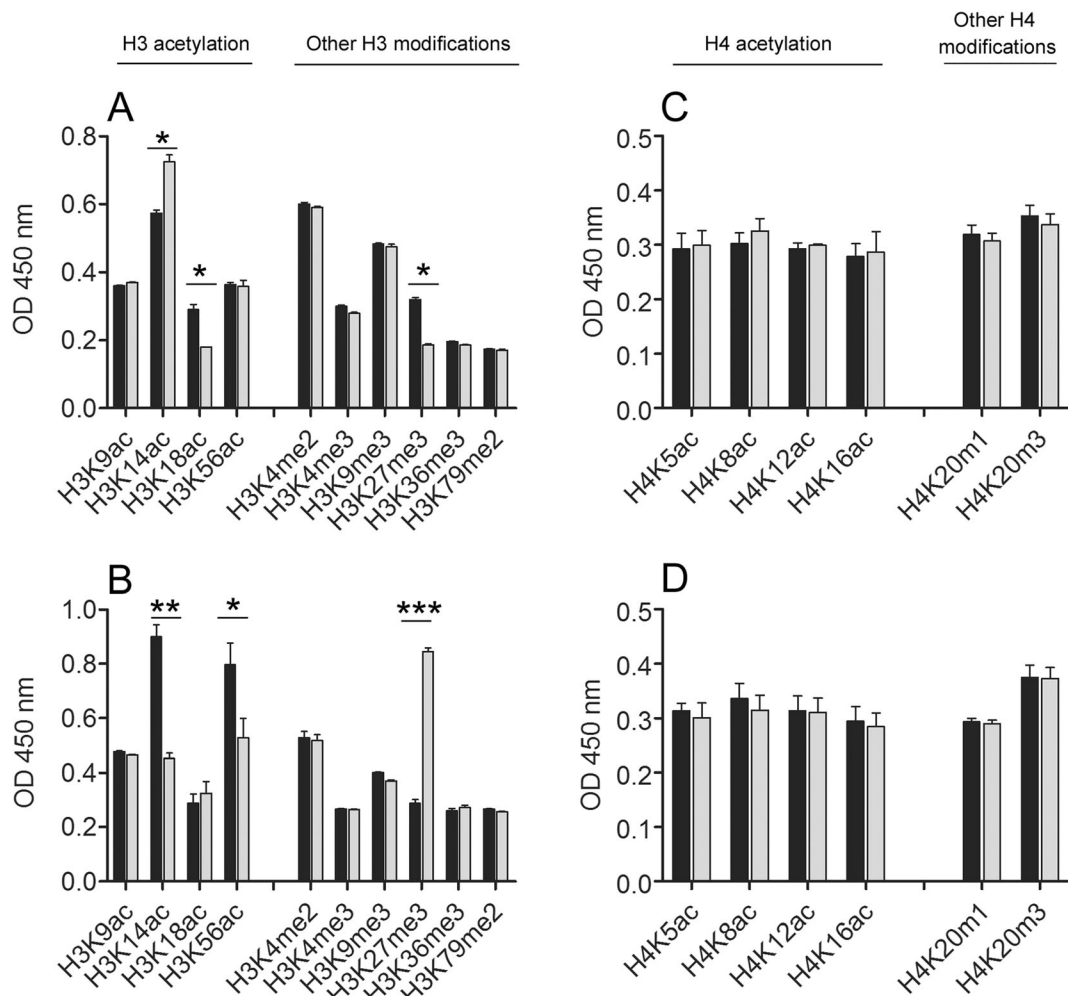


Fig. 8 Effect of feeding gilthead seabream with fish meal (FM)- and soybean meal (SBM)-microdiet (black and gray columns, respectively) from mouth opening to 14 dph (equivalent to 10 d of feeding) under a co-feeding regimen with live feed, on global histone H3 modifications of entire larvae at 14 dph (A) and 36 dph

(B), and on global histone H4 modifications of entire larvae at 14 dph (C) and 36 dph (D). After the initial feeding period (up to 14 dph), all fish were fed with the FM-based and live feed diet up to 36 dph. Values are means + SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

and juveniles of *S. aurata* (Santigosa et al. 2010) and other fish such as Atlantic salmon (Lilleeng et al. 2007; Chikwati et al. 2013b) exhibit a rapid compensatory increase in activities of these enzymes. The premature stage of pancreas development in early *S. aurata* larvae would lie behind their failure to respond to SBM with trypsin synthesis. Strengthening this hypothesis is the fact that we did not observe changes in trypsin expression in *S. aurata* larvae in response to SBM. This is also in line with the thought that regulatory mechanisms for the production of digestive enzymes in early fish larvae are not efficient (Zambonino-Infante and Cahu 2001). However, SBM-induced increase in trypsin activity in

juvenile fish is more marked in the distal intestine and have been attributed to a reduced ability to reabsorb the pancreatic enzymes (Krogdahl et al. 2003) and the up-regulation of trypsin-like activity by immune cells (Lilleeng et al. 2007). Therefore, other plausible explanation for our observations is that these intestinal processes are not fully functional in early larvae.

Conversely, lipase activity was relatively insensitive to SBM in *S. aurata* larvae, as reported before in post-smolt Atlantic salmon (Chikwati et al. 2013b). The observed decrease in pancreatic proteases may be responsible for less lipase inactivation in the digestive tract explaining the stable lipase activity. Other, more

likely explanation, is that regulation of lipase activity in *S. aurata* larvae and other pancreatic enzymes proceeds by distinct mechanisms. A recent study in *S. aurata* larvae supports this hypothesis (Mata-Sotres et al. 2016). The later removal of SBM from diet of *S. aurata* larvae that fed SBM during 14 d lead larvae to resume the activity of most pancreatic enzymes but produced a significant reduction in size of older larvae suggesting that some physiological processes remain altered.

After 10 d of SBM feeding, *S. aurata* larvae did not modify LAP and AP activities. Conversely, a longer SBM feeding period resulted in decreased of both LAP and AP activities. Our observations on LAP activity in hole larvae resembles the decreased in LAP activity the distal intestine of salmon fed SBM (Chikwati et al. 2013b). Observations in salmonids lead to the suggestion that SBM-induced decrease in LAP activity results from increased sloughing of intestinal epithelia cells (Chikwati et al. 2013a) and epithelial proliferation with immature cell population expressing lower levels of brush border enzymes (Bakke-McKellep et al. 2007). This may be also a likely explanation for our result because LAP activity was recovered ones SBM was removed from diet. However, our analysis did not differentiate between intestinal LAP and other LAPs enzymes ubiquitously distributed so results must be taken with caution. The same is true for AP, an enzyme activity distributed all over the larvae though enriched at the intestine. IAP is involved in different pivotal process such as negative regulator of lipid absorption, pH regulation, lipopolysaccharide detoxification/tolerance to commensal microbiota, inflammation attenuation and intestine homeostasis (Lallès 2010) and its activity is known to decrease in fish feeding soybean anti-nutrients (Couto et al. 2015). Interestingly, after a restricted (10 d) exposure to SBM older larvae had more LAP activity in the absence of SBM, while such long-term effect was not observed for AP.

After 10 d of SBM feeding, *S. aurata* larvae increased MMPs gene expression but this did not result in higher activity. A longer SBM feeding period increased both gene expression and activity of MMPs in *S. aurata* larvae. Increased MMP activities (e.g., *mmp9* and *mmp13*) have been well documented during inflammation (Pedersen et al. 2015) and tissue injury (Castillo-Briceño et al. 2009) in fish, but not studied before in *S. aurata* in response to SBM. As in other vertebrates, MMPs are induced in fish by the pro-inflammatory

cytokines IL-1 β and TNF- α (Johnson et al. 2004) and in accordance, we observed increased *tnfa* expression due to SBM feeding of *S. aurata* larvae. However, *tnfa* up-regulation was not sustained after 10 d of feeding, whereas a synchronized behavior for *mmp9* and *illb* expression was observed irrespective of the SBM time of exposure. These observations were not surprising, as *tnfa* from *S. aurata* is relatively irresponsive to some stimuli known to up-regulate *illb* (García-Castillo et al. 2002; Castillo-Briceño et al. 2009). Other studies in fish (Chadzinska et al. 2008) and other vertebrates (Schneck et al. 1998) also pointed to a major functional interplay between fish MMP9 and IL-1 β during both the initiation and the resolution of the inflammatory response. Larvae receiving SBM at first feeding appear later to have, in the absence of SBM, reduced *illb*, *mmp9*, and *l3* expressions, irrespective of the severity of the SBM short-term effects. The exception was *ill10*, whose up-regulation was only observed in old larvae receiving SBM for the shortest period. It is not known if this is a result of different time-course responses of the different cytokines (Pérez-Cordón et al. 2014) or a true lasting effect of early nutrition.

The expression of *PepT1*, mostly in the proximal intestine, is known to be related with di- and tripeptide transport efficiency and growth in fish (Romano et al. 2014). In *S. aurata* juveniles, expression of *PepT1* was shown to be affected by some vegetal proteins and not by others (Terova et al. 2013). Here we showed that *PepT1* expression in *S. aurata* larvae is not affected by SBM at the short-term, but that SBM feeding for either 10 d or 14 d lead to older larvae with slightly higher mean values of *PepT1* expression, although not statistically different to those in larvae fed the FM-diet. However, this observation is noteworthy as we showed that the increased *PepT1* expression of juvenile zebrafish in response to SBM was higher if they shortly faced SBM at first feeding (Perera and Yúfera 2016). On the other hand, we did not observe a significant impact of either 10 or 14 d of SBM feeding on the expression of *fapp2* and *fabp6*. Conversely, *fabp2*, involved in fatty acid transport, decreased in Atlantic salmon at the transcript and protein levels during SBM-induced distal intestinal inflammation (Venold et al. 2013) as a result of a down regulation of regulatory proteins, rapid enterocyte turnover, and reduced lipid digestibility (Krogdahl et al. 2003). Also, *fabp6*, which is thought to be needed for the efficient intracellular transport of bile acids in enterocytes (Gong et al.

1994) did not vary its expression due to SBM or resulted altered later in larval development. Conversely, we found in zebrafish juveniles a decreased expression of *fabp6* after SBM feeding of juveniles, whether or not they were provided with SBM at first feeding (Perera and Yúfera 2016).

Multivariate analysis performed confirmed that the duration of the SBM feeding period drives some features of the physiology of larvae in different directions, mostly evidenced by some genes related with inflammation, tissue remodeling, and nutrient transport. Further studies are necessary to determine if these effects persist in more advanced developmental stages (i.e., true nutritional programming).

We found that SBM feeding of *S. aurata* larvae globally increase H3K14ac, which is a well known activating mark of transcriptional initiation and enhancers expression (Karmodiya et al. 2012), elongation (Johnsson et al. 2009), and DNA repair (Duan and Smerdon 2014), although it can also mark inactive promoters to a lesser extent (Karmodiya et al. 2012). Concurrently, we observed that SBM decreased globally the repressive mark H3K27m3. H3K27me3 is known to occur along with histone activating marks such as H3K14ac at bivalent promoters (Karmodiya et al. 2012) and its decrease may also indicate the up-regulation of regulatory genes. Our results suggest that these chromatin modifications may be related with the transcriptional activation of SBM-affected genes during feeding. After SBM removal from diet, the level of the activating marks H3K14ac and H3K56ac decreased, restoring the global acetylation level. This behavior was expected as histone acetylation is a dynamic process and changes are reverted upon removal of activators or repressors (Katan-Khaykovich and Struhl 2002). Results suggest that after SBM removal from diet, larvae undergo overall gene down-regulation, which is also supported by the later increase of the repressive mark H3K27m3. It is interesting that H3K27m3, which is known to be up-regulated and persistent at advanced stages of inflammation, has been proposed to render affected genes such as cytokines less responsive to further inflammatory stimulation (i.e., reprogramming effect) (Angrisano et al. 2010). We also observed H3K56ac to decrease after SBM removal from diet. This mark is paramount for genome stability and its down-regulation promote DNA repair (Miller et al. 2010).

On the other hand, we demonstrated that early SBM feeding of *S. aurata* larvae resulted in lasting global hypomethylation. Global methylation data are difficult to read as transcriptional activation has been associated with both hypomethylation (Reik and Dean 2001) and hypermethylation (Gavery and Roberts 2013). Hypomethylation has been associated with the re-programming of developmentally silenced genes during tissue regeneration (Thummel et al. 2006) and DNA repair (Reik 2007).

Nutrition is known to affects epigenetic phenomena by nutrients acting as a source of methyl groups or as co-enzymes for one-carbon metabolism (Jaenisch and Bird 2003), or by affecting the activity of histone modifier enzymes (Feil and Fraga 2012). The mechanisms by which SBM exerts the observed epigenetic effects on fish larvae are unknown. It may be related with some SBM component known to act as a histone deacetylase inhibitors such as genistein and other isoflavones (Link et al. 2010), or through the action of microbiota metabolites with histone deacetylase inhibitory properties such as butyrate (Chang et al. 2014), or with the capacity to increase the histone acetylation substrate acetyl-coA, such as acetate (Kendrick et al. 2010). Butyrate and acetate are known to modulate the expression of cytokine genes (Karmodiya et al. 2012; Fusunyan et al. 1999) and intestinal alkaline phosphatase (Hinnebusch et al. 2003) through histone acetylation. Genistein and other isoflavones may also be responsible for the DNA demethylation observed in this study through DNA methyl transferases inhibition (Link et al. 2010). These mechanisms deserved further studies in fish larvae.

Feeding trials with larvae are challenging, largely because of the limited ability to assess feed intake. Accordingly, there are some confounding factors in our experiments associated with possible differences in feed intake of microdiets (e.g. due to less palatability of the SBM-diet). These may lead to uncertainty on the role of SBM as origin of the differences detected. Considering these limitations, we ensure microdiet ingestion by undersupply live food and by providing microdiets at times of high ingestion rate in *S. aurata* larvae (Mata-Sotres et al. 2015). Ingestion of microdiets was confirmed in all cases by the observation of larvae under a microscope. This feeding regime did not affect per se larvae growth as compared to a traditional regime with only live feed. The size of larvae at 15 dph observed this work (4.5–5 mm) is similar to that observed in other studies for larvae of the same age (5–5.5 mm, Polo et al.

1992; 4.8–5 mm, Izquierdo et al. 2013). The same is true at 36 dph (7.5–8 mm, this work; 7–7.5 mm, Izquierdo et al. 2013) for larvae fed with the FM- or SBM-microdiets for the first 10 days. In addition, different results from SBM fed *S. aurata* larvae resemble those reported for juvenile fish of different species after SBM feeding as discussed above, confirming that larvae ingested enough microdiets for dietary treatments to exert the observed effects. It is also pertinent to remark that due to their small size, dissection of individual tissues/organs is usually impractical in fish larval studies. Thus, further studies are needed for sorting the observed results to different *S. aurata* organs, which will assist in advancing the knowledge on particular physiological processes affected by SBM inclusion in early diet.

In conclusion, the present study shows that *i*) there are limitations for expanding SBM feeding of *S. aurata* larvae beyond 10 d from mouth opening (i.e., 14 dph), affecting some key physiological processes and ultimately normal growth, and *ii*) it is worthy to explore the possibility to manage the future responses of *S. aurata* to SBM through early nutritional manipulation, even within a short developmental window of 10 d, given the absence of evident effects on normal development and growth, and the occurrence of apparently lasting effects on the activity of some key enzymes, genes, and chromatin modifications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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