The combination of palm and rapeseed oils emerges as a good dietary alternative for optimal growth and balanced lipid accumulation in juvenile gilthead sea bream reared at an elevated temperature

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Abstract

The aquaculture sector needs to develop new tools to optimally increase production for feeding, while facing future environmental changing conditions. One of the major improvements in this industry has been reducing the usage of fishmeal and fish oil (FO) in diet formulations to promote a more sustainable production. In this line and considering global warming, in the present study, juvenile gilthead sea bream were fed a diet with a 60% of FO substitution by vegetable oils (VO) containing mainly palm oil (P) (80% of the VO content) and maintained at 21 or 28 ºC. Furthermore, in the condition of elevated temperature, fish were fed with two other diets with the same level of FO substitution and containing either 80% of the VO content of rapeseed oil (R) or an equilibrated combination of both VO (named PR). High temperature induced a significant increase in growth, but a reduction in the hepatosomatic and viscerosomatic indexes, and circulating triglycerides. Otherwise, significant differences were not found among the fish fed the three distinct diets at 28 ºC for any of the somatic or plasma parameters analyzed. In the adipose tissue, the higher number of adipocytes of smaller size in fish maintained at 28 ºC compared to fish at 21 ºC, suggested an activation of adipogenesis despite the gene expression of peroxisome proliferator-activated receptor gamma (*pparg*) was significantly decreased. Similarly, lipid droplets of smaller area and downregulation of the fat translocase *cd36* and the fatty acid binding protein *fabp1* were found in the liver of fish reared at 28 ºC compared to those at 21 ºC. Concerning differences among dietary groups, fish fed R diet showed in the liver a more extensive surface area occupied by lipid droplets of bigger size, while contrarily, fish fed PR diet had the smallest area filled with smaller lipid droplets. In adipose tissue, PR diet-fed fish had also adipocytes within the smaller size range. These histological data, together with the significant upregulation of several transcription factors (i.e. *ppars*) and genes related to lipid transport in the two tissues, indicated that the PR diet could be the most equilibrated for gilthead sea bream, to grow at a high rearing temperature while avoiding excessive fat accumulation. Thus, this study highlights the need of fine-tuning the substitution of FO by VO in the elaboration of feeds for this species to assure proper growth and physiological status in a future global warming scenario.

Keywords: fish oil substitution, vegetable oils, adipose tissue, liver, lipid metabolism, temperature, aquaculture.

1. Introduction

Fish production must face the challenge of feeding an increasing world population, which is leading to a major demand of these kind of products, due also to the elevated consumption per capita and their high nutritional value (United Nations, 2016; Godfray *et al.,* 2010). Additionally, the aquaculture sector has to impulse new strategies to increase sustainably the production, assuring a high-quality product, also considering the future environmental challenging conditions.

One of the strategies promoted in the last decades in the aquaculture industry has been to reduce the usage of fishmeal and fish oil (FO) in diet formulations to promote a more sustainable production of fish feeds (Tacon and Metian, 2008). The major approach has been to substitute these limiting products by similar ingredients from plant sources (i.e. vegetable oils, VO), always adjusting the substitution according to the nutritional requirements of each fish species (Kaushik *et al.,* 1995; Rosenlund *et al.,* 2001; Torstensen *et al.,* 2005). These studies proved that the partial substitution of FO by VO up to 66% could guarantee proper growth and fish feed utilization (Benedito-Palos *et al.,* 2008; Betancor *et al.,* 2016; Izquierdo *et al.,* 2000; 2003). Although adverse effects were also described in fish fed diets highly substituted (80 to 100% VO), affecting digestion, absorption, hepatic metabolism, lipid distribution, muscular fatty acid profile and adipose tissue growth (Bell *et al.,* 2001; Bouraoui *et al.,* 2011; Caballero *et al.,* 2002; Jordal *et al*., 2007; Lopes *et al.,* 2017; Montero *et al.,* 2003). Nevertheless, more recent studies performed in gilthead sea bream (*Sparus aurata*) have shown that feeds with less than 7% of marine ingredients could lead to a good growth performance without causing significant metabolic alterations (Simó-Mirabet *et al*., 2019; Gil-Solsona *et al*., 2019).

Unfortunately, fewer studies have evaluated FO substitution in a scenario of altered environmental conditions considering global climate change will increase the mean sea surface temperature more than 2 ºC by the end of this century (IPCC, 2014). In this sense, the influence of different dietary lipid levels or sources together with temperature have been reported on Senegalese sole (*Solea senegalensis*) (Guerreiro *et al.,* 2012) and European sea bass (*Dicentrarchus labrax*) (Vagner *et al.,* 2007). In salmonids, besides the effects on growth and metabolism, changes in fillet fatty acid composition have also been described (Jobling and Bendiksen, 2003; Norambuena *et al.,* 2015; 2016; Tocher *et al.,* 2004; Wijekoon *et al.,* 2014). Consequently, temperature is an important abiotic factor that aquaculture must face of and consider when a VO is selected for a substitution.

Adipose tissue and liver play an important role in lipid metabolism and energetics homeostasis regulation (Choe *et al.,* 2016). The adipose tissue can grow by hyperplasia, which implies the production of new adipocytes via precursor cells (i.e. adipogenesis) and by hypertrophy where lipid accumulation increases cell volume (Otto and Lane, 2005), and both processes can be

affected by VO substitution. In this context, Cruz-Garcia *et al.* (2011) and Torstensen *et al.* (2011) previously demonstrated that highly substituted diets with 66-70% VO and 75-80% plant meal, could lead to hypertrophic adipose tissue growth, therefore causing abnormal fat deposition because of unbalanced lipolysis and lipid accumulation. Besides, these alterations frequently go hand in hand with the presence of a fatty liver (Cruz-Garcia *et al.*, 2011). Thus, due to its function in tissue lipids distribution through lipoproteins, the liver is also a key indicator of the effects caused by dietary FO substitution in fish metabolism and health.

Regarding specifically the fatty acid composition of the different oils used in aquafeeds, FO is rich in n-3 long chain polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, which optimal content and proportion in gilthead sea bream diets has been estimated at 1.9% and 2:1, respectively (Ibeas *et al.*, 1997). In fact, the optimal content of these fatty acids is key given that an absence or a disproportion of these nutrients are known to affect growth and lipid metabolism (Caballero *et al.*, 2003; Montero *et al.*, 2003; Tocher, 2003). On the other hand, the most commonly used VO in feeds formulation are palm (PO), soybean (SO), rapeseed (RO), linseed (LO) and sunflower oils, which are composed by different fatty acids although most of them having n-6 and n-9 PUFA in abundance (Turchini *et al.*, 2010). Otherwise, VO do not have n-3 LC-PUFA, therefore in fish fed diets with a high FO replacement a reduction in the profile and nutritional qualities of the final product has been observed (Rosenlund *et al.*, 2010). Among the VO, RO is generally cheaper and available at greater amounts than FO and is rich in monounsaturated fatty acids (MUFA) (Turchini *et al.*, 2009), which are known to be readily catabolized by β-oxidation (Bell *et al.*, 2001). While PO, that contains about 50% of saturated fatty acids (SFA), is the most abundantly produced VO in the world and one of the cheapest oils in the market, and it has demonstrated to be a potential alternative to FO in salmonids (Ng *et al.*, 2007; Torstensen *et al.*, 2000).

In a recent study by our group in gilthead sea bream fed different VO diets, the fish fed a diet with the combination of PO with other VO, presented higher growth when compared to fish fed diets with blends of several other VO, without affecting the quality of the fillet although accompanied by an excess of adipose tissue (Sánchez-Moya *et al.*, 2020). These data suggested that the inclusion of PO in vegetable diets for gilthead seabream could be suitable at an elevated rearing temperature, when a high metabolic demand occurs. Besides PO has some favorable characteristics such as its resistance to get rancid making the fish feeds more durable. In this context, in some warm-water species, PO also showed some advantages compared to other VO, as for instance an enhancement of flesh quality due to the bioaccumulation in the fillet of antioxidants present in this oil (Ng *et al.*, 2001; Ng *et al.*, 2004; Lim *et al.*, 2001). Added to these interesting properties, both SFA and MUFA, present in PO and RO respectively, are known to be preferred over PUFA for energy production in fish (Henderson, 1996). With all that, the aim of

the present study was to investigate: First, the effects of a high (28 ºC) versus usual (21 ºC) rearing temperature in gilthead sea bream juveniles fed a diet rich in PO. Second, the effects of feeding three different substituted FO diets with either PO, RO or a blend of both VO on fish maintained at a high temperature (28 ºC). Somatic growth parameters and indexes, plasma metabolites, as well as histology and quantitative expression of genes involved in lipid metabolism in adipose tissue and liver were evaluated.

2. Material and methods

2.1. Animals, experimental design and ethics statement.

Juvenile gilthead sea bream (*S. aurata*) were obtained from the fishery Piscimar (Burriana, Spain) and were acclimated to the facilities in the Faculty of Biology at the University of Barcelona for one month. Fish (23.03 ± 0.41 g) were kept in an indoor seawater recirculation system, in 400 or 200 L fiberglass tanks at an initial stocking density of 2,87 Kg/m^3, under a 12 h light/12 h dark photoperiod, and were maintained either at 21 or 28 ºC in two different rooms with controlled temperature and fed three times a day a commercial diet (Optibream, Skretting, Burgos, Spain).

For the experimental trial, three different partially substituted FO diets (with 60 to 65% VO content of the total oils, Table 1) were formulated. Table 2 shows the fatty acid composition of the diets, calculated from the analytical data of the VO used as ingredients. The diets were manufactured by Skretting ARC (Stavanger, Norway), and the minimum requirements of EPA and DHA (Glencross, 2009) were adequately provided by the inclusion of a minimum of a 4.45% of FO in addition to the content included in the fishmeal. Furthermore, the composition of n-3 fatty acids was adjusted to be similar in all diets through the inclusion of slightly different proportions of LO (10-15% of the total oils). It has to be taken into account that besides LO contains more than 40% of n-3 fatty acid series, mainly α-linolenic acid (18:3n-3), PO is rich in SFA and MUFA, mainly palmitic (16:0) and oleic (18:1n-9) acids, respectively, whereas RO has a 50% of MUFA. The diets were administered to four groups of fish for 2 months, from the end of October to the end of December. Two groups of fish were fed with a diet containing PO (P) and were reared at the 2 different temperatures, 21 and 28 ºC; while the remaining groups maintained at 28 ºC were fed with a diet containing either RO (R) or a diet with a combination of PO and RO (PR). Each group of fish was distributed in 3 replicate tanks (1x400 L and 2x200 L) at the corresponding 21 or 28 ºC conditioned rooms. Fish were fed with a constant rate of 2.5% body weight adjusted each week. At the end of the trial, fish were fasted for 24 h before sacrifice in order to avoid contamination of the tissues with contents from the gastrointestinal tract during sampling. Ten fish per group (four or three fish per each 400 L or 200 L tank, respectively) were anesthetized with ethyl 3-aminobenzoate methanesulfonate (MS222) (Sigma–Aldrich, Tres

Cantos, Spain) and blood was extracted from the caudal vein. The blood was centrifuged 10 min at 5000 rpm to separate the plasma, which was stored at -80 ºC until metabolites concentrations were determined. Then, seven fish (three or two fish from the 400 L or 200 L tanks, respectively) were sacrificed by cranial concussion and samples of adipose tissue and liver were snap frozen in liquid nitrogen and stored at -80 ºC until performing gene expression analyses. Furthermore, samples of liver and adipose tissue from four extra fish per condition were taken and fixed in 10% buffered formalin.

All animal handling procedures complied with the Guidelines of the European Union Council (86/609/EU) and were approved by the Ethics and Animal Care Committee of the University of Barcelona (permit numbers CEEA 110/17 and DAAM 9488), following the regulations and procedures established by the Spanish and Catalan governments.

2.2. Growth parameters and plasma analyses

The following parameters and indexes concerning somatic growth were determined or calculated using the indicated formulas: initial body weight (IBW), final body weight (FBW), weight gain (WG) [(FBW-IBW)x100], somatic growth rate (SGR) [(ln (FBW)−ln (IBW))/t×100], body length (BL), condition factor (CF) [(FBW)/(BL)^3×100], hepatosomatic index (HSI) [(Liver weight/FBW)x100], viscerosomatic index (VSI) [(Viscera weight)/FBW)x100] and mesenteric fat index (MFI) [(Mesenteric fat weight/FBW)x100].

Plasma samples were analyzed for metabolites using commercial enzyme kits: non-esterified fatty acids (NEFA-HR2, Wako Chemicals GmbH, Neuss, Germany), and TGs and glycerol (Serum Triglyceride Determination Kit, Sigma-Aldrich, Tres Cantos, Spain), following the manufacturers' indications.

2.3. Histology analyses

Fixed adipose tissue samples were dehydrated in a graded ethanol series and embedded in paraffin. Sections of 4 µm obtained with a microtome (Leica RM2125, Leica Microsystems, Wetzlar, Germany) were stained with a standard haematoxylin and eosin (H&E) protocol and mounted with Entellan.

In the case of liver, pieces of tissue previously fixed from the same fish were transferred to optimal cutting temperature (OCT) compound and frozen. Sections of 10 µm were cut with a cryostat (Leica CM3050, Leica Microsystems, Wetzlar, Germany) and stained with either 0.7% Oil red O (ORO) or 0.5% periodic acid-Shiff (PAS) following previously established protocols (Hui *et al.*, 2017; Mehlem *et al.*, 2013) with little adaptations. Briefly, for ORO staining, slides with 2-4 sections of the tissue were dried at room temperature, rinsed in PBS, placed in warmed propylene glycol and finally in ORO staining solution at 60 ºC. The slides were washed, haematoxylin

staining was performed, followed by washes, and then were consequently mounted with Kaiser's glycerol gelatin. For PAS staining, slides with also 2-4 tissue sections were dried at room temperature, rinsed in PBS, placed in PAS solution, washed and finally submerged in Schiffs' reactive. The slides were then washed, and haematoxylin staining was performed followed by washes, dehydration and mounting with Entellan.

All preparations were observed under a light microscope and photographed (Olympus PM10SP Automatic Photomicrography System). For the quantification of the lipid or glycogen content, 4 images from different tissue sections of each fish were used and analyzed with ImageJ (National Institutes of Health, United States). All reagents for histology staining were purchased from Sigma–Aldrich (Tres Cantos, Spain).

2.4. RNA extraction and cDNA synthesis

Frozen pieces of adipose tissue (100 mg) and liver (50 mg) were homogenized in 1 mL of TRIzol®Reagent (Invitrogen, Alcobendas, Spain), using the Precellys Evolution technology (Bertin Instruments, Montigny-le-Bretonneux, France). Total RNA was extracted according to the manufacturer's recommendations, dissolved in DEPC-treated water (RNase-free), quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain) and stored at −80 °C. To eliminate any residual genomic DNA, total RNA (1 µg) was treated with DNase I (Invitrogen, Alcobendas, Spain) and converted into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Valles, Spain), following the manufacturer's instructions.

2.5. Real-time quantitative PCR (qPCR) analyses

The key genes implicated in adipogenesis and energy metabolism regulation and analyzed by qPCR comprise the following (Table 3): The transcription factors or nuclear receptors: peroxisome proliferator-activated receptors (*ppara*, *pparb*, *pparg*) and liver X receptor (*lxr*); the enzymes: fatty acid synthase (*fas*), lipoprotein lipase (*lpl*) and hormone-sensitive lipase (*hsl*); and the fatty acid transporters: fat translocase/cluster of differentiation (*cd36*), fatty acid transport protein 1 (*fatp1*) and fatty acid binding protein 11 (*fabp11*). Reference genes analyzed were *b-actin*, elongation factor 1 alfa (*ef1a*) and ribosomal protein S18 (*rps18*). qPCR analyses and preliminary validation assays were performed as described in Riera-Heredia *et al*. (2018; 2019). The stability of the reference genes as well as the mRNA levels of expression of the genes of interest calculated relative to the most stable reference genes (geometric mean of *ef1a* and *rps18*) according to the Pfaffl method (Pfaffl, 2001) were determined using the CFX Manager Software implemented in the CFX384 thermocycler (Bio-Rad, El Prat de Llobregat, Spain).

2.6. Statistical analyses

First, data outliers were identified with boxplots and the interquartile method (IQR) with fences (Hubert and Vandervieren, 2008). Then, data normality and homoscedasticity were assessed using Shapiro–Wilk and Levene's test, respectively. Independent samples' Student's *t*-test was used for comparison between two groups (fish fed diet P at different temperatures). For multiple mean comparisons (among the three dietary groups at the same temperature) of normal distributed data, one-way ANOVA was used followed by Tukey's or Dunnett's T3 *post hoc* tests in case of homogeneous or heterogeneous variance data, respectively. When data did not fit normal distribution, the non-parametric Kruskal–Wallis test, followed by Mann–Whitney test, were used. Statistical analyses were performed using SPSS Statistics version 22 (IBM, Armonk, NY, USA). Results are presented as mean ± SEM, and $P < 0.05$ was considered to indicate a statistically significant difference. Graphs were generated using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

3. Results

3.1. Somatic growth parameters and plasma metabolites in response to temperature and diet.

The fish fed the P diet presented significant differences in growth parameters and somatic indexes when the animals reared at the two different temperatures were compared (Table 4). Fish fed with P diet at 28 ºC showed significantly higher FBW, WG, SGR and BL, and significantly lower HSI and VSI values when compared to fish fed diet P at 21 ºC. Nevertheless, significant differences were not observed in CF or MFI, despite lower values were found in fish reared at high temperature regarding mesenteric fat.

When comparing the fish reared at 28 ºC and fed the three different experimental diets, significant differences were not found, although for FBW, WG and HSI slightly higher values were observed in gilthead sea bream fed diet P in comparison with the fish fed the R or PR diets. Contrarily, VSI and MFI values where lower in fish fed with diet P than in those fed the other two diets.

In relation to plasmatic metabolite levels, the effects of temperature in fish fed with the P diet showed significant differences in TGs being lower in animals maintained at 28 ºC (Fig. 1A), while NEFAs presented the same tendency, but the change was not significant (Fig. 1B). Moreover, significant differences were not found in glycerol levels between the fish fed with diet P at the two temperatures tested (Fig. 1C), and neither between the fish fed the different experimental diets and reared at 28 ºC for none of the plasma parameters analyzed (Fig. 1A-C).

3.2. Adipose tissue histological changes in response to temperature and diet.

H&E staining of adipose tissue slices showed differences due to rearing temperature in fish fed diet P. Gilthead sea bream at 28 ºC showed a significantly higher percentage of smaller adipocytes (with less than 0.01 mm^2) compared with fish at 21 ºC, and despite not being significant, also presented a reduced percentage of bigger adipocytes (Figure 2B). Concerning to the differences observed in response to diet in fish reared at 28 ºC, again fish fed the PR diet presented significantly more smaller adipocytes than those animals fed the diet R, showing the fish fed the P diet an intermediate value (Figure 2B).

3.3. Liver histological changes in response to temperature and diet.

The images obtained after ORO staining of the liver sections of fish upon all treatments (P at 21 ºC and P, R and PR at 28 ºC) showed visual differences in lipid accumulation (Fig. 3A). Moreover, significant differences in the number and size of lipid droplets (LD) and the total lipid content were determined. Specifically, when comparing livers from fish fed with the P diet at the two temperatures, those from fish maintained at 28 ºC presented a significantly bigger area occupied by lipids (Fig. 3B), with a higher number of LD (Fig. 3C) but, of smaller size (with less than 10^{-2} mm^2) than those present in fish reared at 21 ºC (Fig. 3E). Regarding livers from fish fed with the P, R or PR diets at 28 ºC, differences in the surface area occupied by LD were observed. The R diet-fed fish, was the group with significantly greater area containing LD, then the P group, and finally the PR group, which showed similar levels as the fish fed diet P and reared at 21 ºC (Fig. 3B). In addition, the fish fed with the R diet was also the one that presented the greatest number of LD (Fig. 3C). But, when analyzing the size distribution of LD (Fig. 3D), the PR diet-fed group presented significantly the highest number of LD of smaller size (less than 10^{-3} mm^2), and a lower percentage of larger LD. Moreover, the fish fed diet R, presented significantly more LD of bigger size (more than 0.1 mm^2) compared to the other groups of fish at 28 ºC. Contrarily, PAS staining did not show significant differences among groups, although fish at 28 ºC fed the R diet seemed to accumulate less glycogen when compared with gilthead sea bream fed the P or PR diets (Figs. 3E and 3F).

3.4. Gene expression changes in adipose tissue and liver in response to temperature.

Gene expression of transcription factors, enzymes and fatty acid transporters was analyzed in adipose tissue and liver samples from fish fed the P diet and reared at 21 or 28 ºC. The transcription factors *pparb*, *pparg* and *lxr* were downregulated in both tissues in the high-temperature group, although only *pparg* was significantly reduced in the adipose tissue (Figs. 4A and 4D). Regarding the enzymes related with lipid metabolism, significant differences in expression were not observed in either tissue (Figs. 4B and 4E). Concerning fatty acid transporters, similar tendencies of reduced expression were found in both tissues with the increase

in temperature, although only *cd36* was significantly downregulated in the two tissues and *fabp11a* in the liver (Figs. 4C and 4F).

3.5. Gene expression changes in adipose tissue and liver in response to diet at 28 ºC.

The same clusters of genes were analyzed in the fish fed the R and PR diets and compared with the animals fed the P diet at 28 ºC. Gene expression of transcription factors *pparb* and *pparg* was significantly increased in the adipose tissue of gilthead sea bream fed PR diet, and *ppara, pparb* and *lxr* in the liver compared with the P diet-fed fish (Figs. 5A and 5D). Moreover, in adipose tissue, *pparb* and *pparg* transcript levels, as well as those of *pparg* and *lxr* in the liver, were also significantly higher in fish fed the PR diet when compared with the R diet-fed fish. Concerning the gene expression of lipid metabolism-related enzymes, in adipose tissue an upregulation of *fas, lpl* and *hsl* expression caused by diet PR was found compared to that in fish fed the P or R diets, although increased mRNA levels were only significant for *hsl* (Fig. 5B). On the other hand, differences were not observed among the three dietary groups in the liver (Fig. 5E). With respect to fatty acid transporters' expression, a generalized upregulation was observed in the adipose tissue of the PR diet-fed fish when compared to the other two groups although only *fabp11* presented significantly higher mRNA levels (Fig. 5C). In the liver, PR diet-fed fish presented a significant upregulation in *cd36* and *fatp1* expression when compared to the other two groups of fish; while R diet-fed fish showed a significant downregulation compared to fish fed P diet in *cd36* expression (Fig. 5F).

4. Discussion

The present study aimed to elucidate the effects of FO substitution by different VO in a future scenario of increased water temperature. Further knowledge is required to take preventive measures to deal with global climate change at the level of feeds formulation to optimize sustainable aquaculture production of gilthead sea bream assuring fish welfare and product quality.

In this context, elevated temperature (28 ºC) significantly increased somatic growth, while decreased VSI, HSI and MFI in gilthead sea bream in comparison with fish reared at 21 ºC feeding the same diet. According to this, studies on Atlantic salmon (Handeland *et al.*, 2000; 2003; 2008), Senegalese sole (Guerreiro *et al*., 2012), sea bass (Vagner *et al*., 2007) and gilthead sea bream (Guillaume *et al.,* 2004) previously demonstrated that growth is increased by elevated temperatures, as expected in agreement with a boosted fish metabolism. Nevertheless, unfavorable conditions for balanced musculoskeletal growth together with accelerated utilization of lipids can be also induced by high temperatures compared to optimal (Balbuena-Pecino *et al*.,

2019). In addition, other factors can be involved in pushing growth enhancement, since temperature is well known to be key in the digestibility of fatty acids, which is lower with increasing saturation and chain length, and reduced temperature (Caballero *et al.,* 2002; Guillaume *et al.,* 2004; Ng *et al.,* 2004; Olsen and Ringø, 1998; Torstensen *et al.,* 2000). Thus, the importance of VO substitution at different temperatures remains partly in the melting point of the fatty acids present in each oil (Guillaume *et al.,* 2004). This is in concordance with the results found in our study, in which fish reared at elevated temperatures presented higher growth rates, possibly as an indicative of greater feed efficiency of dietary lipids at 28 ºC, even when fish were fed the P diet, containing a high content of SFA. Regarding HSI, this parameter was demonstrated to be also influenced by temperature in Kumgang Fat Minnow (*Rhynchocypris kumgangensis*) (Im *et al.,* 2016) according to our findings, where fish at 28 ºC presented lower HSI when compared to fish at 21 ºC. Moreover, in gilthead sea bream reared at a higher temperature, the decrease in VSI and MFI could be linked to enhanced metabolic activity in visceral adipose tissue depots. A similar decrease in visceral mass in response to temperature was also previously shown in other fish species such as sole (Guerreiro *et al*., 2012).

Furthermore, fish fed with diet P at 28 ºC presented lower plasma levels of TGs in comparison with fish maintained at 21 ºC and slightly decreased circulating NEFAs, suggesting better metabolic performance. Ongoing with these considerations, Ng *et al.* (2003) described in rainbow trout fed a PO diet at a low temperature an increased presence of undigested TGs in feces, therefore supporting that a high temperature appears to improve lipid digestibility, promoting quicker TGs absorption in the gut and plasma clearance. Moreover, in salmon, stomach evacuation has been shown to be faster at high temperatures (Handeland *et al.,* 2008). Therefore, the differences observed in our experiment in circulating metabolites could have been also consequence of such faster evacuation, and thus, accelerated nutrient absorption by the intestine and peripheral tissues in the gilthead sea bream maintained at 28 ºC. Besides, other parameters as animal size can also affect the response in front of temperature changes, as seen in juvenile Atlantic cod (*Ghadus morua* L.), in which warm temperatures were shown to be favorable in smaller animals whereas in larger specimens were not (Tirsgaard *et al.,* 2015).

Respecting the effects of temperature in adipose tissue and liver at a histological level, fish maintained at 28 ºC presented more smaller adipocytes as well as smaller hepatic LD than those fish reared at 21 ºC. These data suggested an induction of adipogenesis with increased formation of new adipocytes, and enhanced lipid breakdown or disminished uptake in the liver (although the total surface occupied by LD in the latter was higher at 28 ºC). These observed histological features were not completelly explained by changes in relative gene expression. In fact, elevated temperature downregulated almost all genes involved in fatty acid metabolism in either adipose tissue or liver when compared to fish maintained at a low temperature. In fish fed the P diet and

maintained at 28 ºC, contrarily to the potential adipocyte hyperplasia observed, gene expression of the key regulator of adipogenesis *pparg* was reduced when compared to fish at 21 ºC. In fact, the pattern of expression of this transcription factor in cultured adipocytes of different fish species in the presence of lipids in the medium, has shown only a transient increased response, but no clear changes in the long term during the adipogenic process (Bou *et al.*, 2017; Salmerón *et al.*, 2016; Riera-Heredia *et al*., 2020). On the other hand, downregulated expression of fatty acid transport genes in both adipose tissue and liver seems to be in accordance with the reduced MFI and HSI at 28 ºC. In fact, in addition to the expected increase in metabolism due to elevated temperatures, it has been suggested that fish increase their swimming activity (Kieffer *et al.*, 1998), which in turn should increase energy consumption, especially in skeletal muscle, primarily through lipid β-oxidation (Nordgarden *et al.*, 2003). Moreover, in darkbarbel catfish (*Pelteobagrus vachellii*) it has been described a reduction in cholesterol and TGs levels in the liver at elevated temperatures, suggesting an increase of hepatic lipid metabolism, thereby accelerating the absorption and utilization of lipids and leading to decreased fat deposition and a low HSI (Qiang *et al.*, 2017), in concordance with our data. Altogether, these results suggest that high temperatures, even in fish fed lipid content from VO in the diet, seem to be beneficial to obtain good growth rates and proper metabolic function, as well as fish with healthy adipose tissue, through enhanced adipogenesis as shown in humans (Choe *et al.*, 2016).

With respect to feeding the different substituted diets P, R or PR in fish reared at 28 ºC, differences in growth were not observed despite of fish fed P diet presented slightly increased WG but decreased VSI and MFI. Likewise, significant increased growth in gilthead sea bream fed with PO was seen in a recent study where 10 experimental diets with different VO blends were tested under normal farming conditions (Sánchez-Moya *et al.*, 2020). Actually, several studies have proved in different fish species that PO can improve growth performance or at least not to affect it negatively (Fonseca-Madrigal *et al.*, 2005; Lim *et al.*, 2001; Ng *et al.*, 2003, 2004; Rosenlund *et al.*, 2001; Torstensen *et al.*, 2000). Nevertheless, in our study, this result was not significant, in concordance with others in which fish fed different VO substituted diets (up to 69%) presented similar or poorer growth performance, feed and protein utilization, when feeding PO compared to diets with RO or SO (Rosenlund *et al.*, 2001; Izquierdo *et al.*, 2003, 2005; Fountoulaki *et al.*, 2009). The differences found between studies could be the result of different fish size (Tirsgaard *et al.*, 2015), percentatge of substitution and type of PO fraction used (Lim *et al.*, 2001; Ng and Gibon, 2010),

The tissue lipid composition is known to be affected by the FO substitution with VO (Francis *et al.*, 2006; Izquierdo *et al.*, 2005; Montero *et al.*, 2005). In Atlantic salmon, the effect of different VO replacements was reflected in the total lipid present in the liver being the diet with RO inclusion the responsible of increased lipid accumulation (Bell *et al.*, 2001; Karalazos *et al.*, 2007;

405 Ruyter *et al.*, 2006; Torstensen *et al.*, 2004). In the present study, liver histology showed that fish
406 fed with R diet had more LD (in number and area occupied by LD), which is in agreement with
407 those studies. The possible explanations of this increased lipid accumulation in hepatocytes could
408 be associated to impaired lipoprotein synthesis (Caballero *et al.*, 2006), or an imbalance between
409 hepatic lipid synthesis, oxidation and their export to other tissues (Postic and Girard, 2008). High
410 lipid hepatic levels have been related with unbalanced n-3/n-6 ratios or MUFA content derived
411 from some dietary substitutions of FO or by the increased amount of n-9 oleic acid present in
412 some VO (Robaina *et al.*, 1998; Wassef *et al.*, 2007). In this context, Fountoulaki *et al.* (2009)
413 concluded that high levels of oleic acid were responsible for the excessive hepatic lipid
414 accumulation observed in sea bream, over fish fed soya or RO diets. Otherwise, smaller
415 adipocytes as well as less area occupied by also smaller LD in the liver was found in fish fed PR
416 diet compared to the other dietary groups at 28 ºC. In mammals, a higher presence of smaller
417 adipocytes reflects the possible increased capacity of the tissue to recruit and develop new
418 adipocytes avoiding a damaging hypertrophy (Medina-Gomez and Vidal-Puig, 2005). Contrarily
419 to the present data, in a previous study in gilthead sea bream fed diets with 66% of FO substitution
420 by a mixture of oils with LO as a principal VO, an enlargement of adipocytes was observed
421 impairing the storage capacity of this tissue (Cruz-García *et al.*, 2011). Thus, the specific
422 combination of dietary VO can differentially affect adipocyte dynamics. On the other hand, the
423 reduced hepatic area occupied by also smaller LD in the fish fed diet PR, could possibly be due
424 to increased fatty acid oxidation. In support of this, Yan *et al.*, (2015) observed in large yellow
425 croaker (*Larimichthys crocea*) liver increased lipid accumulation when fatty acid oxidation
426 activity was decreased. At a transcriptional level, fish fed the PR diet showed a general activation
427 of lipid metabolism by the upregulated expression of *pparb*, *pparg*, *hsl* and *fabp11* in adipose
428 tissue and *pparg*, *cd36* and *fatp1* in liver. These results suggested an increase in both, fatty acids
429 uptake and lipogenesis, as well as mobilization and use, although according to the histological
430 data, the lipid turnover would be directed towards catabolism.

431 In a recent study (Torrecillas *et al.*, 2017), a blend of LO, RO and PO was produced as a FO
432 substitution for being a combination assuring a balance of SFA and MUFA in European sea bass
433 tissues. Up to 60% FO substitution in this species by two mixtures of these three VO did not cause
434 a marked effect on growth, lipogenesis and tissue lipid uptake when compared to a 100% FO diet
435 (Richard *et al.*, 2006). In gilthead sea bream impairment of lipogenic activity and lipid content in
436 fish liver was not detected when using a blend of PO and LO (4:1) to replace FO (Bouraoui *et al.*,
437 2011). Recently, in the same species, fish fed with different oil blends demonstrated to avoid
438 damaging effects of individual substitutions of VO, as RO, lowering its proportion by
439 combination with LO or PO (Sánchez-Moya *et al.*,2020), in support of the findings of the present
440 study.

Overall, the combination of fatty acids from PO and RO in the formulation of the PR diet seems to be the most equilibrated regarding histological data and metabolic status, bringing the gilthead sea bream to grow properly and have balanced lipid accumulation even at an elevated rearing temperature. Specifically, this blended diet could be appropriate for periods with higher temperatures (i.e. summer), as in fact, administration of different diets depending on the season or physiological condition of the fish is a common practice in farms also in the case gilthead sea bream production. Thus, the present study confirms the need to take into account the finetuning of plant components in the elaboration of sustainable feeds for gilthead sea bream aquaculture, especially in a future scenario of global warming.

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740

741 **Tables**

742 **Table 1.** Composition of the experimental diets. Palm (P); Rapeseed (R); Palm + Rapeseed (PR).

Ingredients (%)	**P**	**R**	**PR**
Wheat	6.83	6.83	6.83
Corn meal	10	10	10
Wheat gluten	14.79	14.78	14.78
Soya protein	25	25	25
Broad beans	10	10	10
Fish meal	20	20	20
Fish oil	5.34	5.34	4.45
Linseed oil	1.33	2	1.33
Rapeseed oil	0	6.67	3.34
Palm oil	6.67	0	3.55
Phosphate	0.03	0.03	0.03
Composition (%)			
Moisture	7.6	7.7	7.5
Protein	51.9	51.8	51.7
Fat	19.2	18.9	19.4
Ash	5.7	5.7	5.6

743

744

745 **Table 2.** Fatty acid composition of the experimental diets. Palm (P); Rapeseed (R); Palm +
746 Rapeseed (PR).

Fatty acids (%)	P	R	PR
C14:0	3.40	3.00	2.87
C16:0	25.12	9.88	17.43
C16:1 n-7	3.43	3.41	3.02
C16:2 n-6	0.30	0.30	0.26
C18:0	3.02	1.99	2.62
C18:1 n-9	22.98	30.72	27.75
C18:1 n-7	1.44	2.49	1.90
C18:2 n-6	7.13	10.94	9.74
C18:3 n-3	5.50	9.49	9.72
C18:4 n-3	2.40	2.39	2.11
C20:1 (sum. isomers)	3.65	4.16	3.49
C20:4 n-6	0.17	0.17	0.15
C20:4 n-3	0.41	0.41	0.36
C20:5 n-3 (EPA)	5.42	5.43	4.76
C22:1 (sum. isomers)	5.27	5.43	4.70
C22:5 n-3	0.48	0.48	0.43
C22:6 n-3 (DHA)	5.59	5.60	4.91
C24:1 n-9	0.38	0.43	0.36
Unknown	3.91	3.28	3.42
Sum. saturated FA	31.54	14.88	22.92
Sum. monoenes	37.16	46.65	41.22
Sum n-6 FA	7.60	11.41	10.16
Sum n-3 FA	19.18	23.79	22.29
UFA/SFA	2.04	5.50	3.21
n-3/n-6	2.60	2.08	2.19

747 EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; FA: Fatty acids; SFA: Saturated fatty
748 acids; UFA/SFA: Unsaturated fatty acids / Saturated fatty acids.

749 **Table 3.** Primers used for real-time quantitative PCR. F, forward primer; R, reverse primer; Ta,
750 annealing temperature; Acc. Num., accession number.

751

Gene	Primer sequence (5'→3')	Ta (ºC)	Acc. Num.
ppara	**F:** TCTCTTCAGCCCACCATCCC	62	AY590299
	R: ATCCCAGCGTGTCGTCTCC		
pparb	**F:** AGGCGAGGGAGAGTGAGGATGAGGAG	69	AY590301
	R: CTGTTCTGAAAGCGAGGGTGACGATGTTTG		
pparg	**F:** CGCCGTGGACCTGTCAGAGC	66	AY590304
	R: GGAATGGATGGAGGAGGAGGAGATGG		
lxr	**F:** GCACTTCGCCTCCAGGACAAG	62	FJ502320
	R: CAGTCTTCACACAGCCACATCAGG		
fas	**F:** TGGCAGCATACACACAGACC	60	AM952430
	R: CACACAGGGCTTCAGTTTCA		
lpl	**F:** GAGCACGCAGACAACCAGAA	60	AY495672
	R: GGGGTAGATGTCGATGTCGC		
hsl	**F:** GCTTTGCTTCAGTTTACCACCATTTC	60	EU254478
	R: GATGTAGCGACCCTTCTGGATGATGTG		
cd36	**F:** GTCGTGGCTCAAGTCTTCCA	60	ERR12611_isotig20793
	R: TTTCCCGTGGCCTGTATTCC		
fatp1	**F:** CAACAGAGGTGGAGGGCATT	60	ERR12611_isotig43042
	R: GGGGAGATACGCAGGAACAC		
fabp11	**F:** CATTTGAGGAGACCACCGCT	60	ERR12611_isotig32312
	R: ACTTGAGTTTGGTGGTACGCT		
b-actin	**F:** TCCTGCGGAATCCATGAGA	60	X89920
	R: GACGTCGCACTTCATGATGCT		
ef1a	**F:** CTTCAACGCTCAGGTCATCAT	60	AF184170
	R: GCACAGCGAAACGACCAAGGGGA		
rps18	**F:** AGGGTGTTGGCAGACGTTAC	60	AM490061
	R: CTTCTGCCTGTTGAGGAACC		

752

753

Table 4. Growth parameters and somatic indexes from fish fed with the experimental diets P at 21 ºC or P, R and PR at 28 ºC for 2 months. Data are shown as mean ± SEM (n=3 tanks). Asterisks indicate significant differences between fish fed with P diet at different temperatures. Differences were not observed between diets at elevated temperature ($p<0.05$). Initial body weight (IBW); Final body weight (FBW); Weight gain (WG) [(FBW-IBW)x100]; Somatic growth rate (SGR) [(ln (FBW)−ln (IBW))/time×100] (time: 53 days); Body length (BL); Condition factor (CF) [(FBW)/(BL)3×100]; Hepatosomatic index (HSI) [(Liver weight/FBW)x100]; Viscerosomatic index (VSI) [(Viscera weight)/FBW)x100]; Mesenteric fat index (MFI) [(Mesenteric fat weight/FBW)x100]. Palm (P); Rapeseed (R); Palm + Rapeseed (PR).

	21ºC	**28ºC**		
	P	**P**	**R**	**PR**
IBW (g)	24.21 ± 0.57	22.89 ± 0.40	22.80 ± 0.75	22.24 ± 0.77
FBW (g)	54.32 ± 1.78*	68.02 ± 2.34	63.33 ± 2.09	62.21 ± 3.80
WG (%)	124.3 ± 5.2*	197.5 ± 13.3	177.8 ± 2.3	179.2 ± 7.6
SGR (%)	1.52 ± 0.04*	2.05 ± 0.08	1.93 ± 0.02	1.94 ± 0.05
BL (cm)	15.06 ± 0.02*	16.55 ± 0.11	16.37 ± 0.07	16.23 ± 0.22
CF (%)	1.59 ± 0.06	1.5 ± 0.04	1.44 ± 0.03	1.45 ± 0.03
HSI (%)	1.62 ± 0.05*	1.05 ± 0.12	0.90 ± 0.03	0.90 ± 0.05
VSI (%)	7.79 ± 0.51*	5.94 ± 0.17	6.33 ± 0.20	6.34 ± 0.19
MFI (%)	1.64 ± 0.16	1.17 ± 0.15	1.49 ± 0.03	1.43 ± 0.13

766 **Figure legends**

767 **Figure 1.** Plasma metabolites levels in fish fed with the experimental diets P at 21 ºC (n=9) or P
768 (n=8-7), R (n=2-4) or PR (n=7-10) at 28 ºC for 2 months: **(A)** TGs, **(B)** NEFAs and **(C)** Glycerol.
769 Data are shown as mean ± SEM. Asterisks indicate significant differences between fish fed with
770 P diet at different temperatures. Differences were not observed between diets at elevated
771 temperature ($p<0.05$). Although it should be taken into account that only 2 samples of plasma
772 were available for the fish fed diet R after the statistical analysis. Triglycerides (TGs); Non-
773 esterified fatty acids (NEFAs); Palm (P); Rapeseed (R); Palm + Rapeseed (PR).

774 **Figure 2. (A)** Representative images of hematoxylin and eosin (H&E) staining of adipose tissue
775 sections and **(B)** adipocytes size distribution from fish fed with the experimental diets P at 21 ºC
776 (n=4) or P (n=4), R (n=3) or PR (n=4) at 28 ºC for 2 months. Magnification 20X. Data are shown
777 as mean ± SEM. Asterisks indicate significant differences between fish fed with P diet at different
778 temperatures. Different letters indicate significant differences between fish fed the different diets
779 at elevated temperature ($p<0.05$). Palm (P); Rapeseed (R); Palm + Rapeseed (PR).

780 **Figure 3. (A)** Representative images of Oil red O (ORO) staining of liver sections from fish fed
781 with the experimental diets P at 21 ºC (n=4) or P (n=3), R (n=4) or PR (n=4) at 28 ºC for 2 months.
782 **(B)** Surface area occupied by lipid droplets (LD), **(C)** number of LD and **(D)** LD size distribution.
783 **(E)** Representative images of Periodic acid–Schiff (PAS) staining of liver sections from fish fed
784 with the experimental diets P at 21 ºC or P, R or PR at 28 ºC for 2 months. **(F)** Area occupied by
785 glycogen. Data are shown as mean ± SEM (n=3-4). Asterisks indicate significant differences
786 between fish fed with P diet at different temperatures. Different letters indicate significant
787 differences between fish fed the different diets at elevated temperature ($p<0.05$). Images
788 magnification 20X. Palm (P); Rapeseed (R); Palm + Rapeseed (PR).

789 **Figure 4.** Relative expression of transcription factors **(A, D)**, lipid metabolism-related genes **(B,**
790 **E)** and fatty acid transporters **(C, F)** in adipose tissue **(A-C)** (P at 21 ºC n=7; P at 28 ºC n=6-7)
791 and liver **(D-F)** (P at 21 ºC n=6-7; P at 28 ºC n=6-7) of fish fed with P diet at 21 ºC and 28 ºC for
792 2 months. Data are shown as mean ± SEM. Asterisks indicate significant differences between fish
793 reared at different temperatures ($p<0.05$). Palm (P).

794 **Figure 5.** Fold change expression of transcription factors **(A, D)**, lipid metabolism-related genes
795 **(B, E)** and fatty acid transporters **(C, F)** in adipose tissue **(A-C)** (R n=7; PR n=3-6) and liver **(D-**
796 **F)** (R n=2-6; PR n=4-6) of fish fed with the experimental diets R or PR respect to fish fed diet P
797 (dotted line) and reared at 28 ºC for 2 months. Data are shown as mean ± SEM. Asterisks indicate
798 significant differences between fish fed with R or PR diets compared to diet P-fed fish. Hash
799 indicate significant differences between fish fed R or PR diets ($p<0.05$). Although it should be

taken into account that for some genes, only 2 samples of liver tissue were available for the fish fed diet R after the statistical analysis. Palm (P); Rapeseed (R); Palm + Rapeseed (PR).