Effects of different dietary vegetable oils on growth and intestinal performance, lipid metabolism and flesh quality in gilthead sea bream.

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Abstract (400 words)

This study tests the effects of feeding different vegetable oils (VO) in gilthead sea bream with the aim of improving sustainable aquafeeds. Juveniles were fed *ad libitum* with ten isonitrogenous (46%) and isolipidic (22%) diets with a 75% fish oil (FO) replacement, differing in VO composition leading to two experimental blocks: fish fed with VO blends or mono-substituted diets. Growth parameters, skin and muscle colour, muscle texture, plasma metabolites, digestive capacities, and transcript levels of intestinal lipid transport, muscle dynamics and lipid metabolism-related genes in white muscle and adipose tissue were studied. In fish fed high-palm oil diets, final body weight and mesenteric fat significantly increased, while effects were not found in hepatosomatic index, reflecting tissue-specific lipid accumulation. Relative intestinal length increased significantly with dietary soya oil (SO) content, suggesting a compensatory mechanism to improve nutrient absorption capacity. Plasma parameters showed few changes upon dietary treatments. Lipase activity was unaltered, while intestinal *fatp1b* expression increased in animals fed blended diets high in rapeseed oil (RO). In adipose tissue, expression of nuclear receptors *pparβ* and *lxr* was modified by dietary fatty acids (FA) profile; however, regarding lipid metabolism and *β*-oxidation genes, only *lpl* showed significant differences, suggesting that FA uptake and oxidation, but not *de novo* lipogenesis is what appears to determine the increase in adipose tissue mass. In fish fed blended VO diets, *lpl* expression showed a positive correlation with MUFA dietary content, suggesting that some FA present in RO enhance its expression, according to data from fish fed mono-substituted diets. In muscle, fish fed blended VO diets also showed a positive correlation of *lpl* expression with dietary MUFA, whereas in mono-substituted, it was significantly higher in fish fed SO, suggesting other mechanisms are involved in LPL regulation. Concerning *β*-oxidation genes in muscle, significant differences were detected in *cpt1a* expression for fish fed blended VO diets, whereas *hadh* and *cpt1b* were unaltered, suggesting slight FA uptake regulation in mitochondria. Expression levels of genes related to myogenic processes were not greatly modified by dietary lipid sources except for *myogenin* in blended VO diets-fed fish, showing a similar profile as that in body weight and opposite with the differentiation marker *mhc*. This study provides new information regarding the effects of dietary VO, demonstrating moderate effects in lipid homeostasis without adverse effects on growth performance, leading to a transversal view of the responses and interactions from intestine to muscle growth and flesh quality.

1. Introduction

Global aquaculture production reached 80 MT in 2016, which required the use of 15 MT of fishmeal (FM) and fish oil (FO) for animal nutrition (FAO, 2018). However, resources are limited, and overfishing makes necessary to explore alternative nutrient sources to satisfy fish feed requirements while maintaining constant supply and competitive prices (Naylor *et al.*, 2000; Tacon and Metian, 2009). FM and FO are characterized by their optimum nutritional profile, closely to feed requirements of the majority of marine carnivorous species. These are the most appreciated to consume in the First World (FAO, 2018), due to their high valuable fatty acid content, rich in eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids, and other nutritional properties. These long chain polyunsaturated fatty acids (LC-PUFA) are involved in several metabolic pathways, providing fluidity to cell membranes (Pinot *et al.*, 2014), retina and brain development (Innis, 2008; Campoy *et al.*, 2012), transcriptional regulation of lipid metabolism-related genes (Deckelbaum *et al.*, 2006; Houston *et al.*, 2017), and are precursors for broad bioactive molecules (e.g. eicosanoids, resolvins, protectins, etc.) (Serhan *et al.*, 2008). Nonetheless, marine fishes have a reduced or inexistent ability to synthetize *de novo* such LC-PUFA from their precursor, α-linolenic acid (ALA; 18:3n-3) (Seiliez *et al.*, 2003; Morais *et al.*, 2009; Betancor *et al.*, 2014). Therefore, n-3 LC-PUFA diet requirement is significantly higher in these species and is considered a limiting factor for aquafeeds formulation (Tocher & Ghioni, 1999; Sargent et al., 1995, 2002; Mourente et al., 2005). A lack or an imbalance of EPA and DHA may produce several negative alterations (Simopoulos, 2000; Caballero *et al.*, 2003; Montero *et al.*, 2003; Tocher, 2003), being a ratio of 2:1 the optimal for gilthead sea bream (*Sparus aurata*) (Ibeas *et al.*, 1997). FO has been used as the main source of energy and essential fatty acids (EFA) in fish nutrition, but nowadays vegetable oils (VO) are displacing its use (FAO, 2018). There is large amount of bibliography about successful partial FO substitution without compromising growth, survival, fish feed utilization or organoleptic profile (Izquierdo *et al.*, 2000, 2003; Glencross *et al.*, 2003; Benedito-Palos *et al.*, 2008; Pérez *et al.*, 2014; Betancor *et al.*, 2016). However, it has been demonstrated that highly substituted VO diets affect negatively to digestion and absorption processes (Santigosa *et al.*, 2011), and quantitative and qualitative the fatty acid profile. Other effects are alteration of cell size in lipid storage tissues like skeletal muscle, liver and perivisceral adipose tissue (Jobling *et al.*, 1991; Torstensen *et al*, 2000; Bell *et al.*, 2001; Caballero *et al.*, 2002; Montero *et*

97 *al*., 2003; Menoyo *et al*., 2004; Cruz-García *et al*., 2011), and muscle fiber proliferation
98 and hypertrophic growth (Lopes *et al*., 2017). VO, in contrast to FO, do not have n-3 LC-
99 PUFA, and usually have a higher amount of 18C n-6 PUFA, especially linoleic acid (LA;
100 18:2n-6), and monounsaturated fatty acids (MUFA) as oleic acid (OA, 18:1n-9). The
101 inclusion of those n-6 rich VO may produce an imbalance in the n-3/n-6 ratio in the diets,
102 from 9-6:1 in FO to 0.3-1:1 in VO (Guillou *et al*., 1995; Robaina *et al*., 1998; Bransden
103 *et al*., 2003). A reduced n-3/n-6 ratio causes a disproportion in the different eicosanoids,
104 which are potent signalling anti-inflammatory molecules when are derived from n-3
105 PUFA and pro-inflammatory when are derived from n-6 PUFA (Simopoulos, 2016;
106 Adam *et al*. 2017). Kalogeropoulos *et al*. (1992) demonstrated in gilthead sea bream that
107 increasing inclusion of soybean oil (SO), rich in LA and OA, increases fat deposition in
108 liver reducing EPA and DHA content, being growth also negatively affected.
109 Nevertheless, differences on growth were not found when the substitution level was
110 below 60% in agreement with Izquierdo *et al*. (2003), who found similar results also for
111 rapeseed oil (RO), rich in OA, and linseed oil (LO), rich in ALA. Palm oil (PO), rich in
112 saturated fatty acids (SFA) as palmitic acid (PA, 16:0) and OA, is the most VO produced
113 worldwide; however, its use is not as widespread in terms of fish nutrition and
114 investigation (Glencross, 2009), and there is no consensus on its effects among species.
115 Increasing inclusion of PO has shown a reduction in specific growth rate (SGR) at least
116 in fish fed above 60% substitution (Komilus *et al*., 2008; Fountoulaki *et al*., 2009; Huang
117 *et al*., 2016); however, some other authors did not find differences or rather a slight
118 increase (Bell *et al*., 2002; Olurin *et al*., 2004; Ayisi *et al*., 2017). It is therefore generally
119 considered that diets with VO added at high inclusion levels, require the blending of
120 different VO to avoid or reduce deleterious effects and to fulfil the EFA requirements of
121 fish. There are several VO that are used industrially; nonetheless, VO composition and
122 EFA fish requirements are very diverse and for that, further studies are still necessary to
123 optimize aquafeeds formulation (Buddington *et al*, 1997; Glencross, 2009; Tocher, 2015).
124 The aim of the present research was to study in gilthead sea bream the effects of the most
125 common commercial VO, included in diets with 75% FO substitution. Diets were mono-
126 substituted or contained a blend of VO, thus modifying the n-3/n-6 and the UFA/SFA
127 ratios, by changing the n-6 profile depending on the VO added. The questions raised were
128 whether higher n-3/n-6 or UFA/SFA ratios could improve fish growth performance and
129 metabolic status, and how other physiological traits are modified. Growth parameters,
130 skin and muscle colour, muscle texture, plasma metabolites and digestive capacities, as

well as, quantitative expression of genes involved in muscle growth, intestinal lipid transport and lipid metabolism in adipose tissue and white muscle were evaluated.

2. Materials and methods

2.1. Experimental diets

Ten isonitrogenous (46%) and isolipidic (22%) diets were formulated and produced by Skretting ARC (Norway). The complete formulation is presented in Table 1. In all diets, 81.2% of FM was substituted by plant protein sources (wheat, wheat and corn gluten, sunflower meal, soya concentrate and fava beans). Moreover, 75% of FO was replaced by a single, or a blend of VO, either 3 or 4 among PO, RO, SO and LO (Table 1, and schematically represented in Fig 1). Diets with different VO blends were named from A to G. To identify the effects of the VO separately, three mono-substituted diets containing SO, RO or PO, and named H to J, respectively, were also tested. Letters (A-J) were used to keep fish maintenance personnel blind concerning diets specifics. Dietary fatty acid composition changed according to the VO content in the formulation (Table 2). The inclusion of a 4.64% of FO in the diets formulation, in addition to what it is contained in the FM, adequately provided the minimum requirements of EPA and DHA (Glencross, 2009). Furthermore, the composition of n-3 fatty acids was adjusted to be the same in all blended VO diets through the inclusion of different proportions of LO. Notwithstanding, it has to be taken into account that besides LO contains 44% of n-3 series, mainly 18:3n-3 (ALA), PO is rich in SFA and MUFA, mainly PA and OA, respectively, RO has 50% of MUFA, whereas SO is rich in n-6 series (37.3%), 98% of which are LA. Thus, although all diets had the same amount of FM and FO, and the LO content was adjusted to equilibrate the n-3 fraction, the n-3/n-6 ratio differed among diets (from 0.54 to 1.35 in blended VO diets). Concerning the non-essential as well as the EFA, the major differences among diets were in the non-essential ones, although variability in the content of the EFA C18:2n-6 (LA) is also high. Besides, depending on the mixed VO used different UFA/SFA ratios (from 3.07 to 5.46) were obtained.

2.2. Fish, feeding trial and sampling procedures

Juvenile gilthead sea bream from the fish farm “Piscimar” (Burriana, Alicante) were fattened with a commercial diet during 65 days at the Institut de Recerca i Tecnologia Agroalimentàries (IRTA, Sant Carles de la Ràpita, Spain) facilities. After that period, five

hundred ninety-four fish (81.79 ± 0.26 g) were randomly distributed in a semi-recirculating saltwater system of 27 fiberglass tanks (500 L; 22 fish per tank) and acclimatized for 11 days. During the 18-week trial (October-February), fish in triplicate tanks for diets A to G and duplicate ones for H to J, were fed *ad libitum* the corresponding diet twice a day (at 8 a.m. and 14 p.m.). Surplus was removed 2 h after feeding. The temperature was maintained at 21.9 ± 0.85 ºC and photoperiod followed natural changes (11:24 to 10:29 h of daylight), according to the course of the trial.

At the end of the growth trial 9 animals per treatment from A to G groups and 6 animals per treatment from H to J (3 fish per tank) were anaesthetized (MS-222, Sigma, Madrid), measured, weighed and sacrificed by severing the spinal cord 24 h post-feeding. Blood was taken from caudal vessels with EDTA-Li syringes and centrifuged (5000 rpm, 10 min, 4 ºC) to separate the plasma, which was stored at -80 ºC until analyses. Skin and muscle colour were measured. Samples of proximal intestine, including the intestinal content, were collected and rapidly frozen in liquid nitrogen and maintained at -80 ºC until the enzymatic studies were performed. Pieces of white muscle, proximal intestine and perivisceral adipose tissue were snap-frozen in liquid nitrogen and stored at -80 ºC until gene expression analyses. A piece of 2 x 2 cm of white muscle from the dorsal area was also taken from each fish, bagged and kept on Styrofoam boxes under ice for texture evaluation. Additionally, 10 fish per tank were weighed, sacrificed and eviscerated to calculate different somatic parameters.

All procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona following the European Union, Spanish and Catalan Governments-assigned principles and legislation (permit number DAAM 8982).

2.3. Growth performance, feed utilization and somatic indexes

All fish were individually weighed before (IBW, initial body weight) and after the trial (FBW, final body weight) and then measured (BL, body length). Total biomass from all triplicate/duplicate tanks (n=3/2) was obtained to evaluate the weight gain (WG) during the experimental period and to calculate SGR, condition factor (CF) and feed conversion ratio (FCR). The WG was defined as $((FBW-IBW) / IBW) \times 100$. The SGR was calculated as follows: $((lnFBW-lnIBW) / t) \times 100$, where t is the number of feeding days. The CF as $(FBW / BL^3) \times 100$ and the FCR as $g\ total\ feed\ intake / (FBW-IBW)$. Total feed intake (TFI) was calculated as: *food intake (Kg)-food lost (Kg)*. In addition, hepatosomatic (HSI) and mesenteric fat (MFI) index, and relative intestinal length (RIL), were calculated from

n = 9 (A to G) and n = 6 (H to J) fish (three fish per tank), as follows: *HSI = (liver weight / FBW) × 100*; *MFI = (mesenteric fat weight / FBW) × 100;* and *RIL*, which was measured excluding pyloric caeca and expressed in relation to BW in *mm / g fish* (Santigosa et al., 2008).

2.4. Biochemical analysis of plasma parameters

All plasma parameters were analysed in triplicate, n = 9 (A to G) and n = 6 (H to J), with commercial kits following manufacturers' recommendations. Plasma glucose levels were determined by an enzymatic glucose oxidase/peroxidase colorimetric method (Monlab, Barcelona, Spain). Plasma non-esterified fatty acids (NEFA) using an enzymatic colorimetric method assay for quantitative determination (NEFA-HR2, Wako Chemicals GmbH, Neuss, Germany). Plasma triacylglycerols (TAG) were cleaved by an LPL, releasing free fatty acids and one molecule of glycerol that was measured by consecutive oxidating-reducing reactions by colorimetric analysis (Spinreact, Sant Esteve d'en Bas, Spain).

2.5. Muscle and skin colour

Muscle and skin colour were evaluated, n = 9 (A to G) and n = 6 (H to J), just after sacrifice using a portable CR400 Chroma Meter (Konica Minolta, Madrid, Spain) that was calibrated using the white standard provided. Skin measurements were taken along the lateral line at the level of the fore insertion of the dorsal fin, and muscle measurements were taken in the same area after fish were skinned. Colour features were given in Commission Internationale de l'Eclariage (1976): L*, a* and b*, where L* represents lightness (L* = 0 for black, L* = 100 for white), a* indicates red/green (+a* intensity in red and –a* intensity in green) and b* represents yellow/blue (+b* intensity in yellow and –b* intensity in blue).

2.6. Muscle texture measurement

Texture analysis (n=3) was performed at the Departament d'Enginyeria Agroalimentària i Biotecnologia of the Universitat Politècnica de Catalunya (ESAB, Castelldefels, Spain) using a TA.XT2i texture analyser coupled to a Mini Kramer/Ottawa cell blade. A compression test using a load cell of 30 Kg with a test speed and post-speed of 1 mm s^{-1} was used. The blade was at 90º angle and perpendicular to muscle fibres. Total work and maximal strength were measured in all samples 24 h after sampling to reduce the *rigor*

mortis effects. The size of the muscles' pieces (2 x 2 cm) was verified immediately prior to analysis and adjusted when necessary to ensure all the samples had the same area, and the depth was evaluated with a Vernier caliper for subsequent normalisations. Total work is the parameter used to define the force required to cut the sample completely. Maximal strength is defined as the maximal force applied during a complete texture analysis. Data was evaluated with the Exponent 4.0.9 software (Stable Micro Systems) and values were normalized to a depth of 1 cm.

2.7. Intestinal pH content and digestive enzyme analyses

Intestinal pH content of the samples, n = 9 (A to G) and n = 6 (H to J), was measured using a pH meter (Crison, Micro pH 2000). Intestinal sample homogenization and total alkaline protease activity (TPA) were evaluated according to Santigosa *et al*. (2008) with little modifications. Lipase determination was carried out following Santigosa *et al*. (2011a). All analyses were done in triplicate. Briefly, intestinal samples were individually homogenized (Precellys Evolution, Bertin Instruments, Barcelona, Spain) at 4 ºC in 50 mM TrisHCl buffer pH 7.5 at a final concentration of 250 mg/mL. Homogenates were centrifuged (1100 g, 15 min, 4 ºC, Jouan CR411) and supernatants were recovered and stored at -80 ºC. For TPA determination, samples were reacted with 50 mM TrisHCl buffer at the pH of the intestinal content containing 1% casein at 20 ºC. After 30 min, the reaction was stopped by adding 12 % trichloroacetic acid. The samples were maintained at 4 ºC for 1 h and then centrifuged (7500 g, 5 min, 4 ºC). Individual blanks were established for each sample. Supernatant absorbance was measured at 280 nm (Tecan M200, Tecan Trading AG, Switzerland). Bovine trypsin (Sigma Aldrich, Spain, 12100 BAEE units/mg protein, NC-IUB, 1979) was used as a standard. For the lipase determination, the sample was mixed with buffer containing (in mM) 20.5 Tris, 3.6 taurodeoxycholate, 0.9 deoxycholate, 0.8 tartrate, 0.12 DGGR (1,2-*o*-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester), 0.05 $CaCl_2$, 30 mannitol and 1 mg/L colipase (pH 8.3). The increase in absorbance was recorded at 580 nm in the linear zone. Lipase (Sigma Aldrich, Spain, 33944 U/mg protein, 22980 U/mg solid) was used as a standard. One unit hydrolyses 1.0 micro equivalent of fatty acid from triacetin in 1 h at pH 7.4 and 20 ºC (Santigosa *et al*., 2011a). Protein concentration in the homogenates was determined by the Bradford method using bovine serum albumin as a standard.

2.8. RNA extraction and cDNA synthesis

Total RNA extraction was performed from 30 mg proximal intestine or 100 mg of tissue in the case of white muscle and perivisceral adipose tissue, in 1 mL TRIzol® reagent solution (Applied Biosystems, Alcobendas, Spain) following the manufacturer's instructions. Nanodrop 2000 (Thermo Scientific, Alcobendas, Spain) was used to determine RNA concentration and purity. RNA integrity check was performed with 1% agarose gel stained with SYBR-Safe DNA gel stain (Life Technologies, Alcobendas, Spain). RNA samples were then treated with DNase I (Invitrogen, Alcobendas, Spain) following the manufacturer's recommendations to eliminate any residual genomic DNA before cDNA synthesis. Finally, reverse transcription was carried out using the Transcriptor First Strand cDNA synthesis kit (Roche, Sant Cugat, Spain) following the manufacturer's instructions, using anchored-oligo(dT)15 and random hexamer primers.

2.9. Real-time quantitative-PCR (qPCR)

Gene expression (mRNA) analyses were performed by qPCR in a CFX384 real-time system (Bio-Rad, El Prat de Llobregat, Spain), according to the requirements of the MIQE guidelines (Bustin *et al*., 2009). The genes analysed include transcription factors, myogenic regulatory factors (MRFs) and growth-, lipid metabolism-, and intestinal integrity-related genes (for more details see table 3). All pairs of primers used to determine the expression of each gene of interest and the four reference genes had been previously validated for the different gilthead sea bream tissues (Salmerón *et al*., 2013; Pérez-Sánchez *et al*., 2015, Lutfi *et al*., 2018; Balbuena-Pecino *et al*., 2019). For relative expression calculations different reference genes were used depending on the tissue (*ef1α*, *β-actin* and *rps18* for proximal intestine; *ef1α*, *β-actin* and *rpl27a* for adipose tissue and *rpl27a* and *rps18* for white muscle), according to their stability, which was confirmed with the geNorm algorithm implemented in the Bio-Rad CFX Manager 3.1 software. The analyses were performed in triplicate using 2.5 µL of iTaq Universal SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 200 nM of forward and reverse primers (Table 3) and 1 µL of diluted cDNA for each sample in a final volume of 5 µL. The reactions consisted of an initial denaturation step of 3 min at 95 ºC, 40 cycles of 10 s at 95 ºC, 30 s at 56-68 ºC (primer dependent, see Table 3), followed by an amplicon dissociation analysis from 55 to 95 ºC at 0.5 ºC increase each 30 s. Prior to the analyses, a dilution curve with a pool of samples was run to determine the appropriate cDNA dilution for each gene, as well as confirm the specificity of the reaction, and the absence of primer-dimers. The Bio-Rad CFX Manager 3.1 software was used to calculate the

300 expression levels of each gene by the Pfaffl method (Pfaffl, 2001) relative to the
301 geometric mean expression of the most stable reference genes previously mentioned for
302 each tissue.
303
304 **2.10. Statistical analyses**
305 Fish groups were divided in two experimental blocks and analysed separately: those fed
306 with VO blend diets (A, B, C, D, E, F and G) and those fed with mono-substituted diets
307 (H, I and J) in order to better understand the effect of the different VO combinations
308 independently of the possible negative effects of mono-substituted diets. Diets containing
309 only one type of VO are not usually commercially administered but were useful to test
310 the effect of the presence of individual VO. Tank was used as a biological replicate for
311 growth parameters and dietary group average for correlations. Fish was used as a
312 biological replicate for plasma, color, texture, digestive parameters and relative gene
313 expression and tank effect was checked on each parameter, being added as a random
314 factor when significant nested one-way analysis of variance (nested ANOVA). Data were
315 tested for normality by Shapiro-Wilk test and homogenity of variances by Levene's test.
316 In order to detect statistically significant differences experimental values were compared
317 using an ANOVA, and differences among means were tested for significance using a
318 *post-hoc* Tuckey's multiple range test. For data that did not accomplish normality, the
319 Kruskal-wallis non-parametric test was used followed by Mann-Whitney U test to
320 determine differences among diets. Pearson correlation coefficient was used to determine
321 if correlation existed among dietary fatty acid profile with the different studied
322 parameters. Data are shown as mean ± error of the mean (SEM) and the significance level
323 was set by default at 0.05, being less than 0.01 in some cases that permitted it. The
324 software used for statistical analysis was SPSS (IBM-SPSS Statistics v.25.0, SPSS Inc.,
325 Chicago, IL, USA) and the one used for graphic representation was GraphPad (GraphPad
326 Software Inc).
327
328 # 3. Results
329 ## 3.1. Effects of diets on growth performance and feed utilization
330 Biometric parameters were measured at the end of the feeding experimental trial after 18
331 weeks and values are presented in Table 4. Fish fed with the blended VO diets were

comparable in terms of feed intake (TFI or FCR). Significant differences were not found for FBW, WG, SGR, BL and CF, although fish fed diet C, which includes PO in its formulation, had always the highest values in most of these parameters. Despite not having found significant differences between groups, a positive Pearson's correlation for FBW and n-3/n-6 fatty acid ratio in diet (r= 0.787; $P< 0.05$) and between BL and total SFA (r= 0.825; $P< 0.01$) and with n3/n6 ratio (r= 0.769; $P< 0.05$) was observed. Similarly, in fish fed with mono-substituted VO diets, FBW and WG were significantly higher in J group (PO), compared with I group (RO).

Regarding somatic indexes (Table 4), HSI did not show differences neither among fish fed with mono-substituted nor blended VO diets. Nevertheless, in fish fed blended VO, MFI showed significant differences among dietary treatments and a positive Pearson's correlation with the total amount of SFA contained in feed (r= 0.818; $P< 0.05$). Thus, pointing to a higher accumulation of visceral fat in fish fed with diets containing high levels of PO (C) versus those fed with diets without (A, E, and F) or low levels (G) of that specific oil; while the blend of PO and SO tended to diminish this index (D). In a similar way, in mono-substituted diets' groups, the highest value for MFI was observed in fish fed the diet J, containing only PO. Furthermore, RIL was significantly increased in fish fed with blended VO diets with higher inclusion of SO (E) versus animals fed with high levels of PO (C) or with the diet containing the four VO (G). According to this, a positive Pearson's correlation was found between RIL and the total n-6 fatty acid dietary content (r= 0.766; $P< 0.05$).

3.2. Effects of diets on plasma parameters

In fish fed blended VO diets, differences were not found between dietary groups in glycerol, TAG or NEFA plasma levels (Table 5); while TAG were significantly higher in gilthead sea bream fed F diet (RO and SO) than in groups C (PO) and G (containing the four VO). Among fish fed mono-substituted diets, some significant differences were observed. In this sense, glycerol levels were higher in J (PO) group than in H (SO) group, while the highest value for NEFA was found in fish fed diet I (RO) in comparison with those fed the J diet. Despite the differences detected with blended oils in TAG plasma levels, differences were not found between gilthead sea bream fed mono-substituted diets. Concerning glycemia, in fish fed with single VO diets, the J group (PO) showed the

364 highest value, significantly different from that of H group (SO), which showed the lowest
365 level. Among fish fed blended VO diets, the highest values of glycemia were found in
366 fish fed F diet (SO and RO) although group D (SO and PO) showed moderate high values.
367 Glucose values were significantly lower in animals fed B (RO and PO) and E (SO) diets
368 compared to diet F.

369

370 **3.3. Effects of diets on skin and muscle colour and texture**

371 Skin colour values for L* and b* parameters were similar in all dietary groups (Table 6).
372 Only a* parameter presented statistically significant differences in skin colour in blended
373 VO groups, with higher values (less negative, or reddish) in fish fed with D and G diets,
374 containing 3 (30% SO) and 4 oils (15% SO) respectively, compared with group A, with
375 high levels of RO and without SO, which showed the lowest values (greenish). Regarding
376 muscle colour parameters, significant differences among dietary treatments were not
377 observed (Table 6); however, there was a positive Pearson's correlation ($r= 0.829$; $P< 0.05$)
378 between L* value and total n-6 fatty acids in the feed; whereas it was negative ($r= -0.799$;
379 $P< 0.05$) with the total amount of MUFA. Moreover, there was a negative
380 Pearson's correlation ($r=-0.799$; $P<0.05$) between b* value and total n-6 fatty acids in the
381 feed.

382 Regarding muscle texture, total work and maximal strength were significantly higher in
383 the group fed diet F (containing SO, LO and RO) compared to all the other groups except
384 for G (with all VO), which showed intermediary values (Fig 2). Moreover, none of these
385 parameters were affected in fish fed only one type of VO.

386

387 **3.4. Effects of diets on basal digestive enzyme activities**

388 No effect in intestinal pH content was found among fish fed either blended or mono-
389 substituted diets (Fig 3). TPA was measured in proximal intestine (Fig 3), but differences
390 were not found among fish fed blended VO diets or fish fed single VO diets. Moreover,
391 changes were not found in proximal intestine lipase activity in fish fed mono-substituted
392 diets or among fish fed blended oils (1.14 ± 0.19 and 1.88 ± 0.19 U lipase x mg prot^{-1},
393 respectively, Fig 3). Digestive enzymes did not show any correlation considering the
394 dietary profile.

395

396 **3.5. Effects of diets on gene expression in intestine, in adipose tissue and white muscle**

397 Expression levels of the genes analysed in the intestine did not change in general upon
398 the different dietary fatty acid profiles (Fig 4). Only *fatp1b* expression showed a
399 significant increase in fish fed with VO blends when including high levels of RO (A)
400 compared to those fish fed diets with the four VO (G) or the same proportions of SO and
401 RO (F). Moreover, in animals fed with VO blends a significant positive correlation was
402 found for *cd36* gene expression with the ratio of UFA/SFA and a negative one with the
403 amount of SFA (r= 0.828; P< 0.05 and r= -0.785; P< 0.05, respectively).

404 Relative gene expression of the transcription factors analysed in adipose tissue are
405 presented in Fig 5. *pparβ* and *lxr* showed significant differences among fish fed blended
406 VO diets, having the fish fed D diet (SO and PO) the lowest mRNA levels for both genes,
407 and being significantly different from animals fed A diet (RO) for *pparβ* and from animals
408 fed F diet (RO and SO) for *lxr*. Furthermore, there was a negative Pearson's correlation
409 for *pparα* with UFA/SFA ratio (r= -0.843; *P*< 0.05) and positive one with the total amount
410 of SFA (r= 0.762; *P*< 0.05). Regarding fish fed mono-substituted diets, J group (PO)
411 presented an increased expression of *pparβ* in comparison with fish fed diets H (SO) and
412 I (RO) diets; but differences were not found on *lxr* expression in those fish. In addition,
413 sea bream fed I diet (RO) showed a significantly higher level of expression for
414 *pparγ* compared to fish fed H diet (SO), whereas relative expression for *pparα* did not
415 show significant changes in any case.

416 Relative mRNA levels of lipid metabolism- and *β*-oxidation-related genes in adipose
417 tissue are shown in Fig 6. There were significant differences in *fas* for animals fed mono-
418 substituted diets among I (RO) group, with the highest value, and J (PO) group, the
419 lowest; nevertheless, fish fed blended VO diets did not present differences. For *lpl*,
420 notable differences were found for fish fed blended VO diets, having those fed diet B (RO
421 and PO) significantly higher values than those gilthead sea bream fed D, E and F diets,
422 all containing SO. In addition, there was a positive Pearson's correlation between *lpl*
423 relative expression and MUFA content in the diet (r= 0.365; *P*< 0.01). For fish fed mono-
424 substituted diets, I group (RO) showed significant differences respect H (SO) and J (PO)
425 fed fish, which had lower *lpl* relative expression. Furthermore, differences were not found
426 for the lipolytic marker *hsl* relative expression. Regarding the relative expression of β-

427 oxidation markers (*hadh, cpt1a, cpt1b*), VO source in diet did not change either the
428 adipose tissue gene expression profile.

429 In white muscle from fish fed with blended diets (Fig 7), likewise in adipose tissue, *lpl*
430 showed marked differences in relative expression, being the levels higher in fish fed diet
431 A (with high content of RO) in comparison with those fed D and E diets (without RO).
432 In concordance with the results obtained in adipose tissue, *lpl* also showed a positive
433 Pearson's correlation (r= 0.906; $P< 0.01$) with respect to MUFA content in the diet. For
434 fish fed mono-substituted diets, in I (RO) and J (PO) groups, *lpl* mRNA expression was
435 significantly lower than in H group (SO). Nevertheless, differences were not found for
436 the other lipases, *atgl* and *hsl* in this tissue. Despite this, a positive correlation was found
437 between *atgl* expression respect to UFA/SFA ratio (r= 0.821; P< 0.05); while *hsl*
438 correlated positively with the n-3/n-6 fatty acid ratio in diet (r= 0.927; P< 0.01), and
439 negatively with the total amount of n-6 (r= 0.855; P< 0.01). Moreover, in muscle of fish
440 fed with blended VO diets, *cpt1a* relative expression was affected by diet, showing a
441 positive Pearson correlation with the n-3/n-6 ratio (r= 0.783; P< 0.05) and, significant
442 differences between C (high PO) and E (high SO) groups, with lower levels in fish fed E
443 diet; whereas *cpt1b* and *hadh* were not altered.

444 Expression levels of genes related to the myogenic process were not greatly modified by
445 dietary lipid sources except for *myogenin* in fish fed blended VO diets, whose levels were
446 significantly higher in F group (RO and SO) than in D and G groups (containing SO and
447 PO and the four VO, respectively) (Fig 8). Although changes were not found in *mhc*
448 relative expression between fish fed blended VO diets a positive correlation with the
449 dietary MUFA content (r= -0.835; P< 0.01) was observed. The effect of different dietary
450 lipid sources resulted in elevated levels of *mhc* expression in fish fed diet I (RO) in
451 comparison with those from the J group (PO); with the same groups showing opposite
452 significant differences for *myod1* relative expression.

453

454 ## 4. Discussion

455 The aim of this trial was the evaluation of the effects of diets with 75% FO substitution
456 by VO from different sources (i.e. with distinct fatty acids composition) in gilthead sea
457 bream growth, parameters related with intestinal function and lipid metabolism. The
458 present results demonstrated that in the conditions studied, blended VO induces moderate

459 alterations in lipid homeostasis without adverse effects on growth performance and feed
460 utilization, thus supporting the use of these diets for the sustainable production of this
461 important marine species.

462 In fact, growth parameters were similar in all groups of fish fed VO blended diets, with
463 SGR values around 0.96%/day, reaching the C group, fed with the highest content of PO
464 and thus highest SFA levels, the uppermost values of SGR (1.00 ± 0.01%/day) and WG
465 (246.3 ± 4.5%). These results are contrary to our initial hypothesis that a higher UFA/SFA
466 ratio would induce an improvement in growth performance. In the same line, in fish fed
467 mono-substituted diets, also the presence of PO was beneficial, since only the J group
468 (PO) reached similar elevated levels on growth parameters (e.g. SGR 1.00 ± 0.01%/day
469 and WG 246.8 ± 3.4%), being the FBW and WG significantly higher than in animals fed
470 SO or RO diets (H and I groups, respectively). Accordingly, research in the last years has
471 mostly demonstrated that PO can effectively replace FO in the diets of various farmed
472 fish species without compromising growth performance, as long as the fish have a
473 minimum level of FM or FO to supply EFA. Thus, PO can be used to substitute FO in
474 aquafeeds without affecting growth in rainbow trout (*Oncorhynchus mykiss*) and salmon,
475 even at a 100% level of substitution (Fonseca-Madrigal *et al*, 2005; Torstensen *et al*.,
476 2000; Rosenlund *et al*., 2001), and improve growth in catfish (*Clarias gariepinus)* (Lim
477 *et al*., 2001; Ng *et al*., 2004). Nevertheless, in gilthead sea bream, growth was depressed
478 when fish, with a similar weight to the one of the present study, were fed a PO-based diet
479 (69%) during 24 weeks (Fountoulaki *et al*., 2009). Differences can be attributed, at least
480 in part, to variations in the PO fraction used, which may contain different concentrations
481 of key components such as vitamin E, carotenoids, phytosterols and phospholipids (Ng
482 and Gibon, 2011). Besides the presence of antioxidants (vitamin E and carotenoids), PO
483 contains a high proportion of SFA, giving to this VO exceptional resistance to oxidation,
484 which makes it a cost effective way to include plant oils in diets without the damage side
485 effects of oxidation and subsequent rancidity (Ng *et al*, 2008). In addition, the presence
486 of antioxidants can also contribute to the preservation of FO, which is more easily
487 oxidable.

488 Despite the positive effects on growth performance, feeding blended diets containing high
489 levels of PO (C) caused a higher accumulation of visceral fat in fish compared to gilthead
490 sea bream fed with diets without PO (A, E, and F) or low levels of PO (G). Therefore,
491 enhanced growth was accompanied with higher adiposity, focused in the visceral depot,

although without provoking significant effects on HSI values, which indicated that the liver of PO-fed fish did not accumulate further fat, reflecting therefore a good metabolic status of the animals. Regarding other VO, Menoyo *et al.* (2004) and Fontoulaki *et al.* (2009) reported that SO and RO based diets, with imbalances in n-3/n-6 and UFA/SFA ratios, provoked poor growth in gilthead sea bream. These findings are in agreement with our results where mono-substituted diets based on SO (H) and RO (I) reduced fish growth. These negative effects were avoided when SO and RO proportions were lowered by mixing these VO with LO and/or PO, as observed in the gilthead sea bream fed with the blended diets tested confirming the beneficial effect of mixing VO from different origins (Tortensen and Tocher, 2011). Therefore, it can be also concluded that, when the n-3 dietary fatty acid content and profile cover adequately the requirements, as in the present blended diets, an increase in n-3/n-6 ratio causes slight differences in growth performance improvement. This is confirmed by the positive correlation between n-3/n-6 ratio and both FBW and BL observed, although without significant differences between groups in growth parameters.

Concerning intestinal function, the fact that RIL was significantly increased in fish fed blended VO diets with high inclusion of SO (E), together with its positive correlation with the total n-6 fatty acid dietary content, suggested a compensatory mechanism of the intestine to improve nutrients absorption. Similarly, this happens with high-plant protein inclusion in the diet in rainbow trout and gilthead sea bream (Santigosa *et al*., 2008). In any case, in our study, a tendency to a lower protease activity (TPA) in fish fed high proportion of SO (59 and 30%, corresponding to diets E and D, F, respectively) was observed, indicating altogether a less optimal intestinal functionality. Nevertheless, significant changes were not detected in the gene expression profile of the intestinal fatty acid transporters studied, *cd36* and *fabp2* with the exception of *fatp1b*. In mammals, *cd36* gene expression is high in apical membranes of enterocytes and CD36 is known to contribute to the transport and uptake of fatty acids, as well as exerts a regulatory role initiating chylomicron production (Cifarelli and Abumrad, 2018). In the same line, in grass carp *(Ctenopharyngodon idella) cd36* was highly expressed in intestine in comparison with other tissues but its functionality has not been studied (Tian *et al.*, 2017). In the present study, *cd36* was expressed in the intestine of gilthead sea bream without significant differences among dietary treatments, although its expression correlated positively with the ratio UFA/SFA, suggesting a differential stimulation of its

525 transcription by fatty acids. On the other hand, *fatp1b* is not expressed in the intestine of
526 rodents and humans, where *fatp4* is in contrast more abundant. Nevertheless, the levels
527 of intestinal expression of *fatp1b* and *fatp4* were similar in zebrafish (Wang et al., 2019).
528 Thus, it appears that a different tissue distribution and function of FATPs may occur in
529 fish. In our study, the highest expression of *fatp1b* was found in fish fed with VO blends
530 with high levels of RO (A). Although in zebrafish, only the expression of *fabp2*, and not
531 *fabp1*, was modulated by diet in the intestine (Karanth *et al*., 2009), indicating specific
532 differences in these transporters function among fish species.
533 Regarding plasma parameters, few changes were found between groups fed blended VO
534 diets, since only TAG and glucose levels were significantly modified, but were not clearly
535 related with dietary treatments. In the same line, differences observed in circulating
536 metabolites in fish fed mono-substituted VO diets could be probably attributed to slight
537 variations in rates of nutrients absorption, rather than to distinct fatty acid composition
538 profiles of the experimental diets.
539 Furthermore, considering the importance of different quality attributes (i.e. fillet firmness
540 or coloration), especially for consumers, some studies have focused on the evaluation of
541 organoleptic parameters of fish fed VO (Rosenlund *et al*., 2011). The inclusion of VO do
542 not usually affect skin colour or changes are almost undetectable (Menoyo *et al*., 2014;
543 Izquierdo *et al*., 2005). In our experiment, fish fed high levels of RO (I and A diets)
544 showed the lowest values of reddishness, which could be attributable to high MUFA
545 content. Similarly, muscle colour was not affected significantly by diet, unlike the results
546 obtained in Atlantic salmon fed diets with RO content where both, the visual colour in
547 the fresh filet and, the colorimetric values (redness and yellowness) in the smoked
548 product, were reduced (Torstensen *et al*., 2004).
549 Moving to the transcriptional analyses performed in peripheral tissues, adipose tissue
550 from animals fed diet I (RO) showed significantly higher values of *fas* expression than
551 those fed the J (PO) diet, which presented the lowest values. These data suggested that
552 activation of the *de novo* lipogenesis was not the mechanism that induced fat
553 accumulation in those animals fed diets with high proportions of PO. Accordingly,
554 lipogenic enzymes were also decreased in adipose tissue of gilthead sea bream fed a
555 mixture of VO replacing above 33% of FO without modifying total fat and MFI (Bouraoui
556 *et al*., 2011), suggesting that fat accumulation in fish fed blended VO diets depends to a
557 greater extent on lipid uptake than *de novo* lipogenesis. Interestingly, a positive
558 correlation between *lpl* relative expression in adipose tissue and MUFA content in fish

559 fed VO blended diets was observed in the present study, corroborated by the highest value
560 of *lpl* expression observed in the fish fed diet I (RO) in this tissue in relation to the rest of
561 groups fed mono-substituted diets. In gilthead sea bream, expression of *lpl* in adipose
562 tissue is hormonally and seasonally regulated (Albalat *et al*., 2007; Saera-Vila *et al*, 2005)
563 and it is also down-regulated in fish fed plant protein diets. Nonetheless, studies on *lpl* or
564 *fas* modulation by specific dietary lipid components in adipose tissue have not been
565 reported in this species. However, in *Pagrus major*, dietary fatty acids exert a regulatory
566 effect on *lpl* expression in visceral fat (Liang *et al*., 2002). This latter study concluded
567 that the length of fatty acids rather than the degree of unsaturation influences adipose
568 tissue *lpl* expression, as it occurs in mammals (Amri *et al*., 1996; Raclot *et al*., 1997;
569 Takahashi and Takashi, 1999). Therefore, taking all together, some fatty acids more
570 abundant in RO may be enhancers of *lpl* and *fas* expression in gilthead sea bream adipose
571 tissue, supporting increased fatty acid uptake and synthesis upon these conditions.
572 Although mRNA levels of *β*-oxidation-related genes were not modified significantly in
573 adipose tissue, the expression of the transcription factor *pparβ,* associated with oxidative
574 processes (Kidani and Bensinger, 2012), was significantly lower in fish fed diet D, and
575 was accompanied also with a significant lower expression of *lxr*. The transcription factor
576 *lxr* has been related with the lipolytic pathway in adipose tissue in fish, since LXR
577 agonists induced *lxr* expression and raised lipolysis levels in rainbow trout adipocytes
578 (Cruz-Garcia *et al*., 2012). Altogether, the combination of oils present in diet D (mainly
579 SO and PO) appeared to induce low levels of catabolism of TAG and fatty acids, which
580 would agree with the relatively high mesenteric fat content (i.e. MFI) found in these fish.
581 On the contrary, the groups with the highest adiposity, the fish fed diets C or particularly
582 J, both with PO, which showed significantly higher *pparβ* relative expression than the
583 other fish fed mono-substituted diets, pointed out that the equilibrium between fat
584 accumulation and oxidation is what determines the final adipose tissue mass in the animal.
585 In skeletal muscle, genes related to lipid metabolism showed a similar expression profile
586 as that in adipose tissue. Regarding *lpl*, although usually is regulated in a tissue specific
587 manner, as observed in adipose tissue, muscle *lpl* was also upregulated in the group of
588 fish fed with high content of RO (diet A) and depressed in groups fed diets without RO
589 (D and E), considering blended diets; showing also a positive correlation with dietary
590 MUFA content. Thus, the presence of a certain proportion of MUFA in the diet could
591 favour *lpl* expression independently of the tissue. Nonetheless, among mono-substituted
592 diets-fed fish, the group I (RO) showed relatively low values for *lpl* expression,

593 suggesting that other mechanisms are also involved in LPL control, as confirmed by other
594 studies that demonstrated a complex regulation of this protein at different levels (Albalat
595 *et al*., 2007; He *et al*, 2018). Among *β*-oxidation-related genes, only the expression of
596 *cpta1* was significantly affected by the different experimental diets. D (SO and PO) and
597 E (SO) groups were also those presenting the lowest values of *cpt1a* expression (in
598 addition to the lowest *lpl* values), suggesting altogether lower metabolic activity (i.e. fatty
599 acid oxidation and uptake) in the muscle of these fish, which could be related with the
600 lower n-3/n-6 ratios found in those diets.
601 Finally, concerning muscle growth and development, the expression of several related
602 genes were analysed, with the ones involved in myogenic processes not being modified
603 by dietary lipid sources in blended VO groups with the exception of *myogenin*, which
604 showed the highest level of expression in fish fed F diet (RO and SO). This higher
605 expression of *myogenin* was accompanied by higher levels of textural parameters of the
606 fillet, total work and maximal strength, which altogether suggested that those fish muscles
607 had undergone a myogenic hyperplasic process (Johnston *et al.,* 2000). A similar profile
608 between FBW and *myogenin* and *myod1* expression (both of them markers of induced
609 myogenesis), could be observed in groups H, I and J; while *mhc*, a marker of muscle cells
610 already differentiated (Garcia de la serrana *et al.*, 2014), showed the opposite transcript
611 profile. Thus, according to the role of *myod1* and *myogenin*, it appears that a higher
612 growth rate in the group of fish fed diet J (PO) would be here accompanied by an
613 activation of the myogenic process regulated by these factors. In support of this
614 observation, Garcia de la serrana *et al*., (2012) found previously that gilthead sea bream
615 fed with seven experimental diets, containing different proportions of protein and lipid,
616 levels of MyoD positive cells and TOR phosphorylation were significantly higher also in
617 those fish with higher growth. Thus, overall it is interesting to find these changes in
618 myogenic parameters between the fish groups, in which the differences in growth were
619 also more pronounced, pointing out their value as indicators of muscle growth
620 improvement.

621 In conclusion, this study provides new information regarding the importance of feeds
622 formulation in aquaculture, demonstrating that combinations of different VO can be used
623 indistinctly in diets with up to 75% FO substitution with limited physiological differences
624 in gilthead sea bream. However, a diet mono-substituted could result in some negative
625 traits depending on the VO source used. Specifically, even PO, which induced the lowest

626 UFA/SFA ratio of the present blended diets, could be a very valuable candidate plant oil,
627 considering its positive effect on growth, which although accompanied by a higher
628 visceral adiposity, did not cause metabolic alterations. Moreover, increases in n-3/n-6
629 ratios induced by the different profiles of the n-6 fraction in the diets, slightly modified
630 fish growth performance in our experimental conditions. Furthermore, the effects of the
631 different dietary VO analysed in the present work provide a transversal view of the
632 responses and interactions, from intestine to adipose tissue and muscle, including growth,
633 flesh quality, and lipid metabolism in this important aquaculture species. All this
634 information can be useful, either in basic research and applied investigation, confirming
635 some key molecules that can be utilized as markers of the effects of new designed diets,
636 on fish growth and metabolic status.

637

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Tables

Table 1. Ingredients and proximate composition of the 10 experimental diets. Diets A to G contain blends of vegetable oils substituting fish oil; while in the light grey columns are the mono-substituted diets, H to J.

	Diet									
Ingredients (%)	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
Wheat				7.09					7.09	
Corn gluten				3.86					3.86	
Wheat gluten				15.00					15.00	
Sunflower meal				3.00					3.00	
Soya concentrate				30.00					30.00	
Fava beans				6.00					6.00	
Fish meal				15.00					15.00	
Fish oil				4.64					4.64	
Palm oil	-	5.03	9.87	4.81	-	-	2.43	-	-	13.88
Linseed oil	2.02	3.00	3.94	3.37	2.82	2.42	2.7	-	-	-
Rapeseed oil	11.57	5.67	-	-	-	5.80	5.84	-	13.58	-
Soya oil	-	-	-	5.54	10.81	5.39	2.65	13.63	-	-
Vit/Min premix	1.82	1.71	1.60	1.69	1.78	1.8	1.79	1.78	1.83	1.53
Proximate composition (%)										
Dry matter	93.21	93.21	93.21	93.21	93.21	93.21	93.21	93.21	93.21	93.21
Crude protein	46.51	46.46	46.42	46.45	46.49	46.50	46.33	46.49	46.51	46.39
Crude fat	22.9	22.0	22.0	22.4	21.9	22.4	21.9	22.4	22.2	21.8
Ash	5.70	5.66	5.63	5.66	5.68	5.69	5.66	5.68	5.70	5.61

Table 2. Fatty acid composition of the 10 experimental diets. Diets A to G contain blends of vegetable oils substituting fish oil; while in the light grey columns are the mono-substituted diets, H to J.

Fatty acid (%)	Diet									
	A	B	C	D	E	F	G	H	I	J
C14:0	2.21	2.34	2.47	2.33	2.12	2.14	2.27	2.15	2.20	2.63
C16:0	9.36	17.41	24.83	18.98	12.55	10.95	14.17	13.27	9.32	30.81
C16:1n-7	2.48	2.36	2.30	2.36	2.31	2.37	2.40	2.34	2.50	2.31
C16:2n-6	0.30	0.28	0.27	0.28	0.28	0.30	0.29	0.29	0.31	0.27
C18:0	2.47	3.19	3.82	3.51	3.16	2.80	2.99	2.87	2.13	3.73
C18:1n-9	36.19	31.73	26.79	23.02	19.19	27.30	29.81	19.88	38.67	30.57
C18:1n-7	2.67	2.04	1.41	1.62	1.88	2.24	2.15	2.03	2.84	1.43
C18:2n-6	16.52	14.12	12.08	21.98	31.95	24.93	19.10	36.51	17.47	10.89
C18:3n-3	10.31	10.07	9.75	9.83	10.11	10.11	10.04	4.37	6.52	0.98
C18:4n-3	0.74	0.73	0.72	0.70	0.73	0.73	0.74	0.75	0.75	0.70
C20:1 sum. isomers	2.47	2.03	1.72	1.70	1.85	2.15	2.05	1.80	2.59	1.72
C20:4n-6	0.23	0.21	0.24	0.23	0.23	0.22	0.22	0.29	0.25	0.24
C20:4n-3	0.24	0.24	0.24	0.25	0.23	0.22	0.24	0.22	0.24	0.23
C20:5n-3 EPA	3.24	3.12	3.07	3.02	3.02	3.10	3.15	3.02	3.27	3.04
C22:1 sum. isomers	2.32	2.12	2.07	2.18	2.06	2.24	2.17	2.13	2.40	2.10
C22:5n-3	0.52	0.43	0.46	0.43	0.43	0.46	0.46	0.44	0.48	0.45
C22:6n-3 DHA	2.98	2.90	2.95	2.95	2.87	2.99	2.95	2.98	3.07	2.89
C24:1n-9	0.33	0.29	0.24	0.27	0.23	0.27	0.26	0.25	0.34	0.24
SFA not listed	1.01	0.90	0.76	0.87	0.97	1.01	0.96	1.06	1.08	0.81
Monoenes not listed	0.11	0.11	0.11	0.10	0.14	0.12	0.11	0.10	0.13	0.11
n-6 FA not listed	0.29	0.24	0.27	0.21	0.24	0.25	0.22	0.20	0.29	0.22
n-3 FA not listed	0.20	0.18	0.16	0.17	0.20	0.20	0.20	0.22	0.20	0.16
Others	0.38	0.37	0.33	0.32	0.36	0.32	0.36	0.36	0.37	0.36
Sum. SFA	15.05	23.84	31.88	25.69	18.80	16.90	20.39	19.35	14.73	37.98
Sum. MUFA	46.57	40.68	34.64	31.25	27.66	36.69	38.95	28.53	49.47	38.48
Sum. n-6 FA	17.34	14.85	12.86	22.70	32.70	25.70	19.83	37.29	18.32	11.62
Sum. n-3 FA	18.23	17.67	17.35	17.35	17.59	17.81	17.78	12,0	14.53	8.45
UFA/SFA	5.46	3.07	2.03	2.78	4.15	4.75	3.75	4.02	5.59	1.54
n-3/n-6	1.05	1.19	1.35	0.77	0.54	0.69	0.9	0.32	0.79	0.73
Unknown	2.40	2.60	3.0	2.70	2.90	2.60	2.70	2.50	2.60	3.10

EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; FA: Fatty acids; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; UFA/SFA: Unsaturated fatty acids / Saturated fatty acids

Table 3. Primers used for real-time qPCR: sequence, annealing temperature (Ta) and GenBank accession numbers. F: forward, R: reverse.

Type	Gene		Sequence 5'– 3'	Ta	Accession number
Reference	*β-actin*	**F**	TCCTGCGGAATCCATGAGA	60	X89920
		R	GACGTCGCACTTCATGATGCT		
	ef1a	**F**	CTTCAACGCTCAGGTCATCAT	60	AF184170
		R	GCACAGCGAAACGACCAAGGGGA		
	rpl27a	**F**	AAGAGGAACACAACTCACTGCCCCAC	68	AY188520
		R	GCTTGCCTTTGCCCAGAACTTTGTAG		
	rps18	**F**	GGGTGTTGGCAGACGTTAC	60	AM490061.1
		R	CTTCTGCCTGTTGAGGAACCA		
Transcription factors	*pparα*	**F**	TCTCTTCAGCCCACCATCCC	62	AY590299
		R	ATCCCAGCGTGTCGTCTCC		
	pparβ	**F**	AGGCGAGGGAGAGTGAGGATGAGGAG	69	AY590301
		R	CTGTTCTGAAAGCGAGGGTGACGATGTTTG		
	pparγ	**F**	CGCCGTGGACCTGTCAGAGC	66	AY590304
		R	GGAATGGATGGAGGAGGAGGAGATGG		
	lxr	**F**	GCACTTCGCCTCCAGGACAAG	62	FJ502320
		R	CAGTCTTCACACAGCCACATCAGG		
Lipid metabolism	*lpl*	**F**	GAGCACGCAGACAACCAGAA	60	AY495672
		R	GGGGTAGATGTCGATGTCGC		
	fas	**F**	TGGCAGCATACACACAGACC	60	AM952430
		R	CACACAGGGCTTCAGTTTCA		
	atgl	**F**	GTGCTTCAGTCCTGGATGTCTTC	60	JX975711
		R	AGCCTTGCAGGTCCATGTTGA		
	hsl	**F**	GCTTTGCTTCAGTTTACCACCATTTC	60	EU254478
		R	GATGTAGCGACCCTTCTGGATGATGTG		
β Oxidation	*hadh*	**F**	GAACCTCAGCAACAAGCCAAGAG	60	JQ308829
		R	CTAAGAGGCGGTTGACAATGAATCC		
	cpt1a	**F**	GTGCCTTCGTTCGTTCCATGATC	60	JQ308822
		R	TGATGCTTATCTGCTGCCTGTTTG		
	cpt1b	**F**	CCACCAGCCAGACTCCACAG	60	DQ866821
		R	CACCACCAGCACCCACATATTTAG		
Myogenesis	*myf5*	**F**	CTACGAGAGCAGGTGGAGAACT	64	JN034420
		R	TGTCTTATCGCCCAAAGTGTC		
	myod1	**F**	TTTGAGGACCTGGACCC	60	AF478568.1
		R	CTTCTGCGTGGTGATGGA		
	myod2	**F**	CACTACAGCGGGGATTCAGAC	60	AF478569
		R	CGTTTGCTTCTCCTGGACTC		
	myogenin	**F**	CAGAGGCTGCCCAAGGTCGAG	68	EF462191
		R	CAGGTGCTGCCCGAACTGGGCTCG		
	mrf4	**F**	CATCCCACAGCTTTAAAGGC	60	JN034421
		R	GAGGACGCCGAAGATTCAC		
Myostatins Myosin	*mstn1*	**F**	GTACGACGTGCTGGGAGACG	60	AF258448.1
		R	CGTACGATTCGATTCGCTTG		
	mstn2	**F**	ACCTGGTGAACAAAGCCAAC	60	AY046314
		R	TGCGGTTGAAGTAGAGCATG		
	mhc2	**F**	AGCAGATCAAGAGGAACAGCC	60	AY550963.1
		R	GACTCAGAAGCCTGGCGATT		
Fatty acid transporters	*cd36*	**F**	GTCGTGGCTCAAGTCTTCCA	60	ERR12611_isotig20793
		R	TTTCCCGTGGCCTGTATTCC		
	fatp1b	**F**	TGCAGCAGTTTCTTGGATGA	60	ERR12611_isotig43042
		R	TGCAGCTCTTGCGTTCAAAA		
	fabp2	**F**	CGAGCACATTCCGCACCAAAG	60	KF857310
		R	CCCACGCACCCGAGACTTC		
Intestinal integrity	*alpi*	**F**	CCGCTATGAGTTGGACCGTGAT	60	KF857309
		R	GCTTTCTCCACCATCTCAGTAAGGG		
	int-pla2	**F**	CAGTACAACAACTATGGCTGCTTCT	60	JX975709
		R	GGTCCACTTTATCCACAGGTCTTC		

944 *β-actin*, beta actin; *ef1α*, elongation factor 1 alpha; *rpl27a*, ribosomal protein L27a; *rps18,*
945 ribosomal protein S18, *pparα*, peroxisome proliferator-activated receptor alpha; *pparβ*,
946 peroxisome proliferator-activated receptor beta; *pparγ*, peroxisome proliferator-activated
947 receptor gamma; *lxr*, liver X receptor; *lpl*, lipoprotein lipase; *fas*, fatty acid synthase; *atgl,*
948 adipose triglyceride lipase; *hsl*, hormone sensitive lipase; *hadh*, hydroxyacyl-CoA
949 dehydrogenase; *cpt1a*, carnitine palmitoyltransferase 1A; *cpt1b*, carnitine
950 palmitoyltransferase 1B; *myf5; myod1*; *myod*2; *myogenin*; *mrf4*; myostatins (*mstn1*;
951 *mstn2*); *mhc2*, myosin heavy chain 2; *cd36a*, fatty acid translocase; *fatp1b*, fatty acid
952 transport protein; *fabp2*, fatty acid binding protein 2; *alpi*, intestinal-type alkaline
953 phosphatase, *int-pla2*, intestinal phospholipase A2.

Table 4. Growth performance, feed utilization and somatic indexes of gilthead seabream fed during 18 weeks the different experimental diets. Initial body weight (IBW), final body weight (FBW), weight gain (WG), specific growth rate (SGR), body length (BL), condition factor (CF), total feed intake (TFI) and feed conversion ratio (FCR), n = 3 (A to G) and n = 2 (H to J) tanks/diet. Hepatosomatic index (HSI), mesenteric fat index (MFI) and relative intestinal length (RIL). Data are shown as mean ± S.E.M. n = 9 (A to G) and n = 6 (H to J) fish/diet. Different letters in the same row indicate significant differences among groups with fish fed blended and mono-substituted (light grey columns) diets evaluated separately (*P*-value: *<0.05). n.s.: not significant.

	A	B	C	D	E	F	G	*P*	H	I	J	*P*
IBW (g)	81.3 ± 0.4	82.1 ± 0.5	82.2 ± 0.5	81.7 ± 0.2	81.3 ± 0.4	81.8 ± 0.5	82.0 ± 0.4	n.s.	81.9 ± 0.3	82.2 ± 0.2	81.5 ± 0.1	n.s.
FBW (g)	271.3 ± 5.2	272.3 ± 1.5	284.6 ± 3.7	269.1 ± 6.4	269.0 ± 3.5	270.0 ± 5.8	273.3 ± 6.2	n.s.	262.2 ± 12.4ab	254.9 ± 1.7b	282.6 ± 2.8a	*
WG (%)[1]	233.8 ± 6.4	231.6 ± 1.8	246.3 ± 4.5	229.3 ± 7.8	231.0 ± 4.3	229.9 ± 7.1	233.3 ± 7.6	n.s.	220.2 ± 15.1ab	210.3 ± 2.1b	246.8 ± 3.4a	*
SGR (%/days)[2]	0.96 ± 0.02	0.96 ± 0.0	1.00 ± 0.01	0.96 ± 0.02	0.96 ± 0.01	0.96 ± 0.01	0.96 ± 0.02	n.s.	0.93 ± 0.03	0.89 ± 0.02	1.00 ± 0.01	n.s.
BL (cm)	21.6 ± 0.1	21.7 ± 0.1	22.0 ± 0.1	21.6 ± 0.1	21.6 ± 0.0	21.5 ± 0.2	21.8 ± 0.1	n.s.	21.3 ± 0.3	21.2 ± 0.1	21.9 ± 0.1	n.s.
CF (%)[3]	2.67 ± 0.02	2.65 ± 0.01	2.67 ± 0.01	2.65 ± 0.03	2.67 ± 0.05	2.69 ± 0.06	2.64 ± 0.03	n.s..	2.70 ± 0.0	2.62 ± 0.02	2.70 ± 0.02	n.s.
TFI (Kg)	4.83 ± 0.19	4.40 ± 0.3	5.11 ± 0.36	4.61 ± 0.48	4.93 ± 0.61	4.52 ± 0.17	5.00 ± 0.29	n.s.	4.48 ± 0.31	4.70 ± 0.07	5.17 ± 0.9	n.s.
FCR[4]	1.19 ± 0.12	1.27 ± 0.1	1.09 ± 0.06	1.04 ± 0.09	1.19 ± 0.02	1.05 ± 0.07	1.18 ± 0.06	n.s.	1.08 ± 0.03	1.21 ± 0.01	1.20 ± 0.09	n.s.
HSI (%)[5]	1.25 ± 0.08	1.22 ± 0.06	1.21 ± 0.09	1.23 ± 0.06	1.29 ± 0.11	1.22 ± 0.07	1.21 ± 0.04	n.s.	1.23 ± 0.12	1.25 ± 0.10	1.32 ± 0.10	n.s.
MFI (%)[6]	1.09± 0.1b	1.53 ± 0.09ab	1.82 ± 0.17a	1.40 ± 0.25ab	1.34 ± 0.14b	1.36 ± 0.10b	1.04 ± 0.17b	*	1.07 ± 0.16b	1.09 ± 0.13ab	1.63 ± 0.14a	*
RIL[7]	0.56 ± 0.03ab	0.49 ± 0.03ab	0.45 ± 0.02b	0.52 ± 0.03ab	0.62 ± 0.05a	0.52 ± 0.03ab	0.46 ± 0.02b	**	0.53 ± 0.04	0.45 ± 0.03	0.52 ± 0.06	n.s.

[1]WG = ((FBW-IBW)/ IBW) x 100; [2]SGR = ((lnFBW – lnIBW) / t) x 100; [3]CF = (FBW / BL^3) x 100; [4]FCR= (total feed intake / (FBW-IBW)); [5]HSI= (liver weight / FBW) x 100; [6]MFI = (mesenteric fat weight / FBW) x 100; [7]RIL = (mm / g fish).

Table 5. Plasma levels of glycerol, triacylglycerides (TAG), non-esterified fatty acids (NEFA) and glucose of gilthead sea bream fed during 18 weeks the different experimental diets. Data are shown as mean ± S.E.M. n = 9 (A to G) and n = 6 (H to J) fish/diet. Different letters in the same row indicate significant differences among groups with fish fed blended and mono-substituted (light grey columns) diets evaluated separately (*P*-value: *<0.05, **<0.01). n.s.: not significant.

	A	B	C	D	E	F	G	*P*	H	I	J	*P*
Glycerol (mM)	0.1 ± 0.02	0.11 ± 0.02	0.12 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.12 ± 0.01	0.08 ± 0.01	n.s.	0.07 ± 0.01b	0.11 ± 0.01ab	0.15 ± 0.02a	*
TAG (mM)	1.69 ± 0.12ab	1.54 ± 0.11ab	1.34 ± 0.13b	1.45 ± 0.12ab	1.41 ± 0.08ab	1.72 ± 0.09a	1.37 ± 0.08b	**	1.43 ± 0.09	1.39 ± 0.16	1.62 ± 0.13	n.s.
NEFA (mM)	0.34 ± 0.04	0.32 ± 0.04	0.39 ± 0.06	0.35 ± 0.03	0.36 ± 0.03	0.33 ± 0.04	-	n.s.	0.4 ± 0.03a	0.43 ± 0.02a	0.34 ± 0.01b	**
Glucose (mM)	6.58 ± 0.74b	5.86 ± 0.44b	6.5 ± 0.79b	8.04 ± 0.66ab	5.76 ± 0.12b	9.25 ± 0.74a	6.94 ± 0.75b	**	6.55 ± 0.5b	8 ± 0.78ab	9.81 ± 1.13a	*

Table 6. Skin and muscle colour values of gilthead sea bream fed during 18 weeks the different experimental diets. Data are shown as mean ± S.E.M. n = 9 (A to G) and n = 6 (H to J) fish/diet. Different letters in the same row indicate significant differences among groups with fish fed blended and mono-substituted (light grey columns) diets evaluated separately (*p*-value: *<0.05). L*: lightness; a*: redness; b*: yellowness; n.s.: not significant.

		A	B	C	D	E	F	G	*P*	H	I	J	*P*
Skin	L*	59.9 ± 1.5	59.7 ± 0.7	61.2 ± 1.4	59 ± 0.5	59.6 ± 2	63.1 ± 0.8	64.5 ± 2.7	n.s	60.1 ± 2.4	58.3 ± 0.9	58.3 ± 2.8	n.s.
	a*	-2.72 ± 0.21b	-2.03 ± 0.25ab	-1.98 ± 0.32ab	-1.63 ± 0.28a	-1.81 ± 0.34ab	-1.86 ± 0.17ab	-1.54 ± 0.2a	*	-2.25 ± 0.39	-2.96 ± 0.35	-2.52 ± 0.47	n.s.
	b*	7.98 ± 0.37	8.72 ± 0.55	8.81 ± 0.55	8.12 ± 0.44	7.7 ± 0.37	8.77 ± 0.43	8.6 ± 0.35	n.s.	7.42 ± 0.62	9.57 ± 0.61	7.58 ± 0.68	n.s.
Muscle	L*	51.3 ± 1.4	52 ± 1.5	52.5 ± 1.4	53 ± 2.2	57.9 ± 0.7	52.9 ± 2.4	52.04 ± 2.4	n.s.	53.8 ± 1.4	49.9 ± 2.7	50.16 ± 1.9	n.s.
	a*	-1.51 ± 0.06	-1.77 ± 0.12	-1.62 ± 0.1	-1.72 ± 0.09	-1.75 ± 0.07	-1.63 ± 0.04	-1.68 ± 0.09	n.s.	-1.06 ± 0.45	-1.65 ± 0.15	-1.63 ± 0.09	n.s.
	b*	-1.72 ± 0.44	-1.5 ± 0.59	-0.82 ± 0.59	-1.6 ± 0.83	-2.38 ± 0.45	-1.98 ± 0.3	-0.91 ± 0.46	n.s.	-0.27 ± 1.28	-1.64 ± 0.9	-1.54 ± 0.8	n.s.

974 **Figures legends**

975 **Figure 1.** Percentage of inclusion of the different oils in the 10 experimental diets. Fish
976 Oil (FO); Linseed Oil (LO); Rapeseed oil (RO); Soybean Oil (SO) and Palm Oil (PO).

977

978 **Figure 2.** Muscle texture parameters of gilthead seabream fed during 18 weeks the
979 different experimental diets. Data are shown as mean ± S.E.M (n = 3). Different letters
980 indicate significant differences among groups with fish fed blended and mono-substituted
981 VO diets evaluated separately.

982

983 **Figure 3.** Intestinal pH, total protease and lipase activity of gilthead seabream fed during
984 18 weeks the different experimental diets. Data are shown as mean ± S.E.M. n = 9 (A to
985 G) and n = 6 (H to J) fish/diet. Different letters indicate significant differences among
986 groups with fish fed blended and mono-substituted VO diets evaluated separately.

987

988 **Figure 4.** Relative expression of fatty acid transporters and intestinal integrity-related
989 genes in proximal intestine of gilthead seabream fed during 18 weeks the different
990 experimental diets. Data are shown as mean ± S.E.M. n = 9 (A to G) and n = 6 (H to J)
991 fish/diet. Different letters indicate significant differences among groups with fish fed
992 blended and mono-substituted VO diets evaluated separately.

993

994 **Figure 5.** Relative gene expression of transcription factors in visceral adipose tissue of
995 gilthead seabream fed during 18 weeks the different experimental diets. Data are shown
996 as mean ± S.E.M. n = 9 (A to G) and n = 6 (H to J) fish/diet. Different letters indicate
997 significant differences among groups with fish fed blended and mono-substituted VO
998 diets evaluated separately.

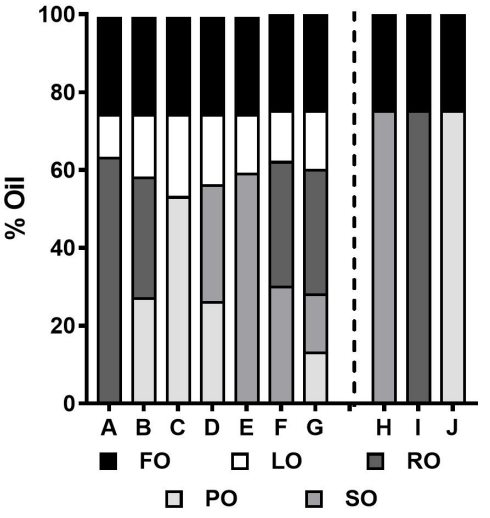
999

1000 **Figure 6.** Relative expression of lipid metabolism- and β-oxidation-related genes in
1001 visceral adipose tissue of gilthead seabream fed during 18 weeks the different
1002 experimental diets. Data are shown as mean ± S.E.M. n = 9 (A to G) and n = 6 (H to J)

fish/diet. Different letters indicate significant differences among groups with fish fed blended and mono-substituted VO diets evaluated separately.

Figure 7. Relative expression of lipid metabolism- and β-oxidation-related genes in white muscle of gilthead seabream fed during 18 weeks the different experimental diets. Data are shown as mean ± S.E.M. n = 9 (A to G) and n = 6 (H to J) fish/diet. Different letters indicate significant differences among groups with fish fed blended and mono-substituted VO diets evaluated separately.

Figure 8. Relative expression of myogenic regulatory factors and muscle growth-related genes in white muscle of gilthead seabream fed during 18 weeks the different experimental diets. Data are shown as mean ± S.E.M. n = 9 (A to G) and n = 6 (H to J) fish/diet. Different letters indicate significant differences among groups with fish fed blended and mono-substituted VO diets evaluated separately.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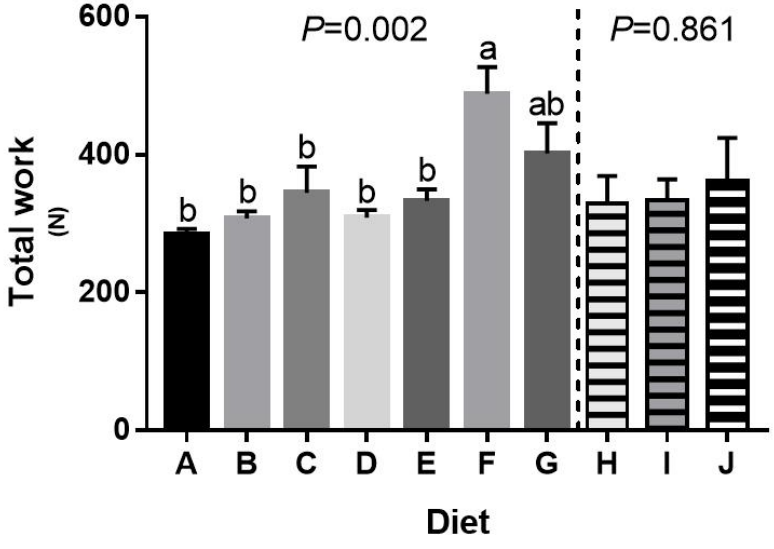
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H
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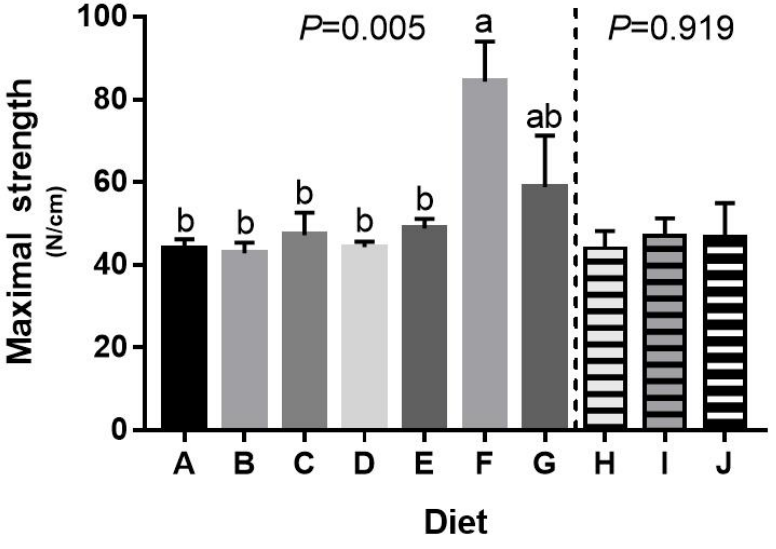
Diet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b
A
B
C
D
E
F
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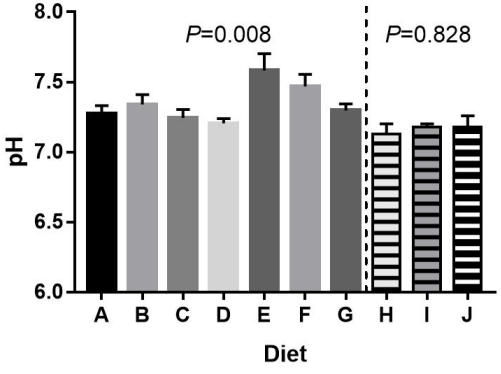
H
I
J
Diet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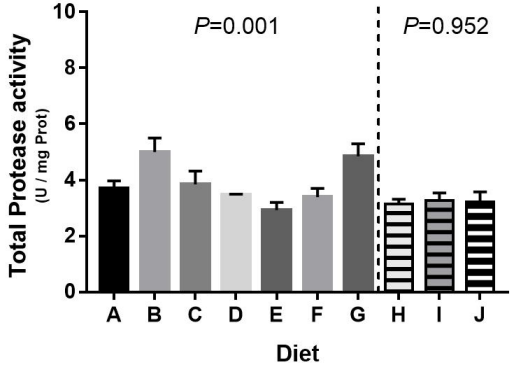
Diet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8
6
4
2
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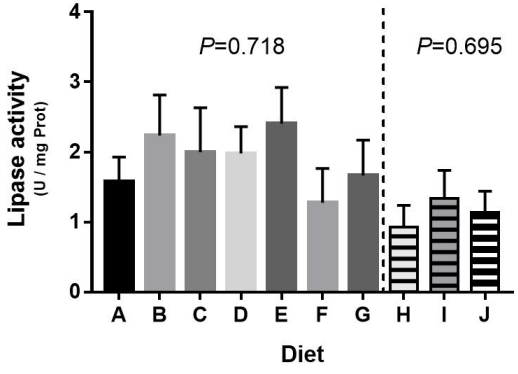
Diet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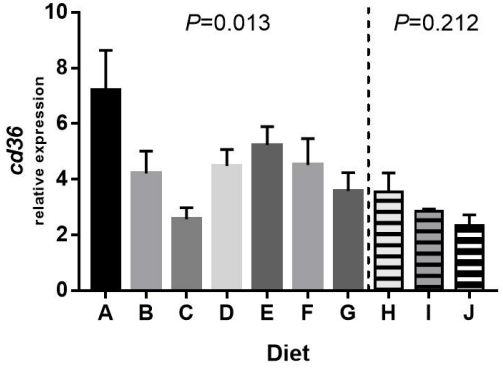
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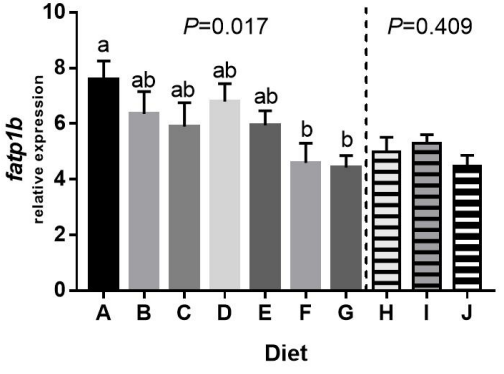
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b
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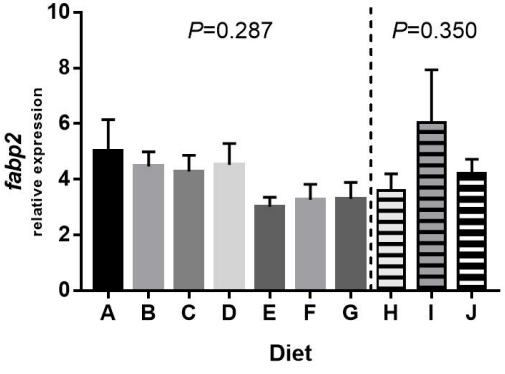
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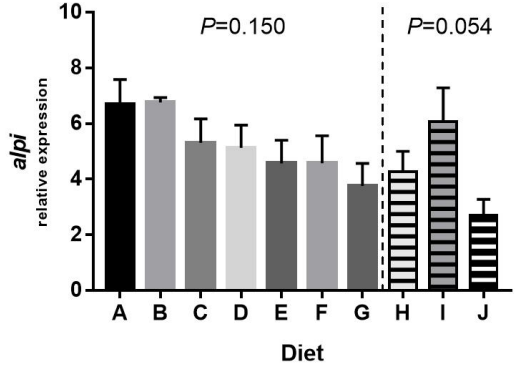
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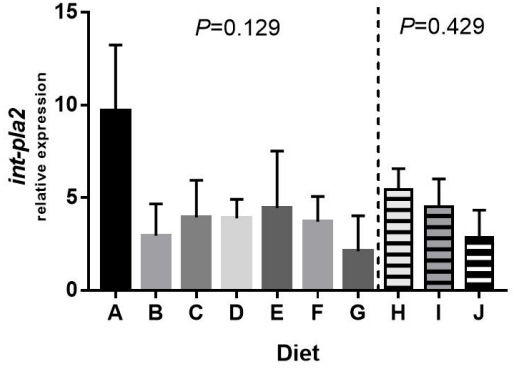
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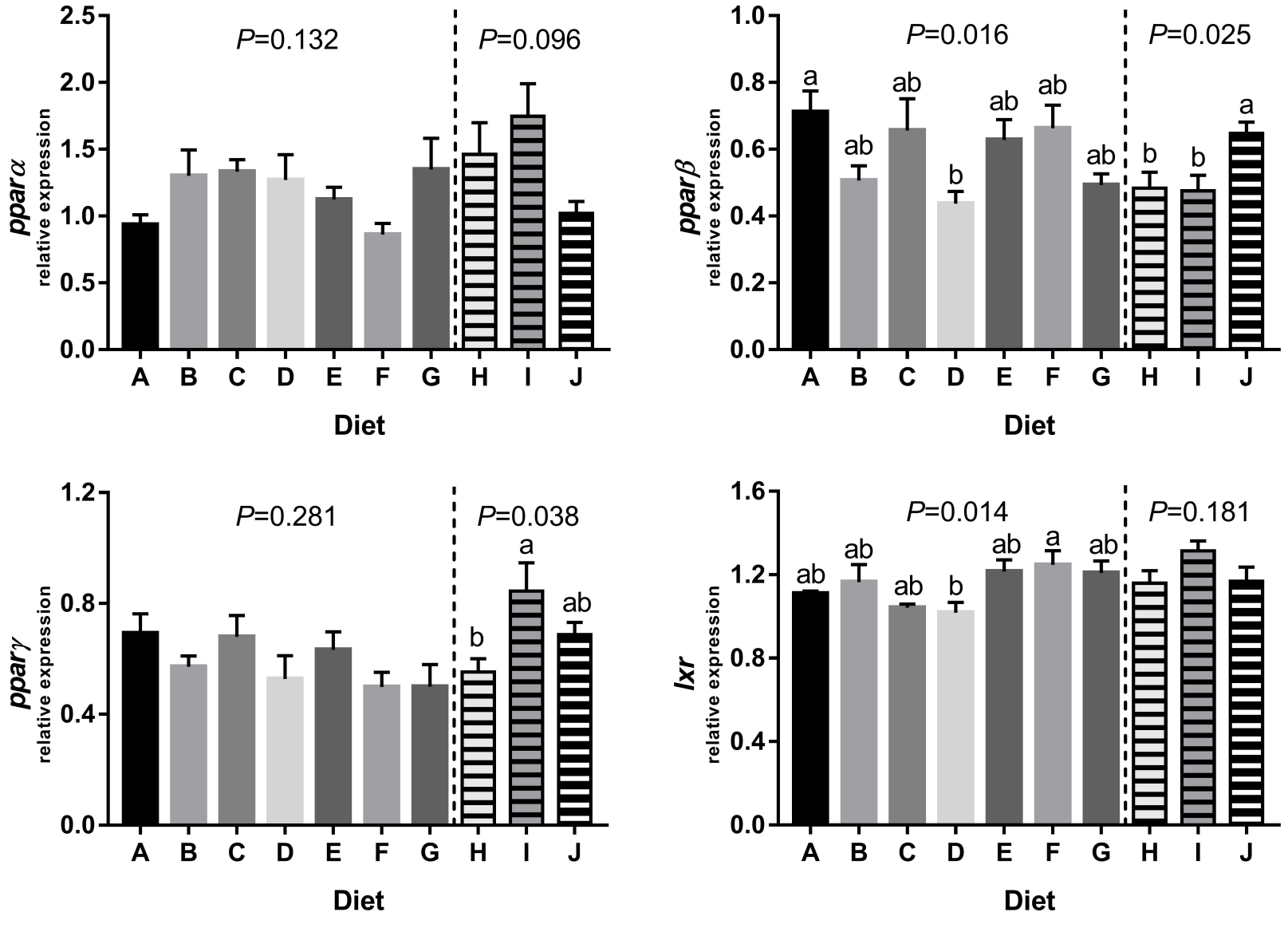
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Diet
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0.0
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A B C D E F G H I J
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0.4
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A B C D E F G H I J
Diet
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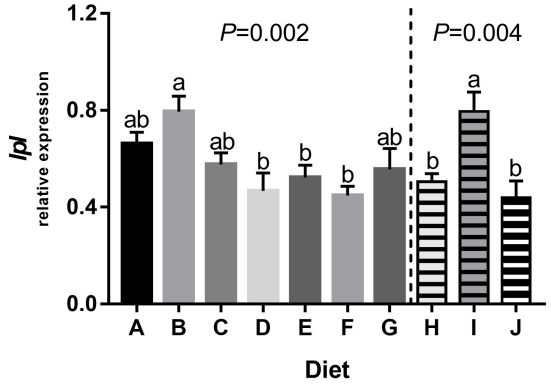
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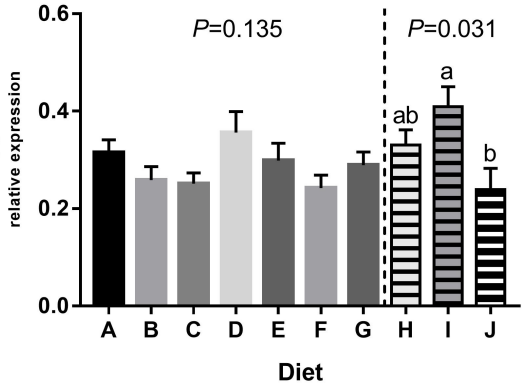
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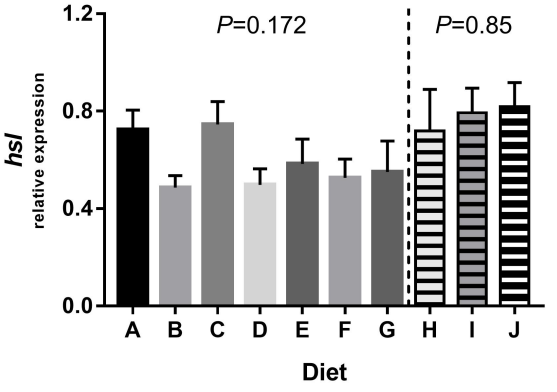
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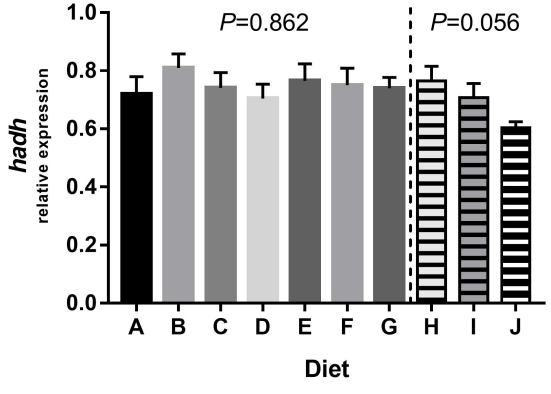
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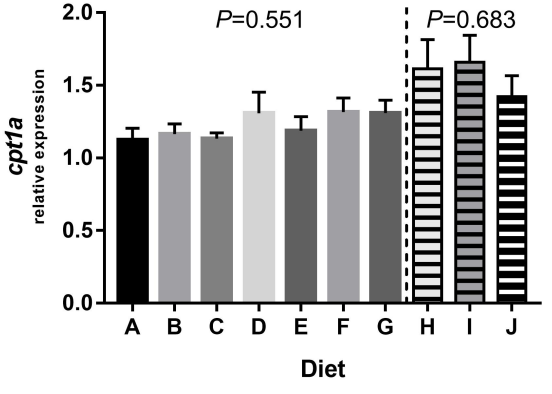
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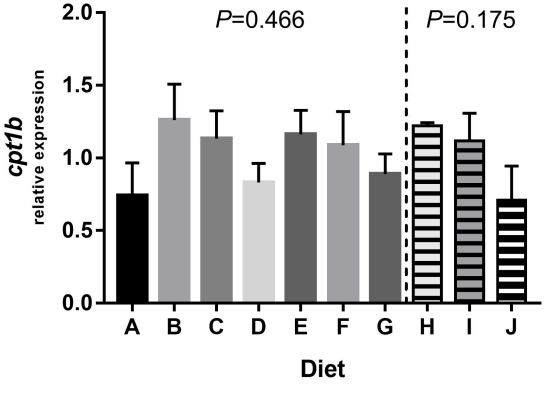
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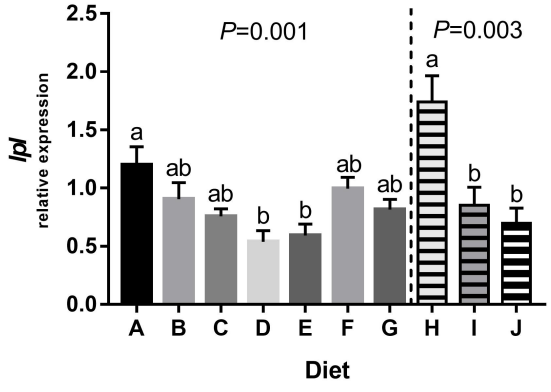
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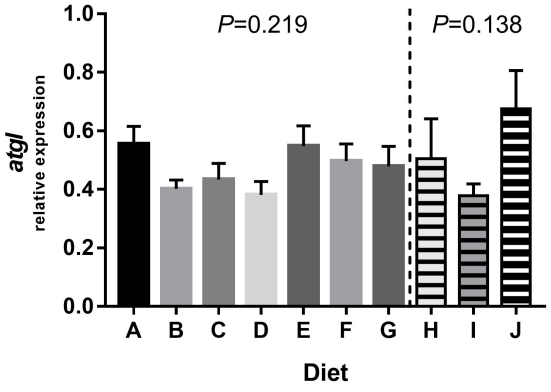
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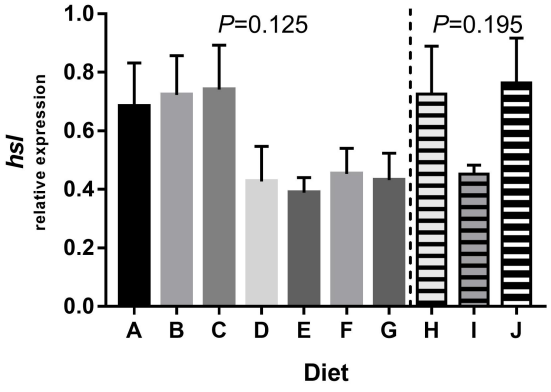
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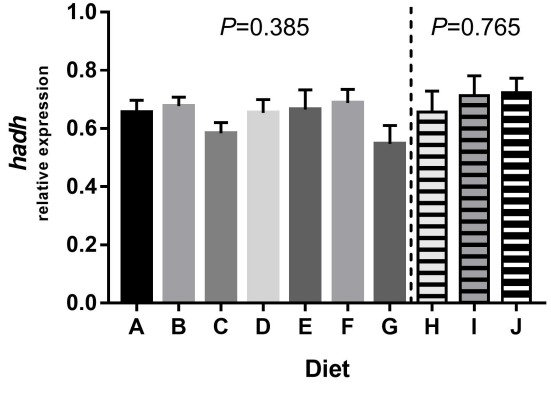
Diet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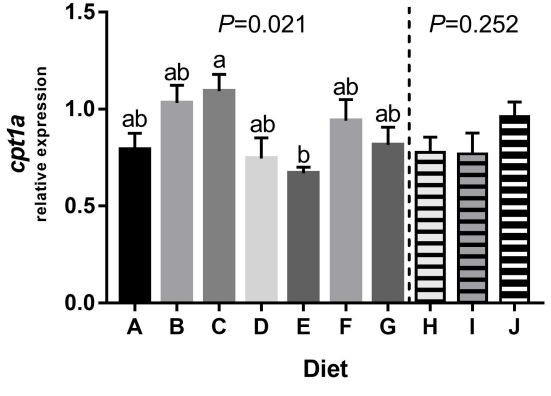
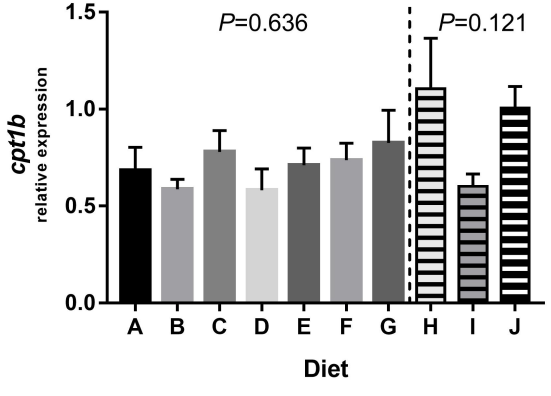
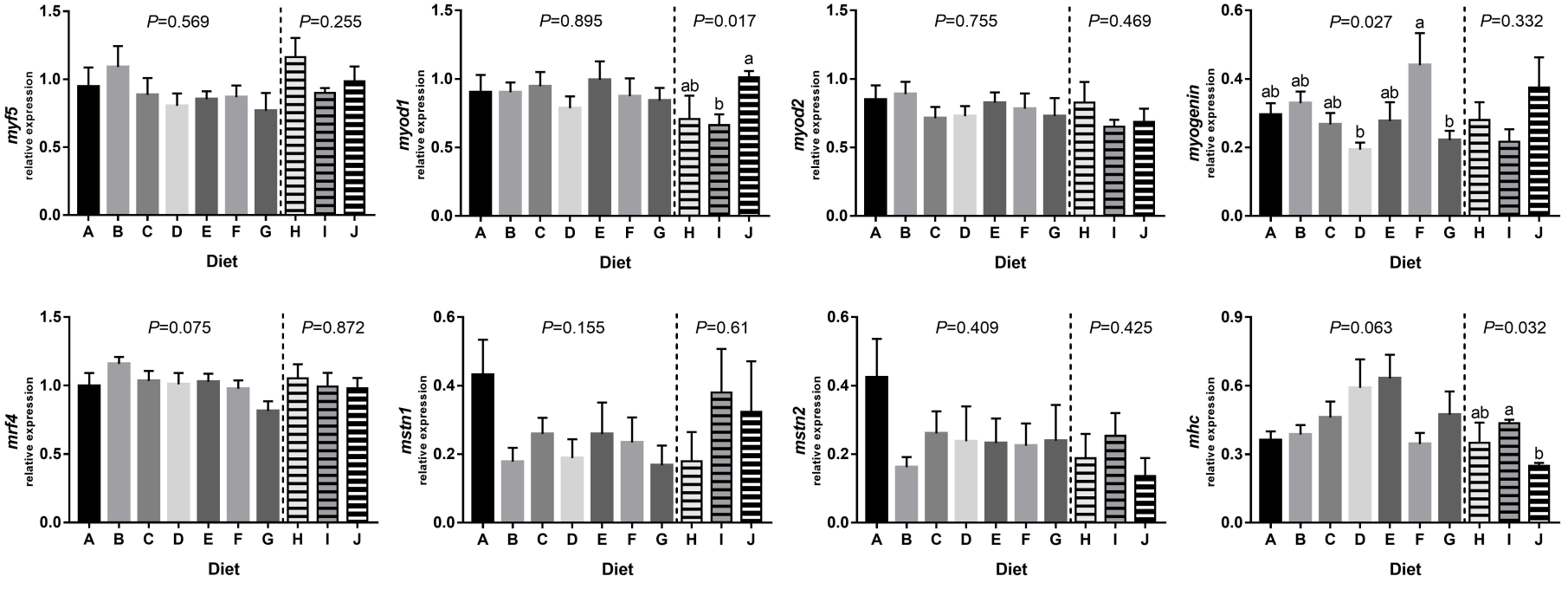
0.8
0.6
0.4
0.2
0.0
A B C D E F G H I J
Diet


cpt1a
relative expression
P=0.021
P=0.252
ab
ab
a
ab
b
ab
ab
1.5
1.0
0.5
0.0
A B C D E F G H I J
Diet


cpt1b
relative expression
P=0.636
P=0.121
1.5
1.0
0.5
0.0
A B C D E F G H I J
Diet


myf5
relative expression
P=0.569
P=0.255
myod1
relative expression
P=0.895
P=0.017
ab
b
a
myod2
relative expression
P=0.755
P=0.469
myogenin
relative expression
P=0.027
P=0.332
ab
ab
ab
b
ab
a
b
mrf4
relative expression
P=0.075
P=0.872
mstn1
relative expression
P=0.155
P=0.61
mstn2
relative expression
P=0.409
P=0.425
mhc
relative expression
P=0.063
P=0.032
ab
a
b
A B C D E F G H I J
Diet