Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry and molecular growth-related markers in gilthead sea bream (*Sparus aurata*)

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Abbreviations: ALAT, alanine aminotransferase; ALP, alkaline phosphatase; ASAT, aspartate aminotransferase; CAPN1, calpain 1; CAPN2, calpain 2; CAPN3, calpain 3; CAST, calpastatin; CAV3, caveolin 3; CDH15, cadherin 15; COXI, cytochrome c oxidase subunit I; CPT1A, carnitine palmitoyltransferase 1A; CS, citrate synthase; CTSB, cathepsin B; CTSD, cathepsin D; CTSL, cathepsin L; CTSS, cathepsin S; CUL2, cullin 2; CUL3, cullin 3; CUL5, cullin 5; DER-1, derlin-1; DES, desmin; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; FAME, fatty acid methyl ester; FE, feed efficiency; FM, fish meal; FO, fish oil; FST, follistatin; GHR-I, growth hormone receptor I; GHR-II, growth hormone receptor II; GLDH, glutamate dehydrogenase; GRP-94, glucose-regulated protein, 94 kDa; GRP-170, glucose-regulated protein, 170 kDa; Hb, haemoglobin; Hc, haematocrit; HDL, high density lipoprotein; HSI, hepatosomatic index; Hsp30, 30 kDa heat shock protein; Hsp90α, 90 kDa heat shock protein alpha 1; Hsp90β, 90 kDa heat shock protein beta; IGF-I, insulin-like growth factor-I; IGF-II, insulin-like growth factor-II; IGFALS, insulin-like growth factor-binding protein complex acid labile subunit; IGFBP1, insulin-like growth factor binding protein 1; IGFBP2, insulin-like growth factor binding protein 2; IGFBP4, insulin-like growth factor binding protein 4; IGFBP7, insulin-like growth factor binding protein 7; IGFR1, insulin-like growth factor receptor I; IGFR2, cation-independent mannose-6-phosphate receptor; IL-1β, interleukin-1 beta; IL-1R1, interleukin-1 beta receptor 1; IL-1R2, interleukin-1 beta receptor 2; IL-6, interleukin-6; IL-6RA, interleukin-6 receptor A; IL-6RB, interleukin-6 receptor B; IL-8, interleukin-8; IL-8RA, interleukin-8 receptor A; IL-10, interleukin-10; IL-10RA, interleukin-10 receptor A; IL-10RB, interleukin-10 receptor B; INSR, insulin receptor; IGF-I, insulin-like growth factor-I; LC-PUFA, long chain polyunsaturated fatty acid; LDL, low density lipoprotein; LXRα, liver X receptor α; MEF2A, myocyte-specific enhancer factor 2A; MEF2C, myocyte-specific enhancer factor 2C; MET, c-met/hepatocyte growth factor receptor; MSI, mesenteric index; MSTN, myostatin; mtHsp10; 10 kDa heat shock protein, mitochondrial; mtHsp60, 60 kDa heat shock protein, mitochondrial; mtHsp70, 70 kDa heat shock protein, mitochondrial; Myf5, myogenic factor 5; Myf6, myogenic factor 6; MyoD1, myoblast determination protein 1; MyoD2, myoblast determination protein 2; ND2, NADH-ubiquinone oxidoreductase chain 2; NDUFAF2, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 2; OXPHOS, oxidative phosphorylation; PAX7, paired box 7; PCNA, proliferating cell nuclear antigen; PGC1α, proliferator-activated receptor gamma coactivator 1 alpha; PPARα, peroxisome proliferator-activated receptor α; PPARγ, peroxisome proliferator-activated receptor γ; PSD12, 26S proteasome non-ATPase regulatory subunit 12; PSMA5, proteasome subunit alpha type-5; PSMB1, proteasome subunit beta type-1; PSMD4, 26S proteasome non-ATPase regulatory subunit 4; RB, respiratory burst; RBC, red blood cells; RIA, radioimmunoassay; SCO1, SCO1 protein homolog mitochondrial; SGR, specific growth rate; SIRT1, sirtuin1; SIRT2, sirtuin2; SIRT3, sirtuin3; SIRT4, sirtuin4; SIRT5, sirtuin5; SOX3, transcription factor SOX3; TG, triglycerides; TNFα, tumor necrosis factor-alpha; TRADD, tumor necrosis factor receptor type 1-associated death domain protein; UBE2A, ubiquitin-conjugating enzyme E2 A; UBE2D2, ubiquitin-conjugating enzyme E2 D2; UBE2L3, ubiquitin-conjugating enzyme E2 L3;

UBE2N, ubiquitin-conjugating enzyme E2N; UCHL3, ubiquitin carboxyl-terminal hydrolase isozyme L3; UCP1, uncoupling protein 1; UCP2, uncoupling protein 2; UCP3, uncoupling protein 3; VLDL, very low density lipoprotein; VO, vegetable oils; VSI, viscerosomatic index.

Abstract

Four isoproteic/isolipidic plant protein-based diets were formulated to assess the lasting effects of feed additives and low fish meal (FM) and fish oil (FO) diet formulations on gilthead sea bream growth performance. FM was included at 23% in the control diet (D1) and at 3% in the other three diets (D2, D3, D4). Added oil was either FO (D1) or a blend of vegetable oils replacing 58% (D2) and 84% (D3, D4 diets) of FO. A commercial sodium butyrate preparation (NOREL, 70-BP) was added to the D4 diet at 0.4%. Each diet was allocated to triplicate groups of juvenile fish fed to satiety over an 8-month feeding trial (May-December). All fish grew efficiently from 15 g of initial body weight to 296–320 g with an overall feed efficiency (FE) of 0.95-1.01, although fish fed D3 and D4 diets showed transient growth impairments over the course of the first four weeks of the trial. Data on biometric indexes, whole body composition, haematology and blood biochemistry revealed a strong effect of sampling time in fish sampled at mid-summer (August) and late autumn (December). In contrast, the diet effect was mostly reduced to a few blood parameters. Low inclusion levels of FM reduced plasma haemoglobin levels (D2, D3), but these effects were reversed by butyrate supplementation (D4). The same phenomena occurred for total cholesterol with the highest circulating concentration of choline and IGF-I in fish fed the D4 diet during their summer growth spurt. At the transcriptional level, gene expression profiling of liver and skeletal muscle with a PCR-array of 87 growth markers provided additional evidence for an overall well-growth condition in all of the experimental groups. Up to 73 genes were found at detectable levels in the liver tissue, but only 13 were differentially expressed. Likewise, 84 genes were actively transcribed in the skeletal muscle, but only nine were differentially expressed in at least one experimental group. Butyrate supplementation reversed the up-regulated expression of inflammatory cytokines (TNFα) and muscle markers of cellular morphogenesis and protein breakdown (CDH15, CAPN3, PSMA5, PSMB1, UBE2N) in the muscle of fish fed the extreme D3 diet. These results support the use of low FM/FO diets alone or supplemented with feed additives, which have the potential to improve or reverse metabolic steady-states.

Keywords: fish meal, fish oil, butyrate, growth, blood biochemistry, molecular markers.

Highlights:

- A very high replacement of fish meal and fish oil is highly feasible through the production cycle of gilthead sea bream.
- A short adaptive period with an intermediate diet is recommended when fish are fed diets with extremely low FM and FO diets.
- Data on haematology, blood biochemistry and growth rate regulated markers are indicative of a health-promoting action of dietary butyrate.

1. Introduction

The availability of wild fishery-derived raw materials is finite and the rapid and sustained growth rate of global aquaculture have forced the industry to explore alternative and more sustainable feed ingredients (Nasopoulou and Zabetakis, 2012; Tacon and Metian, 2008). Much attention has been focused on plant ingredients and there is now accumulating evidence for a large and combined replacement of FM and FO in a wide range of fish species of interest for European aquaculture. This includes Atlantic salmon (Bell et al., 2004; Pratoomyot et al., 2010; Torstensen et al., 2008), rainbow trout (Kaushik et al., 1995; Thanuthong et al., 2011), Atlantic cod (Jobling et al., 2008; Karalazos et al., 2007), turbot (Regost et al., 1999; 2003), European sea bass (Kaushik et al., 2004; Mourente and Bell, 2006) and gilthead sea bream (Benedito-Palos et al., 2007; 2009; Gómez-Requeni et al., 2004; Izquierdo et al., 2005). However, the long-term metabolic consequences of feeding very low FM and FO diets (with less than 10% inclusion levels of raw marine ingredients) on metabolic programming at different developmental stages and on different fish species are still under debate.

The nutritional and quality characteristics of edible fish matter are important aspects to consider (Nasopoulou and Zabetakis, 2012; Turchini et al., 2009). Certainly, high levels of n-3 LC-PUFA are important quality factors in human foods, and farmers are facing increasing pressures to include high levels of EPA (20:5n-3) and DHA (22:6n-3) in the finishing diets of salmonids and freshwater fish, which do not have specific requirements for n-3 LC-PUFA (Bell et al. 2004; Thanuthong et al. 2011). This is more evident in marine fish due to their limited capacity to elongate and desaturate C18 FAs into long chain C20 and C22 PUFAs. However, importantly, this metabolic constraint facilitates the multi-species predictive modelling of fillet FA composition year-round using a dummy regression approach with gilthead sea bream as the reference subgroup category (Ballester-Lozano et al., 2014a; 2014b). This tool is public accessible through an easy-to-use-web interface (www.nutrigroup-iats.org/aquafat) to guarantee a relatively high content of n-3 LC-PUFAs in marine fish meat, ensuring that the human health benefits of consuming farmed fish are retained (Larsen et al., 2011; Lund, 2013).

Early growth studies have also proven that FO can be totally replaced by a blend of VOs in practical gilthead sea bream diets with a 35% inclusion level of FM (Bouraoui et al. 2011). Alternatively, up to 65–70% of FO can be replaced in diets with

a 15–20% inclusion level of FM without detrimental effects or changes in growth performance (Benedito-Palos et al. 2007) or FA composition of phospholipids (Benedito-Palos et al. 2011), which are highly regulated to preserve cell integrity and function. However, to further proceed with low FM inclusion levels, fish feeds should be adequately fortified or supplemented with essential nutrients, which has generated increasing interest for natural feed additives and nutraceuticals in the industry. One example of this is butyrate, a short-chain FA that has received attention for its positive effect on gut health in several models of livestock animals (reviewed by Guilloteau et al., 2010). For instance, oral supplementation of microencapsulated sodium butyrate improves the immunological status and intestinal condition in common carp (Liu et al., 2014). Similarly, a slight but significant growth improvement has been reported in gilthead sea bream with dietary butyrate supplementation (Robles et al., 2013).

Recent progress has been achieved within the ARRAINA EU project to define reliable reference values for a set of biometric indexes, and biochemical, haematological and histochemical markers that have specificity, sensitivity and diagnostic value for the most common nutrient deficiencies arising from the replacement of FM and FO by alternative raw materials (Ballester-Lozano et al., 2015). At the molecular level, important advances have also been made on pathway-focused gene expression analyses using new genomic tools derived from the CSIC-nutrigroup transcriptomic database (www.nutrigroup.iats.org/seabream). Thus, gilthead sea bream PCR arrays are now available for wide transcriptional profiling of the intestine (Pérez-Sánchez et al., 2015) and mitochondria (Bermejo-Nogales et al., 2014b; 2015). Likewise, a set of growth rate regulated makers, including selected markers of the GH/IGF system (12), muscle growth and cell differentiation and proliferation (15), protein breakdown (20), protein folding and assembly (9), inflammatory and anti-inflammatory response (13), energy sensing (5), OXPHOS and mitochondrial respiration uncoupling (10), and master regulators of lipid metabolism (3), has recently been developed and validated for routine and clinical assessment of molecular signatures for growth in growing fish. This new tool together with more conventional biometric and biochemical markers of fish performance were used herein as part of the experimental setup to fully validate the use of extremely low FM and FO diets in gilthead sea bream. At the same time, the potential benefits of dietary butyrate supplementation at the lowest FM/FO inclusion level were explored, with attention focused on the metabolic and molecular profiling of

blood, and liver and skeletal muscle as key target tissues for growth regulation at the systemic and peripheral level.

2. Materials and methods

2.1. Diets

Four isoproteic and isolipidic plant protein-based diets (1.9, 3, 4.5 mm extruded feeds), containing a blend of soya protein, corn gluten, wheat gluten, rapeseed cake, and wheat, were formulated and delivered by BioMar (Denmark). FM was included at 23% in the D1 (control) diet and at 3% in the other three experimental diets (D2, D3 and D4). Fish hydrolysate (CPSP) was added at 2% in all diets. Added oil was either FO (D1 diet) or a blend of VOs (1:1 ratio of rapeseed oil: palm oil) replacing 58% (D2 diet) and 84% (D3 and D4 diets) FO. A commercial butyrate preparation (NOREL, 70-BP) was added to the D4 diet at 0.4%. All diets contained histidine (0.14%), antioxidants (0.045%) and a mineral-vitamin mix (0.5%). Lysine, methionine, choline, lecithin and monocalcium phosphate were balanced in D2, D3 and D4 diets to the values of the control diet. The FA composition of experimental diets varied with the progressive FO replacement, decreasing on a dry matter basis the EPA (20:5n-3) plus DHA (22:6n-3) content from 2.9% (D1) to 1.38% (D2) and 0.6–0.7% (D3, D4) (Table 1).

2.2. Feeding trial and sample collection

Juvenile gilthead sea bream of Atlantic origin (Ferme Marine de Douhet, France) were acclimatised for four weeks to the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS, Spain). During this initial period, fish were fed with a standard diet (Efico YM 568 1.9 mm, BioMar). Then, fish of 13–16 g initial mean body weight were distributed in 2500 L tanks in triplicate groups of 180 fish each. Oxygen content of outlet water remained higher than 75% saturation, and day-length and water temperature followed the natural changes at IATS latitude (40º 5’N; 0º 10’E). Each experimental diet was offered to visual satiety 1–2 times per day and 3–6 days per week from May 2013 to December 2013 (8-month feeding trial), according to the changes in fish size and season. Feed intake was recorded weekly and

fish were counted and group-weighed every 4–6 weeks. No significant mortalities (less than 0.5%) were registered regardless of dietary treatment.

At weeks 13 (August) and 31 (December), overnight fasted fish (4 fish per tank, 12 per experimental condition) were randomly sampled and anaesthetised with 3-aminobenzoic acid ethyl ester (MS-222, 0.1 g/l) for blood and tissue collection. Blood was quickly drawn from caudal vessels with heparinized syringes. One aliquot was used for haematological measurements. The remaining blood was centrifuged at 3,000 *g* for 20 min at 4ºC, and plasma samples were frozen and stored at -20ºC until biochemical assays. Prior to tissue collection, fish were killed by cervical section, and liver, viscera and mesenteric fat were weighed. Liver and a dorsal muscle portions were rapidly harvested, frozen in liquid nitrogen and stored at -80ºC until RNA isolation. Additional specimens of fish (a pool of 10 fish at the beginning and pools of 4 fish per tank at weeks 13 and 31) were ground for whole body composition analyses.

All procedures were carried out according to the national (IATS-CSIC Review Board) and present EU legislation on the handling of experimental animals.

2.3. Chemical analyses

The proximate composition of diets and whole fish were analysed by standard procedures (AOAC, 2005). Moisture content was determined by drying in an oven at 105ºC for 24 h. Diets and freeze-dried triturated fish were blended and used for protein and lipid analyses. Lipid content (from 0.5 g samples) was determined gravimetrically by the Soxhlet method using 50 ml diethyl ether at 120ºC as the extracting solvent (Soxhlet 4001046 Auto extraction apparatus; Selecta, Barcelona, Spain). Protein content (N x 6.25) was determined using the automated Kjeldhal method (Kjeldhal Auto 4002430 Analyser, Selecta).

2.4. Blood haematology and biochemistry

Hb concentration was determined with a HemoCue B-Haemoglobin Analyser® (AB, Leo Diagnostic, Sweden), which uses a modified azide methaemoglobin reaction for Hb quantification. The Hc was measured after centrifugation of blood in heparinised capillary tubes at 13,000 *g* for 10 min. Counts of RBC were made in a Neubauer chamber, using an isotonic solution (1% NaCl).

Plasma glucose was measured by the glucose oxidase method according to manufacturer's instructions (ThermoFisher Scientific, Waltham, Massachusetts, USA). Plasma TGs were determined using lipase/glycerol kinase/glycerol-3-phosphate oxidase reagent. Total plasma cholesterol was determined using a cholesterol esterase/cholesterol dehydrogenase reagent (ThermoFisher Scientific). HDL and LDL/VLDL cholesterol were determined with the EHDL-100 kit from BioAssay Systems (Hayward, California, USA), based on an improved polyethylene glycol precipitation method in which HDL and LDL/VLDL are separated, and cholesterol concentrations are determined using cholesterol esterase/cholesterol dehydrogenase reagent. Total plasma proteins were measured with the Bio-Rad protein reagent (Hercules, California, USA) with bovine serum albumin as standard.

Changes in plasma enzyme activities of ALAT (EC 2.6.1.2), ASAT (EC 2.6.1.1) and GLDH (EC 1.4.1.2) were measured using colorimetric assay kits (EALT-100, EASTR-100, DGLDH-100; BioAssays Systems). Plasma ALP (EC. 3.1.3.1) activity was determined by a fluorimetric assay kit (QFAP-100, BioAssays Systems).

Plasma levels of creatinine (DICT-500), choline (ECHO-100), calcium (DICA-500), chloride (DICL-250), magnesium (DMG-250) and phosphate (DIPI-500) were measured by colorimetric assay kits (BioAssays Systems). Total antioxidant capacity was measured as Trolox activity using a microplate assay kit (709001) (Cayman Chemical, Ann Arbor, Michigan, USA). Plasma lysozyme activity was measured by a turbidimetric assay adapted to microplates (Sitjà-Bobadilla et al., 2005). Induction of the RB activity in blood leukocytes was measured directly from heparinised blood, following the method described by Nikoskelainen et al. (2005) with some modifications (Saera-Vila et al., 2009a).

Plasma GH was determined by a homologous gilthead sea bream RIA as reported elsewhere (Martínez-Barberá et al., 1995). The sensitivity and midrange (ED50) of the assay were 0.15 and 1.8 ng/ml, respectively. Plasma IGFs were extracted

by acid-ethanol cryoprecipitation, and the concentration of IGF-I was measured by means of a generic fish IGF-I RIA validated for Mediterranean perciform fish (Vega-Rubín de Celis et al., 2004). The sensitivity and midrange of the assay were 0.05 and 0.7–0.8 ng/ml, respectively.

2.5. RNA extraction and reverse transcription

Total RNA from liver and skeletal muscle was extracted with a MagMAX-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, USA). RNA yield was 50–100 μg with UV absorbance measures (A260/280) of 1.9–2.1 and RIN (RNA integrity number) values of 8–10, as measured on an Agilent 2100 Bioanalyser, which is indicative of clean and intact RNA. Reverse transcription of 500 ng of total RNA was performed with random decamers, using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Negative control reactions were run without reverse transcriptase.

2.6. Gene expression analyses

Real-time quantitative PCR was carried out with an Eppendorf Mastercycler Ep Realplex real-time PCR system (Eppendorf, Wesseling-Berzdorf, Germany), using a 96-well PCR array layout designed for the simultaneously profiling of a panel of 87 growth rate regulated genes (Table S1), selected as markers of: i) GH/IGF system: GHR-I, GHR-II, IGF-I, IGF-II, IGFBP1, IGFBP2, IGFBP4, IGFBP7, IGFALS, INSR, IGFR1, and IGFR2; ii) muscle growth and cell differentiation and proliferation: MyoD1, MyoD2, Myf5, Myf6, MSTN, MEF2A, MEF2C, FST, CAV3, DES, CDH15, PCNA, PAX7, SOX3, and MET; iii) protein breakdown: CAPN1, CAPN2, CAPN3, CAST, CTSB, CTSD, CTSL, CTSS, PSMD4, PSD12, PSMA5, PSMB1, UCHL3, UBE2A, UBE2D2, UBE2L3, UBE2N, CUL2, CUL3, and CUL5; iv) protein folding and assembly: mtHsp10, Hsp30, mtHsp60, mtHsp70, Hsp90α, Hsp90β, GRP-170, GRP-94, and DER-1; v) inflammatory/anti-inflammatory response: IL-1β, IL-1R1, IL-1R2, IL-6, IL-6RA, IL-6RB, IL-8, IL-8RA, IL-10, IL-10RA, IL-10RB, TNF-α, and TRADD; vi) energy sensing: SIRT1, SIRT2, SIRT3, SIRT4, SIRT5; vii) OXPHOS: PGC1α, CPT1A, CS, ND2, NDUFAF2, COXI, and SCO1; viii) mitochondrial respiration uncoupling: UCP1, UCP2, and UCP3 and ix) lipolytic/lipogenic transcription factors:

LXRα, PPARα, and PPARγ. The array included 34 new sequences for gilthead sea bream, already represented in the sea bream transcriptomic database (www.nutrigroup-iats.org/seabreamdb) and uploaded to GenBank with the accession numbers KM522771–KM522804. Among them, eighteen are full codifying sequences varying in length from 444 to 2235 nucleotides (Table S2).

Housekeeping genes and controls for general PCR performance were included on each array, and all the pipetting operations were performed with the EpMotion 5070 Liquid Handling Robot (Eppendorf) as reported previously in the same fish species for pathway-focused PCR arrays of intestine (Pérez-Sánchez et al., 2015), mitochondria (Bermejo-Nogales et al., 2014b; 2015) and immune-relevant genes (Pérez-Cordón et al., 2014). The specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5ºC/10 s over a temperature range of 55–95ºC) and linearity of serial dilutions of RT reactions. Fluorescence data acquired during the PCR extension phase were normalised using the delta-delta Ct method (Livak and Schmittgen, 2001). β-actin was used as the housekeeping gene in the normalisation procedure. Technical replicates of samples were run initially to test the reproducibility of the array, but the obtained data had a very high reproducibility score and technical plate replicates were finally omitted. For multi-gene analysis comparisons, relative gene expression was referenced on each tissue to the expression level of IGFR2 of control fish (D1 diet) with an arbitrarily assigned value of 1. Data of fold-change were relative to control fish (values > 1, up-regulated genes in fish fed D2/D3/D4 diets; values < 1, down-regulated genes in fish fed D2/D3/D4 diets).

2.7. Statistical analysis

Data on growth performance, organosomatic indexes, whole body composition, blood biochemistry and gene expression were analysed by one- and two-way ANOVA (with diet and sampling time as variable factors) at a significance level of 5%. All analyses were made using the IBM SPSS Statistics package version 19 (Armonk, NY: IBM Corp.).

3. Results

3.1. Growth performance

All fish in the trial grew efficiently from an initial body weight of 15 g to 296–320 g (Figure 1) with overall FE of 0.95–1.01 and SGR of 1.37–1.42% (Table 2). Fish fed D1 and D2 diets were undistinguishable in terms of growth and feed efficiency for almost all of the experimental period. However, fish fed with the lowest FO inclusion level (D3 and D4 diets) showed an impaired FE in comparison to the control group (0.96 *vs.* 0.60-0.62 for D1 and D3-D4, respectively) over the course of the first four weeks of the trial. This detrimental effect was partially reversed thereafter and only a slight (6–7%) reduction in body weight was found at the last recording sampling time (December).

Biometric data in Table 3 indicates that VSI and MSI indexes (calculated as the ratio of organ weight to fish weight) decreased with fish size when comparisons were made between fish sampled in summer (August) and late autumn (December). In contrast, HSI increased with the increase of fish size during the cold season. Dietary treatment did not have significant effects on organosomatic indexes, although the highest HSI was reported in D3 fish ($P = 0.051$) and a partial recovery was observed with butyrate supplementation (D4 fish). Whole body composition remained almost unaltered by dietary treatment, although a pronounced increase in either protein (from 16% to 18%) or lipid (from 9% to 12%) content was found from August to December, regardless of fish feed.

3.2. Blood analyses

Two-way analysis of variance revealed a sampling-time effect (August 2013 *vs.* December 2013) for most of the analysed parameters (Table 4). In contrast, a diet effect ($P < 0.05$) was mostly reduced to Hb concentration, counts of RBC and plasma levels of cholesterol, choline and IGF-I. Noticeably, significant Diet x Time interactions were shown for most of these parameters indicating that the dietary effects were dependent on the time of sampling. Overall, low FM inclusion levels reduced Hb concentration in fish fed D2 and D3 diets, but butyrate feed supplementation reversed this effect in fish fed the D4 diet in the second sampling. A similar trend was shown for total plasma

cholesterol levels. In summer, this metabolic feature was linked to the rise of HDL cholesterol, whereas in December it was linked to the increase of plasma levels of VLDL/LDL cholesterol. Fish fed the D4 diet showed the highest plasma concentration of choline and IGF-I at the summer sampling time (fast growing period), but no differences were shown in the winter sampling (Diet x Time interaction $P < 0.05$). Total cholesterol, choline and IGF-I were strongly affected by sampling time showing 30-75% increase in total cholesterol and 50% reduction in choline and IGF-I levels between August and December irrespective of the dietary treatment.

3.3. Liver and muscle gene expression profiling

Results of gene expression profiling are summarized as Tables S4 and S5. In the liver tissue, up to 73 genes included in the array were found at detectable levels (Table S4). Among them, 13 were differentially expressed in response to dietary treatment. Relative expression of IGF-I was significantly reduced in D3 fish in comparison to the control group, but this effect was partially reversed by butyrate supplementation in D4 fish. Conversely, the expression of a proliferative cell marker (PCNA) was significantly reduced in the D4 group, but not in the D2 and D3 groups. Overall, the expression of molecular chaperones (mtHsp10, mtHsp60, mtHsp70, Hsp90β) was significantly reduced in D2, D3 and D4. The same trend was observed for receptors of inflammatory (IL-6RB, TRADD) and anti-inflammatory (IL-10RB) cytokines, markers of oxidative metabolism (CS, COXI) and transcription factors with a lipolytic (LXRα) or lipogenic role (PPARγ). For an easier interpretation of these results, data relative to fold-changes of differentially expressed genes is shown in Figure 2A.

In the skeletal muscle, 83 out of 87 genes present in the array were actively transcribed (Table S5, but only 9 genes were differentially expressed in at least one experimental group (fold-changes of differentially expressed genes are shown in Figure 2B). Most of the changes in gene transcription were related to intercellular adhesion glycoproteins (CDH15) and protein-breakdown markers of the calpain/calpastatin system (CAPN3) and ubiquitin-proteasome pathway (PSMA5, PSMB1, UCHL3, UBE2N) with the highest expression in fish fed the D3 diet and a recovery of control values with butyrate supplementation in fish fed the D4 diet. At the same time, the expression of the catabolic/lipolytic cytokine TNFα was significantly reduced in D4 fish in comparison to fish fed the D3 diet with intermediate values in control and D2 fish.

The trend for PPARα and PPARγ was a progressive down-regulated expression with the FM/FO replacement, which was exacerbated with butyrate supplementation, since significant differences were only found between D1 and D4 groups.

4. Discussion

In the present study, the long term effects of concurrent and high substitution of FM and FO in gilthead sea bream diets were evaluated. In particular, gilthead sea bream growing from 15 g to approximately 300 g showed very good overall growth performance and feed utilization even when fed diets containing as little as 3% total FM and 2.5% FO. Feeding trials conducted within the AQUAMAX EU Project (2006-2010) indicated that a combined and partial replacement of FM and FO is highly feasible in gilthead sea bream when the theoretical requirements of essential nutrients are met by diet. In these studies, the threshold level for marine feedstuffs was fixed at 25% (with FM level at 20%) on the basis of growth criteria and histopathological scoring of liver and intestine tissue samples (Benedito-Palos et al., 2007; 2008; 2009). Current studies within the ARRAINA EU Project (2012-2016) indicate that histopathological signs of liver and intestine damage were not found in any experimental group when experimental diets were adequately balanced to the control diet in terms of macro and micro-nutrients (Estensoro et al., 2014). Nevertheless, when comparisons are made among groups, the results presented herein highlighted a slight impairment of growth performance and feed utilization (reduced weight, SGR and FE) at the beginning of the experiment with the decrease of marine ingredients in the diets. This adverse effect was almost completely reversed through the first year of the production cycle (8-month feeding trial) in terms of SGR and FE although, the lower performance in the first period reflected in fish weight throughout the trial. These findings highly support a trend of good growth in all experimental groups, although a short adaptive feeding period with intermediate diets, like D2, should be given prior the use of extremely low FM/FO diets (less than 10% marine ingredients) on intensive marine fish farming.

The GH/IGF system is one of the most important endocrine determinants of growth in a vast array of stress and nutritional disorders arising in gilthead sea bream from crowding and handling stress (Rotllant et al., 2001; Saera-Vila et al., 2009b) or changes in ration size and diet composition (Pérez-Sánchez et al., 1995; Gómez-Requeni et al., 2004; Benedito-Palos et al., 2007). It is noteworthy that gene expression

444 profiling of liver and skeletal muscle with selected markers of growth, including GH
445 receptors, IGFs, IGF binding proteins, insulin receptors, and IGF receptors, highlighted
446 almost the same molecular signatures for all the groups in the trial when fish were
447 sampled during the fast growing period for this species at our latitude. The unique
448 exception is the hepatic IGF-I, which was significantly down-regulated in D3 fish in
449 comparison to control fish. This observation was correlated, at the protein level, with
450 low circulating levels of IGF-I, with a summer rebound effect brought about by butyrate
451 supplementation in fish fed the D4 diet. Despite this, overall growth performance was
452 almost the same in all experimental groups, which is perhaps indicative of
453 overlapping/compensatory effects of growth factors at the hepatic, systemic or
454 peripheral level. In previous feeding trials, we also failed to have a significant
455 improvement of weight gain or FE when butyrate (BP-70) was added at a broad range
456 (0.2–0.9%) in gilthead sea bream feeds with 20% FM and 10% FO (Pérez-Sánchez et
457 al., 2013). However, a 5% increase in weight gain was reported by Robles et al. (2013)
458 in an 8-week feeding trial with the addition of 0.3% butyrate in 15% FM and 7.2% FO
459 diets. Likewise, controversial and sometimes negative results have been reported in
460 other fish species, such as carp (Liu et al., 2014), trout (Gao et al., 2011) and salmon
461 (Bjerkeng et al., 1999). However, as reported below, butyrate had some direct or
462 indirect effects on a series of metabolic traits, which might be indicative of changes on
463 nutrient requirements for an adequate overall fish performance.
464 The hypocholesterolemic effect of plant proteins has been observed in a wide
465 range of fish species, including gilthead sea bream (Gómez-Requeni et al., 2004),
466 European sea bass (Messina et al., 2013), trout (Romarheim et al., 2008), salmon
467 (Hartviksen et al., 2014), and tiger puffer (Lim et al., 2011). Likewise, feeding trials
468 conducted by us with semi-synthetic diets highlighted that FO replacement with VOs
469 acts to lower TG and cholesterol factors in gilthead sea bream (Ballester-Lozano et al.,
470 2015). In the same study, diets formulated for deficiencies in phospholipids and
471 vitamins also showed a hypocholesterolemic effect, whereas phosphorus deficient diets
472 had a hypercholesterolemic effect in gilthead sea bream. A hypocholesterolemic effect
473 of VOs has also been reported for FM-based diets in Atlantic salmon (Jordal et al.,
474 2007) and black sea bream (Peng et al., 2008). No effects on plasma cholesterol levels
475 were found in European sea bass and trout using soybean oil diets (Figueiredo-Silva et
476 al., 2005), but a hypocholesterolemic effect was reported by other authors with the use
477 of a blend of VOs containing linseed oil, palm oil and rapeseed oil (Richard et al.

2006a; 2006b). Additionally, experimental evidence in trout (Norambuena et al., 2013) and salmon (Kortner et al., 2014) indicates that fish actively produce cholesterol as an structural component of cell membranes and as a precursor of bile acids and steroid hormones, adjusting their cholesterol production to dietary cholesterol loads. In the present study, all diets were supplemented with a fixed amount of cholesterol (0.113%), but we observed a cholesterol lowering effect that was especially evident with the maximum replacement of FM and FO in fish fed the D3 diet in winter. However, this apparently negative effect was completely reversed by butyrate feed supplementation in fish fed the D4 diet. There is no literature directly related to this, but it is interesting to note that butyrate is able to regulate lipoprotein metabolism, which is closely related to cholesterol metabolism (Marcil et al., 2002; Nazih et al., 2001).

From our results, we can conclude that low FM inclusion levels in diets D2 and D3 were related to a statistically significant reduction in circulating Hb concentration, but, importantly, this effect was reversed by dietary butyrate supplementation. In humans, butyrate was able to induce fetal Hb production in patients with Hb disorders (Atweh et al., 1999; Fathallah et al., 2007). *In vitro* studies also showed a positive modulation of Hb-A synthesis by butyrate in congenital haemolytic anaemia (Reinhardt et al., 2001). Moreover, short chain FAs promoted absorption of minerals (calcium, magnesium and iron) clinically relevant for the treatment and prevention of certain diseases such as anaemia and osteoporosis in humans (Teitelbaum and Walker, 2002). However, the effects of butyrate supplementation on the circulating Hb concentration have not been yet investigated in fish. Therefore, the results presented here support new potential benefits of fish feed additives in order to improve the overall health and welfare condition of livestock fish, which might be related to iron metabolism.

Another effect of dietary butyrate supplementation was the increase of circulating choline levels in fish sampled during the summer growth spurt. Choline is a water-soluble vitamin compound needed for the structural integrity and signalling roles of cell membranes and cholinergic neurotransmission (acetylcholine synthesis). Choline also acts as a major source of methyl groups via its metabolite trimethylglycine (betaine), which participates in the S-adenosyl methionine synthesis pathway (Glier et al., 2014; Miller, 2002). Therefore, choline is considered an essential nutrient for fish (NRC, 1993) that improves growth, digestive and absorptive capacities, and antioxidant defences, enhancing disease resistance and immune function (Mai et al., 2009; Wu and Davis, 2005; Wu et al., 2011; 2013; 2014). All diets were balanced in choline content

but, interestingly, the highest plasma concentration in the summer sampling point was achieved with dietary butyrate supplementation while no differences were shown between the control diet (D1) and D2 or D3 as well as between all treatments in winter. Whether the increase in the summer is indicative of a better metabolic condition as a result of changes in endogenous choline synthesis, nutrient absorption, or consumption rates remains unclear from a functional and mechanistic point of view.

At the molecular level, it is worth noting that the expression of mitochondrial and cytoplasmic chaperones (mtHsp10, mtHsp60, mtHsp70, Hsp90β) were down-regulated in the liver tissue of D2, D3, and D4 fish groups. It is possible that these changes might elicit an improved protein-folding capacity in control fish, which might be indicative of a healthy metabolic condition. Experimental evidence in gilthead sea bream indicates that mitochondrial activity and biogenesis are highly regulated by nutritional and environmental stressors (Calduch-Giner et al., 2014; Bermejo-Nogales et al., 2015; 2014a; 2014b; 2008), and either mtHsp60, mtHsp70 or Hsp90 are considered highly valuable health markers in a vast array of metabolic disorders. Strong support for this notion comes from inbreeding selection of rat strains with "lower power" *vs.* "high power" mitochondria, which demonstrates that most stress and risk factors segregate with low expression levels of genes required for mitochondria biogenesis and oxidative phosphorylation (Wisløff et al., 2005). Alternatively, this might also represent a risk factor contributing to hepatic steatosis development. Indeed, the decrease in mitochondrial oxidative capacity might be the result of an imbalance between fat oxidation and lipogenesis, leading to hepatic fat accumulation in the human syndrome of non-alcoholic fat liver diseases (Byrne, 2010). In our model, this metabolic risk factor is further supported by the down-regulated expression of some mitochondrial catabolic genes (CS, COXI) in combination with the depressed expression of transcriptional master regulators of lipolysis (LXRα) and lipogenesis (PPARγ), which would suggest different allostatic steady-states in fish fed the control diet and fish fed the other three experimental diets. In any case, as reported above, no signs of histopathological damage have been reported in any experimental group, although there was a tendency for higher HSI in fish fed the D3 diet, with a recovery of control values in fish given the butyrate supplement.

Other transcriptionally-mediated effects were related to the up-regulated expression of CDH15 in the muscle tissue of fish fed with the extremely low FM/FO diet (D3). This muscle cadherin is essential for the control of morphogenetic processes,

546 specifically myogenesis, and provides a trigger for terminal muscle cell differentiation
547 with an up-regulated expression in myotube-forming cells, which have been recognized
548 as part of the mechanisms related to the increase of muscle protein mass as a whole
549 (Donalies et al. 1991). In our case, this occurred in conjunction with the up-regulation
550 of calpain 3 and protein markers of the Ub-proteasome pathway (PSMA5, PSMB1,
551 UBE2N), which is prone to increased proteolysis and, thereby, protein turnover. This
552 provides an essential quality control mechanism that selectively eliminates abnormally
553 folded or damaged proteins that have arisen due to biosynthetic errors, damage by
554 oxygen radicals, or by denaturation (especially at high temperatures). Protein turnover is
555 specifically augmented with the net increase of protein accretion rates in humans,
556 rodents and livestock fish, including gilthead sea bream (Houlihan et al., 1993). This
557 offers the possibility of a stricter quality control system with the rise of growth rates,
558 theoretically increasing the immediate disposal of essential amino acids for protein
559 synthesis. In sepsis, certain types of cancer, and burn injuries, the enhancement of the
560 Ub-proteasome pathway appears to be signalled by cytokines (IL-1, TNFα) released
561 from activated macrophages (Holecek, 2012; Merritt et al., 2012). In our case, the
562 relative muscle gene expression of several ILs and IL-receptors was not altered by the
563 dietary intervention, but the up-regulated expression of TNF-α in D3 fish might elicit
564 the activation of muscle protein catabolism in these fish. These transcriptional changes
565 of muscle protein metabolism were not found with the butyrate diet and the muscle gene
566 expression pattern of D4 fish, at least for myogenic and muscle protein breakdown
567 markers, was related to the control group rather than D3 fish. This provides further
568 evidence for a vast array of metabolic effects that short FAs might have on the
569 regulation of fish metabolism, but the ultimate consequences on sensorial flesh quality
570 remain to be discovered.
571
572 **5. Conclusions**
573
574 Data on growth performance, and blood haematology and biochemistry clearly
575 indicate that a very high level of FM and FO replacement is feasible when the
576 theoretical requirements for essential nutrients are met by the diet. However, with the
577 most extreme diet composition (D3, 3% FM & 2.5% FO), an adaptive period may be
578 necessary to avoid initial and transient detrimental effects on growth performance.
579 Apparently, butyrate supplementation did not alter this time course, although it was able

to restore blood levels of Hb and cholesterol. Other butyrate-mediated effects included an increased circulating concentration of choline and IGF-I over the course of the summer growth spurt. Molecular signatures of growth rate-regulated genes remained almost unaltered with the FM/FO replacement, providing further evidence of suitable growth conditions in all experimental groups. However, it is noteworthy that butyrate supplementation was able to reverse the increased expression of inflammatory cytokines and muscle markers of cellular morphogenesis and protein breakdown in fish fed the extreme FM/FO diet. In contrast, at the hepatic level, the transcription-mediated changes in molecular chaperones, oxidative enzymes, immune-relevant genes, and master regulators of lipid metabolism were not returned to control values by dietary butyrate supplementation. Taken together, our results support the use of low FM/FO fish feeds alone or supplemented with feed additives, although a different steady-state level was observed for some of the analysed parameters.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JPS and SK conceived the investigation. JPS, SK, AO and VK contributed to formulate the experimental diets. GFBL conducted diet and body composition analysis. LBP, PS and JCG participated in blood and gene expression analyses and helped to write the manuscript. JPS coordinated the work and took primary responsibility for the final content of the manuscript. All authors read and approved the final manuscript.

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615 **References**

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617 AOAC, 2005. Official methods of analysis of the AOAC International, 18th ed.,
618 Latimer, G.W., Horwitz, W. (Eds.) Association of Official Analytical Chemists,
619 Gaithersburg, MD.
620 Atweh, G.F., Sutton, M., Nassif, I., Boosalis, V., Dover, G.J., Wallenstein, S., Wright,
621 E., McMahon, L., Stamatoyannopoulos, G., Faller, D.V., Perrine, S.P., 1999.
622 Sustained induction of fetal hemoglobin by pulse butyrate therapy in sickle cell
623 disease. Blood 93, 1790-1797.
624 Ballester-Lozano, G.F., Benedito-Palos, L., Mingarro, M., Navarro, J.C., Pérez-
625 Sánchez, J., 2014a. Up-scaling validation of a dummy regression approach for
626 predictive modelling the fillet fatty acid composition of cultured European sea
627 bass (*Dicentrarchus labrax*). Acuac. Res. doi:10.1111/are.12563.
628 Ballester-Lozano, G.F., Benedito-Palos, L., Riaza, A., Navarro, J.C., Rosel, J., Pérez-
629 Sánchez, J., 2014b. Dummy regression analysis for modelling the nutritionally
630 tailored fillet fatty acid composition of turbot and sole using gilthead sea bream
631 as a reference subgroup category. Aquac. Nutr. 20, 421-430.
632 Ballester-Lozano, G.F., Benedito-Palos, L., Estensoro, I., Sitjà-Bobadilla, A., Kaushik,
633 S., Pérez-Sánchez, J., 2015. Comprehensive biometric, biochemical and
634 histopathological assessment of nutrient deficiencies in gilthead sea bream fed
635 semi-purified diets. Br. J. Nutr. 114, 713-726.
636 Bell, J.G., Henderson, R.J., Tocher, D.R., Sargent, J.R., 2004. Replacement of dietary
637 fish oil with increasing levels of linseed oil: modification of flesh fatty acid
638 compositions in Atlantic salmon (*Salmo salar*) using a fish oil finishing diet.
639 Lipids 39, 223-232.
640 Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J.A., Kaushik, S., Pérez-Sánchez, J.,
641 2007. Combined replacement of fish meal and oil in practical diets for fast
642 growing juveniles of gilthead sea bream (*Sparus aurata* L.): networking of
643 systemic and local components of GH/IGF axis. Aquaculture 267, 199-212.
644 Benedito-Palos, L., Navarro, J.C., Sitjà-Bobadilla, A., Bell, J.G., Kaushik, S., Pérez-
645 Sánchez, J., 2008. High levels of vegetable oils in plant protein-rich diets fed to
646 gilthead sea bream (*Sparus aurata* L.): growth performance, muscle fatty acid
647 profiles and histological alterations of target tissues. Br. J. Nutr. 100, 992-1003.
648 Benedito-Palos, L., Navarro, J.C., Bermejo-Nogales, A., Saera-Vila, A., Kaushik, S.,
649 Pérez-Sánchez, J., 2009. The time course of fish oil wash-out follows a simple
650 dilution model in gilthead sea bream (*Sparus aurata* L.) fed graded levels of
651 vegetable oils. Aquaculture 288, 98-105.
652 Benedito-Palos, L., Bermejo-Nogales, A., Karampatos, A.I., Ballester-Lozano, G.F.,
653 Navarro, J.C., Diez, A., Bautista, J.M., Bell, J.G., Tocher, D.R., Obach, A.,
654 Kaushik, S., Pérez-Sánchez, J., 2011. Modelling the predictable effects of
655 dietary lipid sources on the fillet fatty acid composition of one-year-old gilthead
656 sea bream (*Sparus aurata* L.). Food Chem. 124, 538-544.
657 Bermejo-Nogales, A., Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J.A., Sitjà-
658 Bobadilla, A., Pérez-Sánchez, J., 2008. Confinement exposure induces glucose
659 regulated protein 75 (GRP75/mortalin/mtHsp70/PBP74/HSPA9B) in the hepatic
660 tissue of gilthead sea bream (*Sparus aurata* L.). Comp. Biochem. Physiol. 149B,
661 428-438.
662 Bermejo-Nogales, A., Calduch-Giner, J.A., Pérez-Sánchez, J., 2014a. Tissue-specific
663 gene expression and functional regulation of uncoupling protein 2 (UCP2) by
664 hypoxia and nutrient availability in gilthead sea bream (*Sparus aurata*):

665 implications on the physiological significance of UCP1-3 variants. Fish Physiol.
666 Biochem. 40, 751-762.
667 Bermejo-Nogales, A., Nederlof, M., Benedito-Palos, L., Ballester-Lozano, G.F.,
668 Folkedal, O., Olsen, R.E., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2014b.
669 Metabolic and transcriptional responses of gilthead sea bream (*Sparus aurata*
670 L.) to environmental stress: New insights in fish mitochondrial phenotyping.
671 Gen. Comp. Endocrinol. 205, 305-315.
672 Bermejo-Nogales, A., Calduch-Giner, J.A., Pérez-Sánchez, J., 2015. Unraveling the
673 molecular signatures of oxidative phosphorylation to cope with the nutritionally
674 changing metabolic capabilities of liver and muscle tissues in farmed fish. PLoS
675 ONE 10(4), e0122889.
676 Bjerkeng, B., Storebakken, T., Wathne, E., 1999. Cholesterol and short-chain fatty acids
677 in diets for Atlantic salmon *Salmo salar* (L.): effects on growth, organ indices,
678 macronutrient digestibility, and fatty acid composition. Aquac. Nutr. 5, 181-191.
679 Bouraoui L, Sánchez-Gurmaches J, Cruz-García L, Gutiérrez J, Benedito-Palos L,
680 Pérez-Sánchez J, Navarro I., 2011. Effect of dietary fish meal and fish oil
681 replacement on lipogenic and lipoprotein lipase activities and plasma insulin in
682 gilthead sea bream (*Sparus aurata* L.). Aquac. Nutr. 17, 54-63.
683 Byrne, C.D., 2010. Fatty liver: role of inflammation and fatty acid nutrition.
684 Prostaglandins Leukot. Essent. Fatty Acids 82, 265-71.
685 Calduch-Giner, J.A., Echasseriau, Y., Crespo, D., Baron, D., Planas, J.V., Prunet, P.,
686 Pérez-Sánchez, J., 2014. Transcriptional assessment by microarray analysis and
687 large-scale meta-analysis of the metabolic capacity of cardiac and skeletal
688 muscle tissues to cope with reduced nutrient availability in gilthead sea bream
689 (*Sparus aurata* L.). Mar. Biotechnol. 16, 423-435.
690 Donalies, M., Cramer, M., Ringwald, M., Starzinski-Powitz, A., 1991. Expression of
691 M-cadherin, a member of the cadherin multigene family, correlates with
692 differentiation of skeletal muscle cells. Proc. Natl. Acad. Sci. USA. 88, 8024-
693 8028.
694 Estensoro, I., Ballester-Lozano, G., Benedito-Palos, L., Karalazos, V., Mallo, J.J., Sitjà-
695 Bobadilla, A., Kaushik, S., Pérez-Sánchez, J., 2014. Histopathological and
696 transcriptional scoring of intestinal traits in gilthead sea bream (*Sparus aurata*)
697 fed low fish meal and fish oil diets with butyrate supplementation; oral
698 communication. Aquaculture Europe 2014, San Sebastián, Spain.
699 Fathallah, H., Weinberg, R.S., Galperin, Y., Sutton, M., Atweh, G.F., 2007. Role of
700 epigenetic modifications in normal globin gene regulation and butyrate-
701 mediated induction of fetal hemoglobin. Blood 110, 3391-3397.
702 Figueiredo-Silva, A.C., Rema, P., Bandarra, N.M., Nunes, M.L., Valente, L.M.P., 2005.
703 Effects of dietary conjugated linoleic acid on growth, nutrient utilization, body
704 composition, and hepatic lipogenesis in rainbow trout juveniles (*Oncorhynchus*
705 *mykiss*). Aquaculture 248, 163-172.
706 Gao, Y., Storebakken, T., Shearer, K.D., Penn, M., Øverland, M., 2011.
707 Supplementation of fishmeal and plant protein-based diets for rainbow trout
708 with a mixture of sodium formate and butyrate. Aquaculture 311, 233-240.
709 Glier, M.B., Green, T.J., Devlin, A.M., 2014. Methyl nutrients, DNA methylation, and
710 cardiovascular disease. Mol. Nutr. Food Res. 58, 172-182.
711 Gómez-Requeni, P., Mingarro, M., Calduch-Giner, J.A., Médale, F., Martin, S.A.M.,
712 Houlihan, D.F., Kaushik, S., Pérez-Sánchez, J., 2004. Protein growth
713 performance, amino acid utilisation and somatotropic axis responsiveness to fish

meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). Aquaculture 232, 493-510.
Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R., Van Immerseel, F., 2010. From the gut to the peripheral tissues: the multiple effects of butyrate. Nutr. Res. Rev. 23, 366-384.
Hartviksen, M., Bakke, A.M., Vecino, J.G., Ringo, E., Krogdahl, A., 2014. Evaluation of the effect of commercially available plant and animal protein sources in diets for Atlantic salmon (*Salmo salar* L.): digestive and metabolic investigations. Fish Physiol. Biochem. 40, 1621-1637.
Holecek, M., 2012. Muscle wasting in animal models of severe illness. Int. J. Exp. Pathol. 93, 157–171.
Houlihan, D.F., Mathers, E.M., Foster, A., 1993. Biochemical correlates of growth rate in fish. In: Fish Ecophysiology. Rankin, J.C., Jensen, F.B. (Eds). Chapman & Hall, London, 45-71.
Izquierdo, M.S., Montero, D., Robaina, L., Caballero, M.J., Rosenlund, G., Ginés, R., 2005. Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profiles by fish oil feeding. Aquaculture 250, 431-444.
Jobling, M., Leknes, O., Sæther, B.S., Bendiksen, E.Å., 2008. Lipid and fatty acid dynamics in Atlantic cod, *Gadus morhua*, tissues: influence of dietary lipid concentrations and feed oil sources. Aquaculture 281, 87-94.
Jordal, A.E.O., Lie, O., Torstensen, B.E., 2007. Complete replacement of dietary fish oil with a vegetable oil blend affect liver lipid and plasma lipoprotein levels in Atlantic salmon (*Salmo salar* L.). Aquac. Nutr. 13, 114-130.
Karalazos, V., Treasurer, J., Cutts, C.J., Alderson, R., Galloway, T.F., Albrektsen, S., Arnason, J., MacDonald, N., Pike, I., Bell, J.G., 2007. Effects of fish meal replacement with full-fat soy meal on growth and tissue fatty acid composition in Atlantic cod (*Gadus morhua*). J. Agric. Food Chem. 55, 5788-5795.
Kaushik, S.J., Covès, D., Dutto, G., Blanc, D., 2004. Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. Aquaculture 230, 391-404.
Kaushik, S.J., Cravedi, J.P., Lalles, J.P., Sumpter, J., Fauconneau, B., Laroche, M., 1995. Partial or total replacement of fish meal by soybean protein on growth, protein utilization, potential estrogenic or antigenic effects, cholesterolemia and flesh quality in rainbow trout, *Oncorhynchus mykiss*. Aquaculture 133, 257-274.
Kortner, T.M., Bjorkhem, I., Krasnov, A., Timmerhaus, G., Krogdahl, A., 2014. Dietary cholesterol supplementation to a plant-based diet suppresses the complete pathway of cholesterol synthesis and induces bile acid production in Atlantic salmon (*Salmo salar* L.). Br. J. Nutr. 111, 2089-2103.
Larsen, R., Eilertsen, K.-E., Elvevoll, E.O., 2011. Health benefits of marine foods and ingredients. Biotechnol. Adv. 29, 508-518.
Lim, S.J., Kim, S.S., Ko, G.Y., Song, J.W., Oh, D.H., Kim, J.D., Kim, J.U., Lee, K.J., 2011. Fish meal replacement by soybean meal in diets for tiger puffer, *Takifugu rubripes*. Aquaculture 313, 165-170.
Liu, W., Yang, Y., Zhang, J., Gatlin, D.M., Ringo, E., Zhou, Z., 2014. Effects of dietary microencapsulated sodium butyrate on growth, intestinal mucosal morphology, immune response and adhesive bacteria in juvenile common carp (*Cyprinus carpio*) pre-fed with or without oxidised oil. Br. J. Nutr. 112, 15-29.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-$\Delta\Delta C_T$ method. Methods 25, 402-408.

764 Lund, E.K., 2013. Health benefits of seafood; Is it just the fatty acids? Food Chem. 140,
765 413-420.
766 Mai, K.S., Xiao, L.D., Ai, Q.H., Wang, X.J., Xu, W., Zhang, W.B., Liufu, Z.G., Ren,
767 M.C., 2009. Dietary choline requirement for juvenile cobia, *Rachycentron*
768 *canadum*. Aquaculture 289, 124-128.
769 Marcil, V.R., Delvin, E., Seidman, E., Poitras, L., Zoltowska, M., Garofolo, C., Levy,
770 E., 2002. Modulation of lipid synthesis, apolipoprotein biogenesis, and
771 lipoprotein assembly by butyrate. Am. J. Physiol. Gastroint. Liver Physiol. 283,
772 G340-G346.
773 Martínez-Barberá, J.P., Pendón, C., Martí-Palanca, H., Calduch-Giner, J.A., Rodríguez,
774 R.B., Valdivia, M.M., Pérez-Sánchez, J., 1995. The use of recombinant gilthead
775 sea bream growth hormone for radioiodination and standard preparation in
776 radioimmunoassay. Comp. Biochem. Physiol. 110A, 335-340.
777 Merritt, E.K., Cross, J.M., Bamman, M.M., 2012. Inflammatory and protein metabolism
778 signaling responses in human skeletal muscle following burn injury. J. Burn
779 Care. Res. 33, 291–297.
780 Messina, M., Piccolo, G., Tulli, F., Messina, C.M., Cardinaletti, G., Tibaldi, E., 2013.
781 Lipid composition and metabolism of European sea bass (*Dicentrarchus labrax*
782 L.) fed diets containing wheat gluten and legume meals as substitutes for fish
783 meal. Aquaculture 376, 6-14.
784 Miller, D.L., 2002. Health benefits of lecithin and choline. Cereal Foods World 47, 178-
785 184.
786 Mourente, G., Bell, J.G., 2006. Partial replacement of dietary fish oil with blends of
787 vegetable oils (rapeseed, linseed and palm oils) in diets for European sea bass
788 (*Dicentrarchus labrax* L.) over a long term growth study: effects on muscle and
789 liver fatty acid composition and effectiveness of a fish oil finishing diet. Comp.
790 Biochem. Physiol. 145B, 389-399.
791 Nasopoulou, C., Zabetakis, I., 2012. Benefits of fish oil replacement by plant originated
792 oils in compounded fish feeds. A review. LWT - Food Sci. Technol. 47, 217-
793 224.
794 Nazih, H., Nazih-Sanderson, F., Krempf. M., Michel Huvelin, J., Mercier, S., Marie
795 Bard, J. 2001. Butyrate stimulates ApoA-IV-containing lipoprotein secretion in
796 differentiated Caco-2 cells: role in cholesterol efflux. J. Cell. Biochem. 83, 230-
797 238.
798 Nikoskelainen, S., Verho, S., Airas, K., Lilius, E.M., 2005. Adhesion and ingestion
799 activities of fish phagocytes induced by bacterium *Aeromonas salmonicida* can
800 be distinguished and directly measured from highly diluted whole blood of fish.
801 Dev. Comp. Immunol. 29, 525-537.
802 Norambuena, F., Lewis, M., Hamid, N.K.A., Hermon, K., Donald, J.A., Turchini, G.M.,
803 2013. Fish oil replacement in current aquaculture feed: Is cholesterol a hidden
804 treasure for fish nutrition? PLoS ONE. 8(12), e81705
805 NRC (National Research Council), 1993. Nutrient Requirements of Fish. National
806 Academy Press, Washington, DC.
807 Peng, S., Chen, L., Qin, J.G., Hou, J., Yu, N., Long, Z., Ye, J., Sun, X., 2008. Effects of
808 replacement of dietary fish oil by soybean oil on growth performance and liver
809 biochemical composition in juvenile black seabream, *Acanthopagrus schlegeli*.
810 Aquaculture 276, 154-161.
811 Pérez-Cordón, G., Estensoro, I., Benedito-Palos, L., Calduch-Giner, J.A., Sitjà-
812 Bobadilla, A., Pérez-Sánchez, J., 2014. Interleukin gene expression is strongly

813 modulated at the local level in a fish-parasite model. Fish Shellfish Immunol. 37,
814 201-208.
815 Pérez-Sánchez, J., Martí-Palanca, H., Kaushik, S., 1995. Ration size and protein intake
816 affect growth hormone (GH) levels, hepatic GH-binding and plasma insulin-like
817 growth factor-I immunoreactivity in a marine teleost, gilthead sea bream (*Sparus*
818 *aurata*). J. Nutr. 125, 546-552.
819 Pérez-Sánchez, J., Bermejo-Nogales, A., Grammes, F., Pérez-Cordón, G., Øverland, M.,
820 Calduch-Giner, J.A., Mallo, J.J., Sitjà-Bobadilla, A., Ballester-Lozano, G., 2013.
821 Effects of butyrate feed supplementation on gilthead sea bream (*Sparus aurata*)
822 growth performance and intestinal health: A transcriptomic approach; poster.
823 Aquaculture conference "To the next 40 years of sustainable global
824 aquaculture", Las Palmas de Gran Canaria, Spain.
825 Pérez-Sánchez, J., Benedito-Palos, L., Estensoro, I., Petropoulos, Y., Calduch-Giner.
826 J.A., Browdy, C.L., Sitjà-Bobadilla. A., 2015. Effects of dietary NEXT
827 ENHANCE®150 on growth performance and expression of immune and
828 intestinal integrity related genes in gilthead sea bream (*Sparus aurata* L.). Fish.
829 Shellfish Immunol. 44, 117-128.
830 Pratoomyot, J., Bendiksen, E., Bell, J.G., Tocher, D.R., 2010. Effects of increasing
831 replacement of dietary fishmeal with plant protein sources on growth
832 performance and body lipid composition of Atlantic salmon (*Salmo salar* L.).
833 Aquaculture 305, 124-132.
834 Regost, C., Arzel, J., Kaushik, S.J., 1999. Partial or total replacement of fish meal by
835 corn gluten meal in diet for turbot (*Psetta maxima*). Aquaculture 180, 99-117.
836 Regost, C., Arzel, J., Robin, J., Rosenlund, G., Kaushik, S.J., 2003. Total replacement
837 of fish oil by soybean or linseed oil with a return to fish oil in turbot (*Psetta*
838 *maxima*): 1. Growth performance, flesh fatty acid profile, and lipid metabolism.
839 Aquaculture 217, 465-482.
840 Reinhardt, D., Ridder, R., Kugler, W., Pekrun, A., 2001. Post-transcriptional effects of
841 interleukin-3, interferon-gamma, erythropoietin and butyrate on in vitro
842 hemoglobin chain synthesis in congenital hemolytic anemia. Haematologica 86,
843 791-800.
844 Richard, N., Kaushik, S., Larroquet, L., Panserat, S., Corraze, G., 2006a. Replacing
845 dietary fish oil by vegetable oils has little effect on lipogenesis, lipid transport
846 and tissue lipid uptake in rainbow trout (*Oncorhynchus mykiss*). Br. J. Nutr. 96,
847 299-309.
848 Richard, N., Mourente, G., Kaushik, S., Corraze, G., 2006b. Replacement of a large
849 portion of fish oil by vegetable oils does not affect lipogenesis, lipid transport
850 and tissue lipid uptake in European seabass (*Dicentrarchus labrax* L.).
851 Aquaculture 261, 1077-1087.
852 Robles, R., Lozano, A.B., Sevilla, A., Márquez, L., Nuez-Ortín, W., Moyano, F.J.,
853 2013. Effect of partially protected butyrate used as feed additive on growth and
854 intestinal metabolism in sea bream (*Sparus aurata*). Fish Physiol. Biochem. 39,
855 1567-1580.
856 Romarheim, O.H., Skrede, A., Penn, M., Mydland, L.T., Krogdahl, A., Storebakken, T.,
857 2008. Lipid digestibility, bile drainage and development of morphological
858 intestinal changes in rainbow trout (*Oncorhynchus mykiss*) fed diets containing
859 defatted soybean meal. Aquaculture 274, 329-338.
860 Rotllant, J., Balm, P.H.M., Pérez-Sánchez, J., Wendelaar-Bonga, S.E., Tort, L., 2001.
861 Pituitary and interrenal function in gilthead sea bream (*Sparus aurata*) after
862 handling and confinement stress. Gen. Comp. Endocrinol. 121, 333-342.

Saera-Vila, A., Benedito-Palos, L., Sitjà-Bobadilla, A., Nácher-Mestre, J., Serrano, R., Kaushik, S., Pérez-Sánchez, J., 2009a. Assessment of the health and antioxidant trade-off of gilthead sea bream (*Sparus aurata*) fed alternative diets with low levels of contaminants. Aquaculture 296, 87-95.

Saera-Vila, A., Calduch-Giner, J.A., Prunet, P., Pérez-Sánchez, J. 2009b. Dynamics of liver GH/IGF axis and selected stress markers in juvenile gilthead sea bream (*Sparus aurata*) exposed to acute confinement. Differential stress response of growth hormone receptors. Comp. Biochem. Physiol. 154A, 197-203.

Sitjà-Bobadilla, A., Peña-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S., Pérez-Sánchez, J., 2005. Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Aquaculture 249, 387-400.

Tacon, A.G.J., Metian, M., 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. Aquaculture 285, 146-158.

Teitelbaum, J.E., Walker, W.A., 2002. Nutritional impact of pre- and probiotics as protective gastrointestinal organisms. Annu. Rev. Nutr. 22, 107-138.

Thanuthong, T., Francis, D.S., Senadheera, S.D., Jones, P.L., Turchini, G.M., 2011. Fish oil replacement in rainbow trout diets and total dietary PUFA content: I) effects on feed efficiency, fat deposition and the efficiency of a finishing strategy. Aquaculture 320, 82-90.

Torstensen, B.E., Espe, M., Sanden, M., Stubhaug, I., Waagbø, R., Hemre, G.I., Fontanillas, R., Nordgarden, U., Hevrøy, E.M., Olsvik, P., Berntssen, M.H.G., 2008. Novel production of Atlantic salmon (*Salmo salar*) protein based on combined replacement of fish meal and fish oil with plant meal and vegetable oil blends. Aquaculture 285, 193-200.

Turchini, G.M., Torstensen, B.E., Ng, W.K., 2009. Fish oil replacement in finfish nutrition. Rev. Aquacult. 1, 10-57.

Vega-Rubín de Celis, S., Gómez-Requeni, P., Pérez-Sánchez, J., 2004. Production and characterization of recombinantly derived peptides and antibodies for accurate determinations of somatolactin, growth hormone and insulin-like growth factor-I in European sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol. 139, 266-277.

Wisløff, U., Najjar, S.M., Ellingsen, O., Haram, P.M., Swoap, S., Al-Share, Q., Fernström, M., Rezaei, K., Lee, S.J., Koch, L.G., Britton, S.L., 2005. Cardiovascular risk factors emerge after artificial selection for low aerobic capacity. Science 307, 418-20.

Wu, G.B., Davis, D.A., 2005. Interrelationship among methionine, choline, and betaine in channel catfish Ictalurus punctatus. Journal of the World Aquaculture Society. 36, 337-345.

Wu, P., Feng, L., Kuang, S.Y., Liu, Y., Jiang, J., Hu, K., Jiang, W.D., Li, S.H., Tang, L., Zhou, X.Q., 2011. Effect of dietary choline on growth, intestinal enzyme activities and relative expressions of target of rapamycin and eIF4E-binding protein2 gene in muscle, hepatopancreas and intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian). Aquaculture 317, 107-116.

Wu, P., Jiang, J., Liu, Y., Hu, K., Jiang, W.D., Li, S.H., Feng, L., Zhou, X.Q., 2013. Dietary choline modulates immune responses, and gene expressions of TOR and eIF4E-binding protein2 in immune organs of juvenile Jian carp (*Cyprinus carpio* var. Jian). Fish Shellfish Immunol. 35, 697-706.

912 Wu, P., Jiang, W.D., Liu, Y., Chen, G.F., Jiang, J., Li, S.H., Feng, L., Zhou, X.Q., 2014.
913 Effect of choline on antioxidant defenses and gene expressions of Nrf2 signaling
914 molecule in the spleen and head kidney of juvenile Jian carp (Cyprinus carpio
915 var. Jian). Fish Shellfish Immunol. 38, 374-382.
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Figure legends

Figure 1. (A) Seasonal changes of temperature (solid line) and day length (dash line). (B) Body weight over the course of the trial of fish fed experimental diets; arrows indicate tissue sampling times. Values are the mean of triplicate tanks.

Figure 2. (A) Fold-changes (experimental/ control group) of differentially expressed genes ($P < 0.05$) in the liver tissue. (B) Fold-changes (experimental/control group) of differentially expressed genes ($P < 0.05$) in skeletal muscle. Values > 1 indicate up-regulated genes in fish fed the experimental diets (D2, D3, D4); values < 1 indicate down-regulated genes in fish fed the experimental diets (D2, D3, D4).

Table 1. Ingredients and chemical composition of experimental diets.

Ingredient (%)	Diet			
	D1	D2	D3	D4
Fish meal	23.0	3.0	3.0	3.0
Fish hydrolysate (CPSP)	2.0	2.0	2.0	2.0
Soya protein	16.0	25.0	25.0	25.0
Corn gluten	15.0	25.0	25.0	25.0
Wheat gluten	4.00	7.30	7.30	7.30
Rapeseed cake	12.00	9.70	9.90	9.90
Wheat	11.08	6.80	6.64	6.24
Fish oil	15.60	6.56	2.50	2.50
Rapeseed oil	0	4.40	6.50	6.50
Palm olein	0	4.40	6.50	6.50
Monocalcium phosphate	0.303	2.097	2.097	2.097
Histidine	0.136	0.136	0.136	0.136
Mineral-vitamin mix[1]	0.500	0.500	0.500	0.500
Cholesterol	0.113	0.113	0.113	0.113
Amino-acid and micronutrient mix[2]	0.20	2.92	2.74	2.74
Antioxidants	0.045	0.045	0.045	0.045
Yttrium	0.03	0.03	0.03	0.03
BP-70	0	0	0	0.40
Proximate composition				
Dry matter (DM, %)	91.65	91.79	91.80	92.34
Crude protein (% DM)	45.48	46.73	46.12	46.03
Crude fat (% DM)	19.80	19.56	20.13	19.40
EPA+DHA (% DM)	2.90	1.38	0.67	0.63

[1]Supplied the following (g/kg mix, except as noted): calcium 689, sodium 108, iron 3, manganese 1, zinc 1, cobalt 2 mg, iodine 2 mg, selenium 20 mg, molybdenum 32 mg, retinyl acetate 1, DL-cholecalciferol 2.6, DL-α tocopheryl acetate 28, menadione sodium bisulphite. Ascorbic acid 16, thiamin 0.6, riboflavin 1.7, pyridoxine 1.2, vitamin B12 50 mg, nicotinic acid 5, pantothenic acid 3.6, folic acid 0.6, biotin 50 mg.
[2] Contains methionine, lysine, choline, lecithin

Table 2. Growth performance of juvenile gilthead sea bream fed the experimental diets from May to December (31 weeks).

Diet	Mean body weight		Weight	SGR (%)[1]	Feed intake	FE[2]
	Initial	Final	gain (%)		(g dry/fish)	
Week 4 *(May-June)*						
D1(FM23/FO15)	15.0±0.04	23.7±0.12^{a}	58.2±1.1^{a}	1.70±0.01^{a}	9.03±0.25	0.96±0.06^{a}
D2(FM3/FO6)	15.3±0.05	22.9±0.20^{a}	49.9±0.2^{b}	1.50±0.02^{b}	9.05±0.15	0.84±0.03^{b}
D3(FM3/FO2.5)	15.2±0.03	20.5±0.13^{b}	34.3±0.45^{c}	1.09±0.01^{c}	8.77±0.18	0.60±0.04^{c}
D4(FM3/FO2.5/BUT)	15.4±0.04	20.9±0.21^{b}	36.1±0.5^{c}	1.14±0.03^{d}	9.01±0.34	0.62±0.02^{c}
Week 13 *(June-August)*						
D1(FM23/FO15)	23.7±0.12^{a}	87.6±0.87^{a}	272.1±3.71	2.08±0.01	54.8±1.16	1.15±0.09
D2(FM3/FO6)	22.9±0.20^{a}	82.7±0.04^{ab}	256.7±0.24	2.01±0.01	53.4±0.49	1.1±0.01
D3(FM3/FO2.5)	20.5±0.13^{b}	76.0±0.30^{c}	271.3±1.54	2.08±0.01	51.6±0.89	1.07±0.02
D4(FM3/FO2.5/BUT)	20.9±0.21^{b}	77.2±1.72^{bc}	269.3±8.35	2.07±0.03	56.8±4.42	1.01±0.05
Week 31 *(August-December)*						
D1(FM23/FO15)	87.6±0.87^{a}	318.3±0.16^{a}	263.2±4.71^{a}	1.02±0.01	213.1±6.96	1.08±0.03
D2(FM3/FO6)	82.7±0.04^{ab}	321.6±1.17^{a}	298.2±3.98^{b}	1.10±0.07	225.9±0.3	1.06±0.01
D3(FM3/FO2.5)	76.0±0.30^{c}	298.1±4.64^{b}	302.7±2.22^{b}	1.10±0.01	213.1±2.87	1.05±0.01
D4(FM3/FO2.5/BUT)	77.2±1.72^{bc}	296.7±4.62^{b}	291.7±1.97^{b}	1.08±0.01	215.1±0.80	1.03±0.01
Overall *(May-December)*						
D1(FM23/FO15)	15.0±0.04	318.3±0.16^{a}	2027±1.08^{a}	1.42±0.01	298.2±8.46	1.01±0.03
D2(FM3/FO6)	15.3±0.05	321.6±1.17^{a}	2004±7.6^{a}	1.41±0.01	307.7±0.47	0.99±0.01
D3(FM3/FO2.5)	15.2±0.03	298.1±4.64^{b}	1856±30.5^{b}	1.37±0.01	292.7±2.60	0.96±0.01
D4(FM3/FO2.5/BUT)	15.4±0.04	296.7±4.62^{b}	1828±30.1^{b}	1.37±0.01	296.1±1.2	0.95±0.01

Values are the mean ± SEM of dietary triplicates. Different column superscript letters indicate significant differences within each experimental period (Holm-Sidak test, $P<0.05$).

[1]Specific growth rate = 100 x (ln final weight – ln initial weight)/days

[2]Feed efficiency = weight gain/feed intake

Table 3. Organosomatic indexes and whole body composition of juvenile gilthead sea bream sampled in August (week 13) and December (week 31).

	August 2013				December 2013				Two-way ANOVA (P-values)		
	D1	D2	D3	D4	D1	D2	D3	D4	Diet	Time	Diet x Time Interaction
Viscera (g)	8.52±0.19	7.26±0.24	7.80±0.28	7.46±0.29	22.6±0.85	21.63±0.88	21.41±1.01	20.8±0.65	0.064	<0.001	0.785
Mesenteric fat (g)	1.64±0.14	1.40±0.13	1.35±0.09	1.47±0.17	4.54±0.51	4.28±0.52	3.44±0.44	3.64±0.37	0.113	<0.001	0.371
Liver (g)	1.49±0.04	1.25±0.04	1.35±0.04	1.30±0.04	6.42±0.90	7.07±0.26	7.24±0.47	6.68±0.33	0.414	<0.001	0.148
Intestine length (cm)	11.7±0.60	11.4±0.60	11.8±0.70	11.2±0.70	13.3±0.51	13.33±0.60	14.20±0.71	13.04±0.46	0.545	<0.001	0.950
VSI (%)[1]	9.04±0.15	8.40±0.20	9.40±0.26	8.81±0.22	6.67±0.37	6.38±0.19	6.47±0.18	6.26±0.18	0.099	<0.001	0.318
MSI (%)[2]	1.79±0.13	1.61±0.13	1.67±0.11	1.72±0.19	1.34±0.15	1.35±0.13	1.03±0.12	0.99±0.15	0.526	<0.001	0.505
HSI (%)[3]	1.56±0.07	1.47±0.07	1.63±0.07	1.51±0.07	1.87±0.27	2.09±0.07	2.29±0.07	2.07±0.09	0.051	<0.001	0.069
Whole body composition (% wet weight)											
Moisture	66.7±0.27	66.8±0.72	67.8±0.02	66.3±0.26	64.08±0.34	62.80±0.58	62.85±0.72	64.13±0.34	0.342	<0.001	0.133
Crude protein	16.1±0.49	16.2±0.39	15.9±0.17	16.3±0.03	18.06±0.05	18.58±0.82	18.01±0.55	17.72±1.56	0.928	<0.001	0.738
Crude lipid	8.98±0.09	9.09±0.40	8.96±0.34	9.38±0.13	12.60±0.73	12.65±1.31	12.07±0.02	11.63±0.01	0.932	0.004	0.917

Biometric data are the mean ± SEM of 12 fish (4 per each dietary triplicate). Data on whole body composition are the mean of 4 pooled fish per each dietary triplicate.

[1]Viscerosomatix index = (100 x viscera weight)/fish weight

[2]Mesenteric index = (100 x mesenteric fat weight)/fish weight

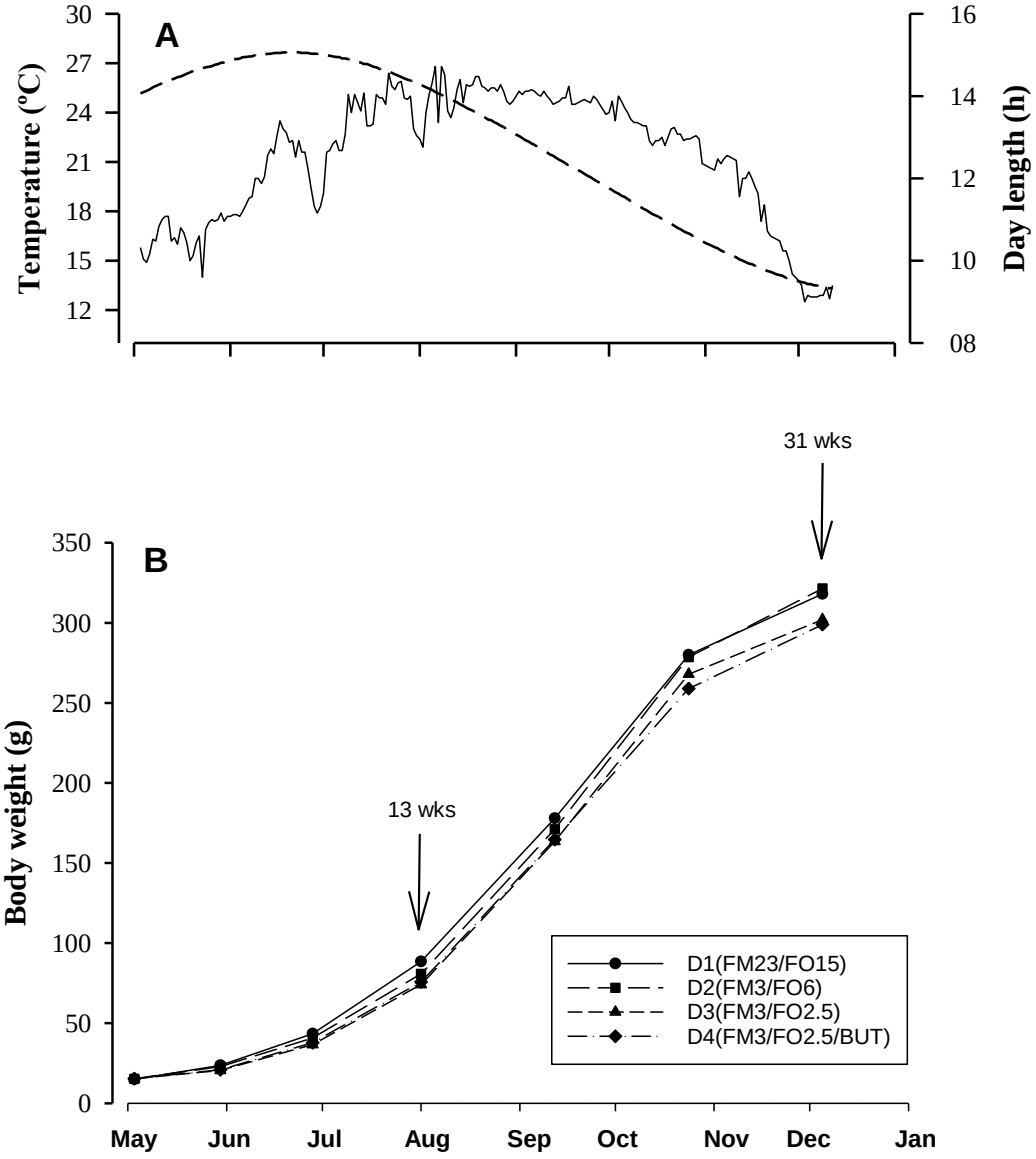
[3]Hepatosomatic index = (100 x liver weight)/fish weight

Table 4. Haematology and blood biochemistry of juvenile gilthead sea bream sampled in August (week 13) and December (week 31).

	August 2013				December 2013				Two-way ANOVA (P-values)		
	D1	D2	D3	D4	D1	D2	D3	D4	Diet	Time	Diet x Time interaction
Haemoglobin (g/dl)	6.45±0.25^{a}	5.09±0.21^{b}	5.55±0.28^{b}	6.37±0.33^{a}	7.74±0.22^{a}	6.11±0.23^{b}	6.15±0.18^{b}	6.93±0.27^{ab}	<0.001	<0.001	0.159
Haematocrit (%)	34.5±1.95	36.7±1.42	35.5±2.12	34.3±1.78	33.4±1.03	33.5±1.49	30.0±2.19	35.7±2.32	0.602	0.061	0.259
RBC x 10^{-6}/ml	2.39±0.12	2.47±0.09	2.39±0.11	2.28±0.09	3.31±0.10^{a}	2.66±0.07^{b}	2.59±0.09^{b}	2.92±0.11^{b}	0.007	<0.001	<0.001
Glucose (mg/dl)	46.8±1.61	53.5±5.12	53.7±2.28	48.9±2.12	46.1±1.95	48.9±2.2	49.1±1.73	47.4±3.41	0.294	0.080	0.621
Triglycerides (mM)	0.56±0.03	0.47±0.04	0.65±0.07	0.63±0.05	4.97±2.80	0.891±0.09	0.951±0.09	3.58±0.94	0.309	0.033	0.323
Total cholesterol (mg/dl)	138.8±11.5^{ab}	141.8±5.99^{ab}	113.5±4.66^{a}	148.9±9.29^{b}	243.5±14.7^{a}	187.8±10.6^{b}	186.4±9.6^{b}	248.2±18.2^{a}	<0.001	<0.001	0.040
HDL cholesterol (mg/dl)	92.4±10.8^{ab}	102.9±5.07^{b}	68.2±2.72^{a}	103.3±7.72^{b}	157.6±7.8^{ab}	164.4±5.5^{a}	139.1±5.4^{b}	107.9±5.8^{c}	<0.001	<0.001	<0.001
VLDL/LDL cholesterol (mg/dl)	21.4±2.55	22.2±2.39	25.1±2.71	26.6±2.24	69.2±13.9^{a}	55.04±9.4^{a}	53.9±3.7^{a}	109.4±7.4^{b}	<0.001	<0.001	<0.001
Total proteins (g/l)	40.9±1.39	41.5±1.67	40.8±1.89	42.2±1.36	49.2±1.26	50.78±1.49	47.18±0.88	50.7±0.96	0.306	<0.001	0.782
ALAT (U/l)	1.34±0.14	1.18±0.27	1.30±0.19	1.28±0.21	1.68±0.20	1.68±0.42	1.48±0.21	1.29±0.20	0.777	0.101	0.886
ASAT (U/l)	16.1±3.62	18.7±3.68	19.7±4.19	17.9±3.7	13.5±2.15	16.9±3.13	11.02±0.99	10.4±2.18	0.661	0.025	0.217
GLDH (U/l)	0.68±0.24	0.94±0.20	0.49±0.08	0.57±0.15	0.74±0.20	0.53±0.12	0.85±0.32	0.72±0.10	0.505	0.734	0.932
ALP (U/l)	96.5±3.61	101.9±5.41	97.7±7.01	99.0±4.85	81.9±4.34	89.8±7.01	78.4±5.34	92.6±7.45	0.316	0.008	0.213
Creatinine (mg/dl)	0.11±0.02	0.1276±0.01	0.125±0.02	0.120±0.03	0.223±0.02	0.189±0.03	0.173±0.02	0.161±0.02	0.865	0.008	0.077
Choline (µM)	15.5±0.97^{a}	16.1±1.07^{a}	16.2±1.24^{a}	20.8±1.27^{b}	7.05±0.62	7.75±0.54	7.12±0.39	6.77±0.69	0.050	<0.001	0.012
Calcium (mg/dl)	13.5±0.37	13.9±0.54	12.3±0.21	13.4±0.06	10.9±0.69^{a}	12.7±0.45^{ab}	13.4±0.46^{b}	14.7±1.02^{b}	0.010	0.383	<0.001
Chloride (mg/dl)	461.3±13.6	468.3±6.7	473.8±9.3	442.8±7.2	429.9±10.8	448.8±10.7	459.4±11.3	429.7±15.3	0.065	0.050	0.915
Magnesium (mg/ml)	1.92±0.07	2.07±0.08	1.89±0.08	1.98±0.07	1.76±0.05	1.83±0.03	1.72±0.04	1.96±0.0.08	0.197	0.004	0.016
Phosphate (mg/dl)	11.3±1.27	11.9±1.55	12.1±1.60	12.1±1.59	11.2±0.25	10.1±0.31	10.40±0.31	10.98±0.12	0.997	0.517	0.966
Antioxidant capacity (Trolox mM)	0.92±0.03	0.91±0.03	0.84±0.03	0.72±0.08	1.12±0.04	1.14±0.03	1.104±0.02	1.14±0.04	0.153	<0.001	0.097
Lysozyme (U/l)	190.5±32.9	208.8±117.4	118.8±30.1	125.7±46.6	56.5±12.7	85.7±28.9	51.8±18.1	32.5±19.1	0.622	<0.001	0.361
Respiratory burst (IRLU)	388.1±40.8	539.9±51.4	758.6±247.1	485.1±52.2	3093.6±743.9	4882.9±671.7	5170.4±1235.5	2926.8±672.3	0.131	<0.001	0.336
GH (ng/ml)	6.37±0.93	6.74±1.50	8.00±1.39	8.71±0.92	2.19±0.68	2.09±0.71	2.78±0.71	2.65±0.85	0.091	<0.001	0.483
IGF-I (ng/ml)	94.0±4.61^{ab}	97.2±4.65^{a}	87.4±4.54^{a}	116.4±4.48^{b}	50.7±4.38	48.4±4.60	43.8±2.61	47.7±5.51	0.050	<0.001	0.016

Data are the mean ± SEM of 10-12 fish. Different superscript letters indicate significant differences within a sampling time (SNK test, P<0.05).

Figure 1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27
24
21
18
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12
16
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12
10
08
B
Body weight (g)
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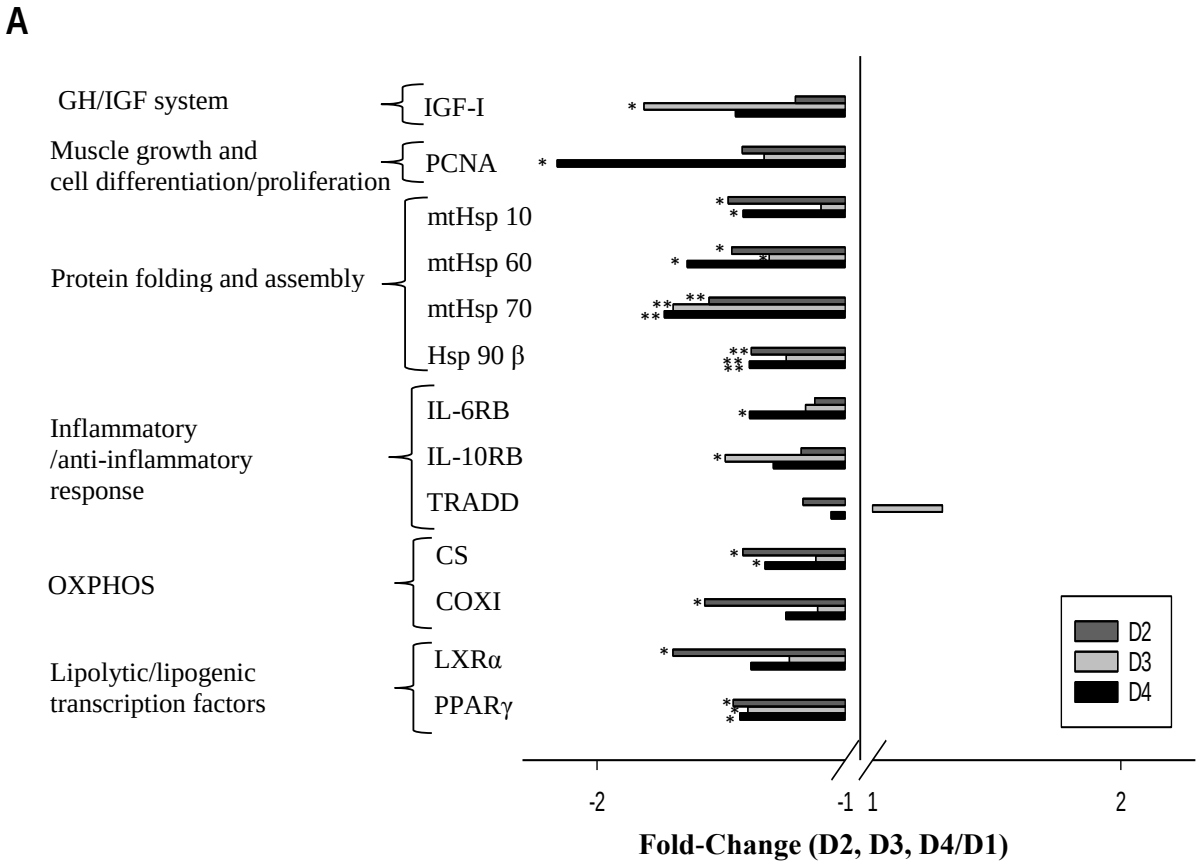
300
250
200
150
100
50
0
13 wks
31 wks
D1(FM23/FO15)
D2(FM3/FO6)
D3(FM3/FO2.5)
D4(FM3/FO2.5/BUT)
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan

Figure 2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PCNA
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/anti-inflammatory
response
IL-6RB
IL-10RB
TRADD
OXPHOS
CS
COXI
Lipolytic/lipogenic
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PPARγ
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D3
D4
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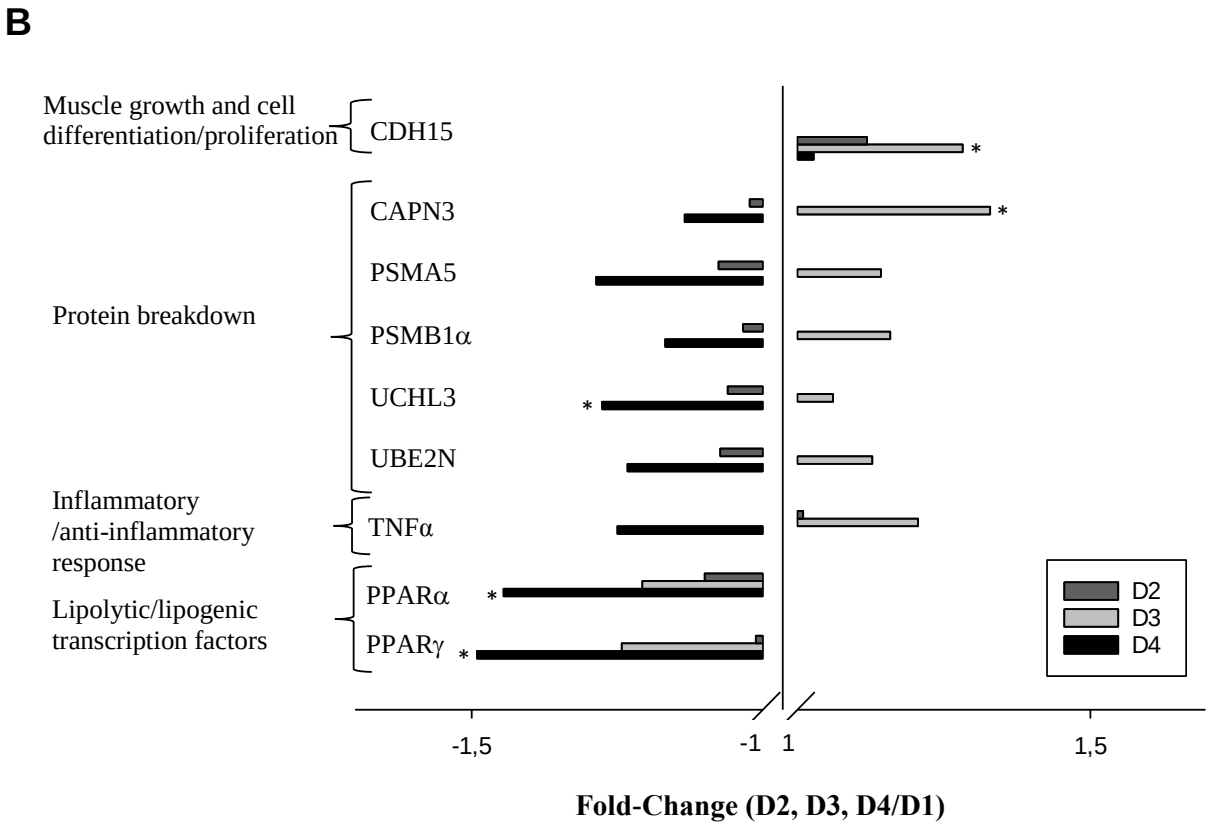
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CAPN3
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UCHL3
UBE2N
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response
TNFα
Lipolytic/lipogenic
transcription factors
PPARα
PPARγ
D2
D3
D4
-1,5
-1
1
1,5
Fold-Change (D2, D3, D4/D1)

Table S1. PCR-array layout of 87 genes with extra-wells for housekeeping genes and general controls of PCR performance.

	1	2	3	4	5	6	7	8	9	10	11	12
A	GHR-I	IGFALS	MSTN	PAX7	CTSD	UBE2A	Hsp30	IL-1β	IL-10	SIRT4	SCO1	PPC1
B	GHR-II	INSR	MEF2A	SOX3	CTSL	UBE2D2	mtHsp60	IL-1R1	IL-10RA	SIRT5	UCP1	PPC2
C	IGF-I	IGFR1	MEF2C	MET	CTSS	UBE2L3	mtHsp70	IL-1R2	IL-10RB	PGC1α	UCP2	PPC3
D	IGF-II	IGFR2	FST	CAPN1	PSMD4	UBE2N	Hsp90α	IL-6	TNF-α	CPT1A	UCP3	PPC4
E	IGFBP1	MyoD1	CAV3	CAPN2	PSD12	CUL2	Hsp90β	IL-6RA	TRADD	CS	LXRα	NPC
F	IGFBP2	MyoD2	DES	CAPN3	PSMA5	CUL3	GRP-170	IL-6RB	SIRT1	ND2	PPARα	NPC
G	IGFBP4	Myf5	CDH15	CAST	PSMB1	CUL5	GRP-94	IL-8	SIRT2	NDUFAF2	PPARγ	ACTB
H	IGFBP7	Myf6	PCNA	CTSB	UCHL3	mtHsp10	DER-1	IL-8RA	SIRT3	COXI		ACTB

Position	Symbol	Description	Accession No.
A1	GHR-I	Growth hormone receptor I	AF438176
B1	GHR-II	Growth hormone receptor II	AY573601
C1	IGF-I	Insulin-like growth factor-I	EF563837
D1	IGF-II	Insulin-like growth factor-II	EF563836
E1	IGFBP1	Insulin-like growth factor binding protein 1	KM522771
F1	IGFBP2	Insulin-like growth factor binding protein 2	AF377998
G1	IGFBP4	Insulin-like growth factor binding protein 4	KM658998
H1	IGFBP7	Insulin-like growth factor binding protein 7	KM522772
A2	IGFALS	Insulin-like growth factor-binding protein complex acid labile subunit	KM522773
B2	INSR	Insulin receptor	KM522774
C2	IGFR1	Insulin-like growth factor receptor I	KM522775
D2	IGFR2	Cation-independent mannose-6-phosphate receptor	KM522776
E2	MyoD1	Myoblast determination protein 1	AF478568
F2	MyoD2	Myoblast determination protein 2	AF478569
G2	Myf5	Myogenic factor 5	JN034420
H2	Myf6	Myogenic factor 6	JN034421
A3	MSTN	Growth/ differentiation factor 8 (Myostatin)	AF258448
B3	MEF2A	Myocyte-specific enhancer factor 2A	KM522777
C3	MEF2C	Myocyte-specific enhancer factor 2C	KM522778
D3	FST	Follistatin	AY544167
E3	CAV3	Caveolin 3	KM522779
F3	DES	Desmin	KM522780
G3	CDH15	Cadherin 15	KM522781
H3	PCNA	Proliferating cell nuclear antigen	KF857335
A4	PAX7	Paired box 7	KM522782
B4	SOX3	Transcription factor SOX3	KM522783
C4	MET	c-met/hepatocyte growth factor receptor	KM522784
D4	CAPN1	Calpain 1	KF444899
E4	CAPN2	Calpain 2	KF444900
F4	CAPN3	Calpain 3	KM522785
G4	CAST	Calpastatin	KM522786
H4	CTSB	Cathepsin B	KJ524457

Table S1. Continued.

Position	Symbol	Description	Accession No.
A5	CTSD	Cathepsin D	AF036319
B5	CTSL	Cathepsin L	KM522787
C5	CTSS	Cathepsin S	KM522788
D5	PSMD4	26S proteasome non-ATPase regulatory subunit 4	KM522789
E5	PSD12	26S proteasome non-ATPase regulatory subunit 12	KM522790
F5	PSMA5	Proteasome subunit alpha type-5	KM522791
G5	PSMB1	Proteasome subunit beta type-1	KM522792
H5	UCHL3	Ubiquitin carboxyl-terminal hydrolase isozyme L3	KM522793
A6	UBE2A	Ubiquitin-conjugating enzyme E2 A	KM522794
B6	UBE2D2	Ubiquitin-conjugating enzyme E2 D2	KM522795
C6	UBE2L3	Ubiquitin-conjugating enzyme E2 L3	KM522796
D6	UBE2N	Ubiquitin-conjugating enzyme E2N	KM522797
E6	CUL2	Cullin 2	KM522798
F6	CUL3	Cullin 3	KM522799
G6	CUL5	Cullin 5	KM522800
H6	mtHsp10	10 kDa heat shock protein, mitochondrial	JX975224
A7	Hsp30	30 kDa heat shock protein	KM522801
B7	mtHsp60	60 kDa heat shock protein, mitochondrial	JX975227
C7	mtHsp70	70 kDa heat shock protein, mitochondrial	DQ524993
D7	Hsp90α	90 kDa heat shock protein alpha 1	KM522802
E7	Hsp90β	90 kDa heat shock protein beta	KM522803
F7	GRP-170	Glucose-regulated protein, 170 kDa	JQ308821
G7	GRP-94	Glucose-regulated protein, 94 kDa	JQ308820
H7	DER-1	Derlin-1	JQ308825
A8	IL-1β	Interleukin-1 beta	AJ419178
B8	IL-1R1	Interleukin-1 beta receptor 1	JX976615
C8	IL-1R2	Interleukin-1 beta receptor 2	AM296027
D8	IL-6	Interleukin-6	EU244588
E8	IL-6RA	Interleukin-6 receptor A	JX976616
F8	IL-6RB	Interleukin-6 receptor B	JX976617
G8	IL-8	Interleukin-8	JX976619
H8	IL-8RA	Interleukin-8 receptor A	JX976620
A9	IL-10	Interleukin-10	JX976621
B9	IL-10RA	Interleukin-10 receptor A	JX976622
C9	IL-10RB	Interleukin-10 receptor B	JX976623
D9	TNF-α	Tumor necrosis factor-alpha	AJ413189
E9	TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain protein	KM522804
F9	SIRT1	Sirtuin 1	KF018666
G9	SIRT2	Sirtuin 2	KF018667
H9	SIRT3	Sirtuin 3	KF018668
A10	SIRT4	Sirtuin 4	KF018669
B10	SIRT5	Sirtuin 5	KF018670
C10	PGC1α	Proliferator-activated receptor gamma coactivator 1 alpha	JX975264

Table S1. Continued.

Position	Symbol	Description	Accession No.
D10	CPT1A	Carnitine palmitoyltransferase 1A	JQ308822
E10	CS	Citrate synthase	JX975229
F10	ND2	NADH-ubiquinone oxidoreductase chain 2	KC217559
G10	NDUFAF2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 2	KC217598
H10	COXI	Cytochrome c oxidase subunit I	KC217652
A11	SCO1	SCO1 protein homolog, mitochondrial	KC217649
B11	UCP1	Uncoupling protein 1	FJ710211
C11	UCP2	Uncoupling protein 2	JQ859959
D11	UCP3	Uncoupling protein 3	EU555336
E11	LXRα	Liver X receptor α	FJ502320
F11	PPARα	Peroxisome proliferator-activated receptor α	AY590299
G11	PPARγ	Peroxisome proliferator-activated receptor γ	AY590304
A12-D2	PPC	Positive PCR control (serial dilutions of standard gene)	AY590304
E12-F12	NPC	Negative PCR control	
G12-H12	ACTB	β-actin	X89920

GH/IGF system: GHR-I, GHR-II, IGF-I, IGF-II, IGFBP1, IGFBP2, IGFBP4, IGFBP7, IGFALS, INSR, IGFR1, IGFR2
Muscle growth and differentiation: MyoD1, MyoD2, Myf5, Myf6, MSTN, MEF2A, MEF2C, FST, CAV3, DES, CDH15, PCNA, PAX7, SOX3, MET
Protein breakdown: CAPN1, CAPN2, CAPN3, CAST, CTSB, CTSD, CTSL, CTSS, PSMD4, PSD12, PSMA5, PSMB1, UCHL3, UBE2A, UBE2D2, UBE2L3, UBE2N, CUL2, CUL3, CUL5
Protein folding and assembly: mtHsp10, Hsp30, mtHsp60, mtHsp70, Hsp90α, Hsp90β, GRP-170, GRP-94, DER-1
Inflammatory/anti-inflammatory response : IL-1β, IL-1R1, IL-1R2, IL-6, IL-6RA,IL-6RB, IL-8, IL-8RA, IL-10, IL-10RA, IL-10RB, TNF-α, TRADD
Energy sensing: SIRT1, SIRT2, SIRT3, SIRT4, SIRT5
Oxidative phosphorylation: PGC1α, CPT1A, CS, ND2, NDUFAF2, COXI, SCO1
Mitochondrial respiration uncoupling: UCP1, UCP2, UCP3
Transcription-related factors of lipid metabolism: LXRα, PPARα, PPARγ

Table S2. Characteristics of new assembled sequences according to BLAST searches.

Contigs	Size (nt)	Annotation[a]	Best match[b]	E[c]	CDS[d]
C2_29775	1283	IGFBP1	AEI25510	6e-38	<1-221
C2_31065	912	IGFBP7	XP_008302045	2e-133	<1-732
C2_8119	2720	IGFALS	XP_008295360	0.0	55-1788
C2_27455	1259	INSR	CAA12279	0.0	<2->1259
C2_94457	462	IGFR1	XP_008281734	9e-8	<1->462
C2_6981	2246	IGFR2	XP_008279839	0.0	96->2246
C2_29713	1018	MEF2A	XP_006628936	4e-54	362->634
C2_20859	861	MEF2C	XP_008283713	1e-173	<3->861
C2_894	1619	CAV3	XP_008295093	2e-98	183-638
C2_3681	2168	DES	XP_008279286	0.0	94-1527
C2_26597	1082	CDH15	XP_008291154	0.0	<1->1082
C3_c43349	509	PAX7	XP_007552599	4e-44	<1-339
C3_c8164	1162	SOX3	AFV74658	0.0	226-1122
C2_25143	423	MET	XP_008276200	1e-64	<1->423
C2_2905	1863	CAPN3	ACY78226	0.0	244->1863
C2_12340	258	CAST	XP_008318906	8e-30	<1->258
C2_302	1560	CTSL	ADJ21807	0.0	76-1086
C2_386	1227	CTSS	BAK55650	0.0	51-1064
C2_3430	1469	PSMD4	XP_005472747	0.0	40-1167
C2_1392	2208	PSMD12	XP_008274826	0.0	117-1487
C2_89	1171	PSMA5	ACQ58745	3e-175	126-851
C2_45131	245	PSMB1	XP_008284963	1e-49	<2->245
C2_660	1389	UCHL3	XP_003451662	4e-141	120-818
C2_659	1293	UBE2A	XP_003452937	1e-103	147-605
C2_5227	1243	UBE2D2	XP_003451814	0.0	260-703
C2_6947	1839	UBE2L3	XP_003976109	3e-87	16-480
C2_17030	926	UBE2N	XP_003972864	4e-102	171-632
C2_4824	3012	CUL2	CBN81753	0.0	117-2351
C2_4406	2400	CUL3	XP008279409	0.0	<1-1902
C2_13502	1347	CUL5	XP_008331257	0.0	<1-1347
C2_77908	448	Hsp30	XP_008422086	3e-60	1->448
C2_4132	2694	Hsp90α	XP_008297037	0.0	160-2337
C2_42	2781	Hsp90β	AAQ95586	0.0	135-2324
C2_2326	1432	TRADD	XP_008302445	3e-174	65-946

[a]Gene identity determined through BLAST searches: IGFBP1, Insulin-like growth factor binding protein 1; IGFBP7, Insulin-like growth factor binding protein 7; IGFALS, Insulin-like growth factor-binding protein complex acid labile subunit; INSR, Insulin receptor; IGF1R, Insulin-like growth factor receptor I; IGF2R, Insulin-like growth factor receptor II; MEF2A, Myocyte-specific enhancer factor 2A; MEF2C, Myocyte-specific enhancer 2C; CAV3, Caveolin 3; DES, Desmin; CDH15, Cadherin-15; PAX7, Paired box protein 7; SOX3, Transcription factor SOX3; MET, c-met/hepatocyte growth factor receptor; CAPN3, Calpain 3; CAST, Calpastatin; CTSL, Cathepsin L; CTSS, Cathepsin S; PSMD4, 26S proteasome non-ATPase regulatory subunit 4; PSMD12, 26S proteasome non-ATPase regulatory subunit 12; PSMA5, Proteasome subunit alpha type-5; PSMB1A, Proteasome subunit beta type 1-A; UCHL3, Ubiquitin carboxyl-terminal hydrolase isozyme L3; UBE2A, Ubiquitin-conjugating enzyme E2 A; UBE2D2, Ubiquitin-conjugating enzyme E2 D2; UBE2L3, Ubiquitin-conjugating enzyme E2 L3; UBE2N, Ubiquitin-conjugating enzyme E2 N; CUL2, Cullin 2; CUL3, Cullin 3; Hsp30, 30kDa heat shock protein; Hsp90α, 90kDa heat shock protein alpha 1; Hsp90β, 90kDa heat shock protein beta; TRADD, Tumor necrosis factor receptor type 1-associated DEATH domain protein.

[b]Best BLAST-X protein sequence match (lowest E value).

[c]Expectation value.

[d]Codifying sequence.

Table S3. Forward and reverse primers for real-time PCR.

Gene name	Symbol		Primer sequence
Growth hormone receptor I	GHR-I	F	ACC TGT CAG CCA CCA CAT GA
		R	TCG TGC AGA TCT GGG TCG TA
Growth hormone receptor II	GHR-II	F	GAG TGA ACC CGG CCT GAC AG
		R	GCG GTG GTA TCT GAT TCA TGG T
Insulin-like growth factor-I	IGF-I	F	TGT CTA GCG CTC TTT CCT TTC A
		R	AGA GGG TG TGG CTA CAG GAG ATA C
Insulin-like growth factor-II	IGF-II	F	TGG GAT CGT AGA GGA GTG TTG T
		R	CTG TAG AGA GGT GGC CGA CA
Insulin-like growth factor binding protein 1	IGFBP1	F	ACA AAC CAA AAC AGT GCG AGT CCT C
		R	CCG TTC CAA GAG TTC ACA CAC CAG
Insulin-like growth factor binding protein 2	IGFBP2	F	AGC GAT GTG TCC TGA GAT AGT GAG
		R	GCA CCG TGG CGT GTA GAC C
Insulin-like growth factor binding protein 4	IGFBP4	F	GGC ATC AAA CAC CCG CAC AC
		R	ATC CAC GCA CCA GCA CTT CC
Insulin-like growth factor binding protein 7	IGFBP7	F	GCC ACA GCT CCG ATC ATC GTC ACT
		R	AGC CAC TCA CAT TGT AGA CCT CAC CTG
Insulin-like growth factor binding protein complex acid labile subunit	IGFALS	F	GCT CGG AAC TTC ACT CAA GTC CCA TC
		R	AGT TGC CAT CCA GCC AGA TAG AAT GA
Insulin receptor	INSR	F	ACG GAC AGC AAG AAG GCA GAG AAT C
		R	GGC TTC AAC GGT CGG ATC AGG T
Insulin-like growth factor receptor I	IGFR1	F	TCA ACG ACA AGT ACG ACT ACC GCT GCT
		R	CAC ACT TTC TGG CAC TGG TTG GAG GTC
Cation-independent mannose-6-phosphate receptor	IGFR2	F	ACA TTC GGG CAG CAC TCC TAA GAT
		R	CCA GTT CAC CTC GTA GCG ACA GTT
Myoblast determination protein 1	MyoD1	F	ATG GAG CTG TCG GAT ATC TCT TTC
		R	GAA GCA GGG GTC ATC GTA GAA ATC
Myoblast determination protein 2	MyoD2	F	CCA ACT GCT CTG ATG GCA TGA TGG ATT TC
		R	GAC CGT TTG CTT CTC CTG GAC TCG TAT G
Myogenic factor 5	Myf5	F	GCA TGG TTG ACA GCA ACA GTC CAG TGT
		R	TGT CTT ATC GCC CAA AGT GTC GTT CTT CAT
Myogenic factor 6	Myf6/MRF 4/herculin	F	GCA GCA ATG ACA AAC CAG AGA GAC GGA ACA
		R	GAG GCT GGA GGA CGC CGA AGA TTC A
Growth/ differentiation	MSTN/GD	F	AAG AGC AGA TCA TCT ACG GCA AGA TCC
		R	TCA AGA GCA TCC ACA ACG GTC TAC CA

factor 8	F-8		
Myocyte-specific enhancer factor 2A	MEF2A	F R	ATG GAC GAG AGG AAC AGG CAG GTT A GGC TAT CTC ACA GTC ACA TAG TAC GCT CAG
Myocyte-specific enhancer factor 2C	MEF2C	F R	TAG CAA CTC CCA CTC TAC CAG GAC AAG GGA ATA CTC GGC ACC ATA AGA AGT CG
Follistatin	FST	F R	GGA CCA GAC AAA CAA CGC ATA TTG CAT AGA TGA TCC CGT CGT TTC CAC
Caveolin 3	CAV3	F R	CAC CAC CTT CAC TGT GTC CAA CGA CGG GGA TGC CAA AGA C
Desmin	DES	F R	ATT CAC CAG AAG GAG GAG GCT GAG AAC AAC GAG TGG CAT TGT CAA CAT CGG CTC TGA
Cadherin-15	CDH15	F R	AAC GCT TAT CTG AGC TAC TCT ATC ATT GG CTG GTT GTT GAT ACC GAA CAT TGT
Proliferating cell nuclear antigen	PCNA	F R	CGT ATC TGC CGT GAC CTG T AGA ACT TGA CTC CGT CCT TGG
Paired box protein 7	PAX7	F R	GAA CGT GAG CTT GTC CAC CCA GAG G GCC GAG TGG TCT CCC AGT TTC ATC C
Trascription factor SOX3	SOX3	F R	ACA TGA AGG AGC ACC CGG ATT ATA AAT ACC GGG CAA AGA ATA CTT GTC TTT CTT GAG CAA
Hepatocyte growth factor receptor	MET	F R	GCC ACA GGA AAC AAG ATT ACT AAA GTC CCT AGC AAA CAG GAA GTA CAG GTG GTA AGC
Calpain 1	CAPN1	F R	CAG AAC CAC AAC GCC GTG AAG TTT AGG CAC TGG GCT TTA AGA CTC TCG
Calpain 2	CAPN2	F R	CAT CTA TAA GAA GAA CGA CTC GGA CAA CTC TGT TGA GGC TGA AAC CTG CGT CTT TA
Calpain 3	CAPN3	F R	TAC GAA GAG GAT GAC GAC CCA GAG GCA TCA GAG CCA CAA CGA GAG T
Calpastatin	CAST	F R	CCC AAA CCC GAG CCC ACC AT GAC AAG AAG TCC AGA GCG TCT CCA GTA
Cathepsin B	CTSB	F R	TGA TTC CCA TGT CGG TTG TC GGG TCT ACT GCC ATT CAC AT
Cathepsin D	CTSD	F R	CAC ACT GGG AGA CCT GCA CTA TGT CAA TG ATT GCC AAC TTG AAG TCC GTC CAT ACC
Cathepsin L	CTSL	F R	GGG AAC GGA TGA CCA GCC TTG T CGG TGT CAT TGG CAG AGT TGT AGT TG

Cathepsin S	CTSS	F	CCA CAT GGG AGA CCT GAC ACC AGA GGA GAT
		R	TCA GTG GGA GGA ATG AAT GTG GCG AAA GAC
26S proteasome non-ATPase regulatory subunit 4	PSMD4	F	CAT CCA CAC CTG CTC TAC CAG ACT TCA
		R	CGT AGG CGA TCT GTT CAT CCT CTG TCA T
26S proteasome non-ATPase regulatory subunit 12	PSMD12	F	CCT GCC ACA GAT GTC TTC TCT TAT TCA
		R	GCC ATT ATT CTG ATG TTA TGC TCC ACC A
Proteasome subunit alpha type-5	PSMA5	F	TGA CAA GAT CGG AGT ATG ACA GAG GTG TGA
		R	CCT CGA TGG CAT ATT CAA CCT GGA ACA ATC
Proteasome subunit beta type-1	PSMB1	F	TGG ATG AAG AGG GCA AAG GAG CAG TGT A
		R	TTG TAT GTG TCT CTC TGG TAG GAG CCC ACT
Ubiquitin carboxyl-terminal hydrolase isozyme L3	UCHL3	F	CAG TGA CAG AGA AGT ATG AGA CAT TCA A
		R	CGT TTC CAA TAG TTT GCT TAA TGA AGT AGA
Ubiquitin-conjugating enzyme E2 A	UBE2A	F	CAT CAT GGT GTG GAA TGC AGT CAT ATT TGG
		R	GGG TTT GTT GGG GTA TTC TTC TGT GAA CT
Ubiquitin-conjugating enzyme E2 D2	UBE2D2	F	TCT GCT GTG CGA CCC AAA CC
		R	GAT GCG GGC GAT CTC GGG TA
Ubiquitin-conjugating enzyme E2 L3	UBE2L3	F	CCT CAT TGC ATT GGT GAA CG
		R	TTG AGT ATT CTT CTG CTA GGT CAG
Ubiquitin-conjugating enzyme E2 N	UBE2N	F	TTG CCT CGT AGG ATT ATT AAG GAG AC
		R	CCT GAA ATG ACC ACA TGG AAG TAA CG
Cullin 2	CUL2	F	GGC ATC CGA GGC ACC AGT AAC C
		R	TGA ACC TCC AGC ACT GAC TCC ACA A
Cullin 3	CUL3	F	AAA GGA GGA TGG TTC AGA AGT TGG
		R	CAG TAT GTG CTT ACG GGT GTT AGA G
Cullin 5	CUL 5	F	CAA ACT CAA GAG GCA GGT GTT GTC GTA
		R	GTA GAA TAG TGT TCC CTC TGC GAA GTC T
10 kDa heat shock protein, mitochondria	mtHsp10	F	CAT GCT GCC AGA GAA GTC TCA AGG
		R	AGG TCC CAC TGC CAC TAC TGT
30kDa heat shock protein	Hsp30	F	ATC TCT CAA CAA GAC CAC ACA ACA CT
		R	TAC AGG CCA GTA CAA GTC CAT GAA TG
60 kDa heat shock protein, mitochondrial	mtHsp60	F	TGT GGC TGA GGA TGT GGA TGG AGA G
		R	GCC TGT TGA GAA CCA AGG TGC TGA G
70 kDa heat shock protein, mitochondrial	mtHsp70/ GRP-75	F	TCC GGT GTG GAT CTG ACC AAA GAC
		R	TGT TTA GGC CCA GAA GCA TCC ATG

90kDa heat shock protein alpha 1	Hsp90a	F	CTC ACA GTT CAT CGG CTA CCC TAT CA
		R	AAC TTC CTC TTC CTT CTC TCC CTC ATC AAG
90kDa heat shock protein beta	Hsp90b	F	AGA ATA ACA TCA AGC TGT ACG TCA GGA GAG
		R	CAC CAC ACC ACG GAC AAA GTT CAG ATA CTC
Glucose-regulated protein, 170 kDa	GRP-170	F	CAG AGG AGG CAG ACA GCA AGA C
		R	TTC TCA GAC TCA GCA TTT CCA GAT TTC
Glucose-regulated protein, 94 kDa	GRP-94	F	AAG GCA CAG GCT TAC CAG ACA G
		R	CTT CAG CAT CAT CGC CGA CTT TC
Derlin-1	DER-1	F	ACT GCC TCG GTT GCC TTT CC
		R	TGG CTG TCA CAA GTC TCC AGA TAT G
Interleukin-1 beta	IL-1β	F	GCG ACC TAC CTG CCA CCT ACA CC
		R	TCG TCC ACC GCC TCC AGA TGC
Interleukin-1 beta receptor 1	IL-1R1	F	GAA GCT GTA CGA CGC CTA C
		R	CTC CAC TGC CTT ACT GTA TCC
Interleukin-1 beta receptor 2	IL-1R2	F	CCT GAC CTC TCC GTG ACC TCT AA
		R	TGG CTG CTG CTG CTG ATG A
Interleukin-6	IL-6	F	TCT TGA AGG TGG TGC TGG AAG TG
		R	TCT TGA AGG TGG TGC TGG AAG TG
Interleukin-6 receptor A	IL-6RA	F	GCA GTG CTC GTA CTC TTC
		R	CTC CGC TCT TCC TCA TTG
Interleukin-6 receptor B	IL-6RB	F	CAG TGT CGG AGT ATG TGG TTG AGT
		R	CCC TCT GCC AGT CTG TCC AA
Interleukin-8	IL-8	F	CAG CAG AGT CTT CAT CGT CAC TAT TG
		R	AGG CTC GCT TCA CTG ATG G
Interleukin-8 receptor A	IL-8RA	F	CTT GTT TCA TCT GAC GAT AG
		R	AAG AGG ATG CTT GTG TAG
Interleukin-10	IL-10	F	AAC ATC CTG GGC TTC TAT CTG
		R	GTG TCC TCC GTC TCA TCT G
Interleukin-10 receptor A	IL-10RA	F	GAG GAC AAT GAA GAG GAA GAC AGG AG
		R	TGT TCG TAG CGG AGT TGG ACT
Interleukin-10 receptor B	IL-10RB	F	AGA CCC ACA GGC TTC AGA T
		R	GCA GCG TCA CCA GGT TAG
Tumor necrosis factor - alpha	TNF-α	F	CAG GCG TCG TTC AGA GTC TC
		R	CTG TGG CTG AGA GCT GTG AG
Tumor necrosis factor	TRADD	F	GAG GGA AAG TTC ATC GTG TTC AAA GTC ATC

receptor type 1-associated DEATH domain protein		R	AAC GGA TCA GCA TCA TGG ACC TTA AGT A
Sirtuin1	SIRT1	F	GGT TCC TAC AGT TTC ATC CAG CAG CAC ATC
		R	CCT CAG AAT GGT CCT CGG ATC GGT CTC
Sirtuin2	SIRT2	F	GAA CAA TCC GAC GAC AGC AGT GAA G
		R	AGG TTA CGC AGG AAG TCC ATC TCT
Sirtuin3	SIRT3	F	CTG CCA AGT CCT CAT CCC
		R	CTT CAC CAG ACG AGC CAC
Sirtuin4	SIRT4	F	GGC TGG CGG AGT CGG ATG
		R	TCC TGA ATA CAC CTG TGA CGA AGA C
Sirtuin5	SIRT5	F	CAG ACA TCC TAA CCC GAG CAG AG
		R	CCA CGA GGC AGA GGT CAC A
Proliferator-activated receptor gamma coactivator 1 alpha	PGC1α	F	CGT GGG ACA GGT GTA ACC AGG ACT C
		R	ACC AAC CAA GGC AGC ACA CTC TAA TTC T
Carnitine palmitoyltransferase 1A	CPT1A	F	GTG CCT TCG TTC GTT CCA TGA TC
		R	TGA TGC TTA TCT GCT GCC TGT TTG
Cytrate synthase	CS	F	TCC AGG AGG TGA CGA GCC
		R	GTG ACC AGC AGC CAG AAG AG
NADH-ubiquinone oxidoreductase chain 2	ND2	F	TAG GTT GAA TGA CCA TCG TA
		R	GGC TAA GGA GTT GAG GTT
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 2	NDUFAF2	F	AGG CAG CAT ACC GAT AGA G
		R	ACT CAT TCT TCA GCA ACT CCT
Cytochrome c oxidase subunit I	COXI	F	GTC CTA CTT CTT CTG TCC CTT CCT GTT CT
		R	AGG TTT CGG TCT GTA AGG AGC ATT GTA ATC
SCO1 protein homolog, mitochondrial	SCO1	F	ACA ACA ACA AGC CCA CCA AGA
		R	GAC AGT GAG TGA ACC CGA AGT AGA T
Uncoupling protein 1	UCP1	F	GCA CAC TAC CCA ACA TCA CAA G
		R	CGC CGA ACG CAG AAA CAA AG
Uncoupling protein 2	UCP2	F	CGG CGG CGT CCT CAG TTG
		R	AAG CAA GTG GTC CCT CTT TGG TCA T
Uncoupling protein 3	UCP3	F	AGG TGC GAC TGG CTG ACG
		R	TTC GGC ATA CAA CCT CTC CAA AG
Liver X receptor α	LXRα	F	GCA CTT CGC CTC CAG GAC AAG
		R	CAG TCT TCA CAC AGC CAC ATC AGG
Peroxisome proliferator-	PPARα	F	TCT CTT CAG CCC ACC ATC CC

activated receptor α		R	ATC CCA GCG TGT CGT CTC C
Peroxisomeproliferator-activated receptor γ	PPARγ	F	CGC CGT GGA CCT GTC AGA GC
		R	GGA ATG GAT GGA GGA GGA GGA GAT GG

Table S4. Relative gene expression of growth-related genes in the liver of gilthead sea bream sampled in August 2013 (week 13). Data are the mean ± SEM of 6-7 fish. All data are referenced to the expression level of IGFR2 of control fish (D1 diet) with an arbitrarily assigned value of 1. Different superscript letters in each row indicate significant differences among dietary treatments (SNK test, P<0.05).

	D1	D2	D3	D4	P-value
GHR-I	19.41±0.99	18.53±2.07	19.63±2.12	20.12±3.42	0.898
GHR-II	13.88±2.54	13.06±2.10	11.29±0.97	14.85±1.92	0.601
IGF-I	68.1±8.65^{a}	56.71±8.96^{ab}	37.6±4.30^{b}	47.21±4.07^{ab}	0.018
IGF-II	28.09±5.30	30.36±2.76	20.83±4.96	23.21±2.85	0.335
IGFBP1	0.11±0.03	0.11±0.01	0.14±0.01	0.10±0.01	0.212
IGFBP2	17.13±2.19	14.59±1.41	13.55±1.18	13.96±1.49	0.452
IGFBP4	6.19±1.06	4.18±0.58	4.60±0.28	4.45±0.63	0.204
IGFBP7	6.44±0.71	5.15±0.48	4.57±0.73	4.31±0.53	0.069
IGFALS	176.7±42.1	129.1±10.2	101.8±12.1	126.7±14.3	0.214
INSR	3.05±0.11	2.25±0.26	2.68±0.25	2.56±0.14	0.101
IGFR1	0.25±0.02	0.19±0.01	0.24±0.01	0.21±0.01	0.057
IGFR2	1.04±0.06	0.90±0.06	0.95±0.05	0.96±0.07	0.469
MEF2A	2.94±0.26	2.32±0.12	2.50±0.25	2.23±0.15	0.083
MEF2C	0.28±0.02	0.25±0.02	0.35±0.05	0.25±0.02	0.136
PCNA	8.73±1.13^{a}	6.17±1.25^{ab}	6.58±0.83^{ab}	4.04±0.53^{b}	0.017
MET	4.11±0.59	3.81±0.21	3.82±0.59	3.47±0.31	0.785
CAPN1	2.94±0.26	2.27±0.25	3.16±0.33	2.24±0.23	0.051
CAPN2	1.24±0.17	1.04±0.04	1.37±0.10	1.18±0.08	0.212
CAPN3	0.09±0.02	0.08±0.01	0.13±0.03	0.08±0.01	0.292
CAST	4.19±0.53	3.93±0.41	5.01±0.35	4.25±0.44	0.374
CTSB	30.61±2.51	33.9±3.15	37.67±3.65	33.74±4.19	0.593
CTSD	4.67±1.50	4.14±1.02	2.69±0.54	3.16±0.23	0.461
CTSL	86.43±10.19	77.26±10.64	77.91±9.76	74.30±12.19	0.875
CTSS	2.12±0.12	2.38±0.35	2.65±0.32	2.44±0.28	0.635
PSMD4	2.53±0.26	2.06±0.22	2.64±0.12	2.03±0.24	0.142
PSD12	3.59±0.30	3.70±0.34	4.20±0.33	3.10±0.39	0.184
PSMA5	3.31±0.34	2.47±0.50	3.91±0.37	2.68±0.60	0.174
PSMB1A	7.31±0.48	6.55±0.52	8.78±0.34	6.39±1.06	0.059
UCHL3	2.07±0.14	1.50±0.17	2.29±0.19	1.82±0.30	0.107
UBE2A	2.15±0.26	1.73±0.11	1.98±0.13	2.10±0.18	0.407
UBE2D2	6.00±0.37	4.92±0.32	4.76±0.22	5.04±0.48	0.118
UBE2L3	12.31±0.86	10.04±0.63	12.95±0.64	10.28±1.07	0.059
UBE2N	3.83±0.15	3.23±0.26	3.86±0.07	3.56±0.42	0.373
CUL2	1.06±0.09	0.91±0.06	1.06±0.06	0.94±0.07	0.331
CUL3	0.99±0.08	0.86±0.07	0.98±0.06	0.81±0.05	0.140
CUL5	0.43±0.03	0.42±0.04	0.44±0.03	0.38±0.03	0.564
mtHsp10	7.30±0.61^{a}	4.96±0.54^{b}	6.65±0.54^{ab}	5.17±0.53^{b}	0.019
Hsp30	0.04±0.01	0.05±0.01	0.07±0.02	0.05±0.01	0.417
mtHsp60	2.39±0.31^{a}	1.64±0.17^{b}	1.83±0.08^{b}	1.46±0.11^{b}	0.011
mtHsp70	4.91±0.44^{a}	3.17±0.24^{b}	2.90±0.22^{b}	2.84±0.22^{b}	<0.001

	D1	D2	D3	D4	P-value
Hsp90β	185.3±3.99[a]	134.4±7.32[b]	149.7±7.70[b]	133.67±4.38[b]	<0.001
GRP-170	6.10±0.62	5.80±0.76	6.36±0.42	4.85±0.44	0.268
GRP-94	15.10±2.50	13.20±1.81	11.78±1.51	9.33±0.72	0.118
DER-1	11.09±0.70	10.09±0.91	10.38±0.62	8.72±0.79	0.185
IL-1β	0.01±0.01	0.01±0.01	0.03±0.01	0.01±0.01	0.325
IL-1R1	2.36±0.26	2.46±0.28	3.50±0.80	3.63±0.94	0.206
IL-1R2	0.01±0.01	0.01±0.01	0.04±0.02	0.01±0.01	0.053
IL-6RA	8.42±1.03	6.65±0.51	8.48±1.44	8.50±0.80	0.495
IL-6RB	7.40±0.42[a]	6.59±0.55[ab]	6.38±0.52[ab]	5.34±0.21[b]	0.020
IL-8	0.03±0.01	0.03±0.01	0.04±0.01	0.03±0.01	0.463
IL-8RA	0.15±0.03	0.14±0.04	0.22±0.07	0.15±0.04	0.576
IL-10	0.05±0.01	0.04±0.01	0.04±0.01	0.03±0.01	0.613
IL-10RA	0.20±0.05	0.16±0.02	0.19±0.05	0.14±0.01	0.518
IL-10RB	5.83±0.42[a]	4.95±0.57[ab]	3.93±0.27[b]	4.52±0.54[ab]	0.048
TNF-α	0.13±0.01	0.11±0.01	0.11±0.01	0.11±0.01	0.555
TRADD	1.10±0.05[a]	0.94±0.06[a]	1.41±0.14[b]	1.04±0.08[a]	0.010
SIRT1	0.51±0.03	0.53±0.04	0.57±0.06	0.55±0.04	0.764
SIRT2	2.01±0.13	1.80±0.15	1.81±0.06	1.77±0.16	0.598
SIRT3	0.34±0.03	0.30±0.04	0.35±0.03	0.23±0.01	0.300
SIRT4	0.14±0.01	0.13±0.01	0.13±0.01	0.13±0.01	0.738
SIRT5	2.14±0.16	1.86±0.17	1.73±0.13	1.72±0.15	0.217
PGC1α	0.18±0.03	0.19±0.03	0.17±0.03	0.22±0.04	0.812
CPT1A	2.81±0.36	1.72±0.23	2.16±0.30	2.34±0.20	0.082
CS	6.50±0.48[a]	4.60±0.32[b]	5.81±0.34[ab]	4.91±0.35[b]	0.008
ND2	184.9±9.65	182.6±18.13	235.7±17.7	204.4±20.76	0.161
NDUFAF2	2.42±0.15	2.39±0.11	2.32±0.15	2.31±0.13	0.922
COXI	406.8±22.1[a]	259.7±47.5[b]	366.0±31.3[ab]	328.1±22.7[ab]	0.028
SCO1	0.49±0.06	0.43±0.05	0.44±0.02	0.35±0.03	0.136
UCP1	108.0±11.71	92.37±20.75	109.8±14.42	112.3±13.36	0.795
UCP2	0.02±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.300
LXRα	5.66±0.78[a]	3.34±0.33[b]	4.62±0.41[ab]	4.10±0.36[ab]	0.038
PPARα	16.77±1.13	14.43±2.06	19.06±1.70	16.42±1.36	0.276
PPARγ	5.50±0.57[a]	3.79±0.55[b]	3.95±0.17[b]	3.86±0.37[b]	0.037

Table S5. Relative gene expression of growth-related genes in the skeletal muscle of gilthead sea bream sampled in August 2013 (week 13). Data are the mean ± SEM of 6-7 fish. All data are referenced to the expression level of IGFR2 of control fish (D1 diet) with an arbitrarily assigned value of 1. Different superscript letters in each row indicate significant differences among dietary treatments (SNK test, P<0.05).

	D1	D2	D3	D4	P-value
GHR-I	8.22±0.46	7.12±0.96	6.81±0.58	7.52±0.55	0.480
GHR-II	3.63±0.37	2.60±0.37	2.96±0.76	2.55±0.50	0.501
IGF-I	0.59±0.06	0.65±0.13	0.69±0.09	0.61±0.04	0.789
IGF-II	3.2±0.32	3.21±0.28	3.11±0.26	2.76±0.36	0.705
IGFBP1	0.31±0.05	0.40±0.05	0.45±0.05	0.31±0.05	0.062
IGFBP4	0.49±0.07	0.67±0.15	0.45±0.05	0.70±0.17	0.419
IGFBP7	4.61±0.53	4.31±0.47	3.97±0.29	3.61±0.67	0.540
INSR	1.44±0.09	1.21±0.07	1.42±0.09	1.31±0.16	0.451
IGFR1	1.41±0.12	1.17±0.12	1.27±0.07	1.17±0.13	0.384
IGFR2	0.83±0.13	0.65±0.03	0.60±0.02	0.61±0.08	0.189
MyoD1	15.23±1.23	12.22±0.72	16.74±1.16	15.07±1.70	0.059
MyoD2	8.04±1.16	7.02±1.01	8.75±1.18	6.83±0.78	0.517
Myf5	1.05±0.09	1.07±0.06	1.12±0.05	0.97±0.08	0.544
Myf6	1.03±0.09	0.96±0.07	1.23±0.04	1.08±0.22	0.530
MSTN	5.21±1.35	4.51±0.45	6.17±1.82	5.70±0.96	0.493
MEF2A	36.17±2.2	34.08±1.95	39.18±2.17	37.30±3.13	0.543
MEF2C	8.77±0.44	7.80±0.42	8.55±0.51	8.09±0.57	0.523
FST	0.84±0.15	0.88±0.22	0.88±0.03	0.89±0.20	0.995
CAV3	73.81±4.50	71.88±2.71	87.10±6.41	77.70±3.76	0.128
DES	194.1±15.23	202.2±8.80	204.1±12.37	198.8±22.59	0.972
CDH15	1.62±0.10^{a}	1.80±0.11^{ab}	2.07±0.09^{b}	1.65±0.12^{a}	0.020
PCNA	2.73±0.29	2.57±0.20	3.00±0.20	2.26±0.26	0.204
PAX7	0.12±0.01	0.15±0.02	0.14±0.02	0.15±0.01	0.383
SOX3	0.06±0.03	0.02±0.01	0.04±0.01	0.03±0.01	0.690
MET	0.37±0.04	0.34±0.04	0.33±0.03	0.26±0.02	0.145
CAPN1	3.92±0.22	3.91±0.34	4.21±0.30	3.85±0.29	0.808
CAPN2	5.16±0.35	5.56±0.71	5.32±0.33	4.96±0.54	0.850
CAPN3	13.76±0.77^{a}	13.45±0.86^{a}	18.24±0.94^{b}	12.13±1.51^{a}	0.003
CAST	13.91±1.15	11.21±0.50	14.24±1.83	12.36±0.87	0.312
CTSB	7.12±0.58	7.19±0.45	5.98±0.37	6.10±0.69	0.262
CTSD	0.91±0.07	0.96±0.06	0.86±0.05	0.73±0.09	0.151
CTSL	11.36±0.90	11.32±1.19	10.78±0.65	10.22±0.79	0.776
CTSS	2.04±0.27	2.32±0.24	1.74±0.30	1.57±0.23	0.224
PSMD4	1.97±0.10	1.92±0.05	2.11±0.08	1.75±0.17	0.179
PSD12	5.95±0.40	5.85±0.50	6.71±0.30	5.12±0.53	0.105
PSMA5	3.10±0.23^{ab}	2.88±0.19^{ab}	3.52±0.20^{b}	2.41±0.31^{a}	0.023
PSMB1A	7.85±0.57^{ab}	7.59±0.20^{ab}	9.04±0.36^{b}	6.72±0.69^{a}	0.028
UCHL3	4.02±0.19^{a}	3.79±0.07^{ab}	4.23±0.30^{a}	3.15±0.26^{b}	0.013
UBE2A	4.29±0.86	3.46±0.14	3.69±0.35	3.06±0.27	0.389
UBE2D2	1.98±0.19	1.92±0.07	2.16±0.17	1.61±0.12	0.091

	D1	D2	D3	D4	P-value
UBE2L3	17.42±1.00	15.68±1.02	18.37±1.15	15.48±0.82	0.153
UBE2N	13.03±0.88^{ab}	12.14±0.30^{ab}	14.60±1.05^{b}	10.57±1.07^{a}	0.025
CUL2	2.05±0.16	1.84±0.04	2.15±0.08	1.75±0.11	0.051
CUL3	3.02±0.15	2.65±0.14	3.27±0.12	2.72±0.25	0.076
CUL5	0.68±0.07	0.67±0.04	0.81±0.11	0.60±0.06	0.228
mtHsp10	5.39±0.79	5.60±0.63	6.83±0.84	4.86±0.67	0.300
Hsp30	0.08±0.06	0.08±0.02	0.11±0.07	0.08±0.04	0.966
mtHsp60	2.10±0.19	2.17±0.19	2.58±0.22	1.85±0.20	0.110
mtHsp70	4.44±0.38	4.21±0.30	5.40±0.49	4.48±0.38	0.197
Hsp90α	60.02±8.51	69.10±8.46	73.07±8.37	57.80±3.82	0.434
Hsp90β	29.87±2.55	31.26±2.86	31.34±2.05	27.65±2.45	0.690
GRP-170	1.83±0.15	1.81±0.13	1.98±0.09	1.60±0.16	0.282
GRP-94	3.23±0.29	3.04±0.16	2.96±0.26	2.41±0.14	0.085
DER-1	9.18±0.55	9.48±0.37	10.84±0.67	8.76±0.60	0.079
IL-1β	0.05±0.01	0.04±0.01	0.05±0.01	0.05±0.01	0.659
IL-1R1	0.61±0.04	0.62±0.09	0.55±0.06	0.47±0.07	0.367
IL-1R2	0.02±0.01	0.02±0.01	0.01±0.01	0.01±0.01	0.086
IL-6	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.456
IL-6RA	0.44±0.03	0.44±0.07	0.47±0.02	0.38±0.06	0.596
IL-6RB	2.91±0.16	2.87±0.12	3.01±0.21	2.40±0.22	0.122
IL-8	0.04±0.01	0.04±0.01	0.04±0.01	0.04±0.01	0.787
IL-10	0.05±0.01	0.06±0.01	0.05±0.01	0.04±0.01	0.356
IL-10RA	0.06±0.01	0.06±0.01	0.05±0.01	0.04±0.01	0.438
IL-10RB	0.73±0.05	0.91±0.05	0.76±0.04	0.73±0.08	0.125
TNFα	0.10±0.01^{ab}	0.10±0.01^{ab}	0.12±0.01^{b}	0.08±0.01^{a}	0.025
TRADD	0.29±0.02	0.32±0.03	0.34±0.02	0.27±0.03	0.251
SIRT1	0.61±0.04	0.61±0.02	0.61±0.02	0.55±0.06	0.672
SIRT2	1.27±0.11	1.19±0.04	1.26±0.07	1.06±0.11	0.314
SIRT3	0.17±0.01	0.17±0.02	0.19±0.02	0.15±0.01	0.310
SIRT4	0.14±0.01	0.12±0.01	0.14±0.01	0.12±0.01	0.254
SIRT5	1.89±0.15	1.74±0.13	1.95±0.08	1.64±0.12	0.282
PGC1α	0.32±0.09	0.24±0.08	0.28±0.06	0.32±0.10	0.896
CPT1A	5.66±0.59	5.16±0.66	4.50±0.36	6.52±0.99	0.226
CS	42.9±3.49	38.41±2.72	40.39±2.24	39.05±3.97	0.769
ND2	182.3±24.2	224.6±11.9	231.1±26.5	205.0±14.1	0.354
NDUFAF2	2.09±0.15	2.00±0.11	2.04±0.14	1.80±0.15	0.462
COXI	553.6±72.1	503.9±22.8	551.4±24.6	503.8±11.4	0.711
SCO1	0.32±0.03	0.29±0.02	0.31±0.03	0.33±0.04	0.812
UCP2	0.64±0.10	0.61±0.09	0.91±0.18	0.72±0.16	0.365
UCP3	14.36±2.62	13.30±3.07	20.36±2.42	16.72±3.42	0.363
LXRα	0.72±0.05	0.70±0.02	0.65±0.04	0.60±0.04	0.137
PPARα	1.98±0.10^{a}	1.80±0.12^{ab}	1.64±0.11^{ab}	1.37±0.16^{b}	0.013
PPARγ	1.64±0.14^{a}	1.62±0.48^{ab}	1.32±0.24^{ab}	1.10±0.10^{b}	0.015