Enhanced dietary formulation to mitigate winter thermal stress in gilthead seabream (*Sparus aurata*): a 2D-DIGE plasma proteome study

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Abstract

Low water temperatures during winter are common in farming of gilthead seabream in the Mediterranean. This causes metabolic disorders that in extreme cases can lead to a syndrome called “winter disease”. An improved immunostimulatory nutritional status might mitigate the effects of this thermal metabolic stress. A trial was set-up to assess the effects of two different diets on gilthead seabream physiology and nutritional state, through plasma proteome and metabolites. Four groups of 25 adult gilthead seabream were reared during winter months, being fed either with a control diet (CTRL) or with a diet called “winter feed” (WF). Proteome results show a slightly higher number of proteins up-regulated in plasma of fish fed the WF. These proteins are mostly involved in the immune system and cell protection mechanisms. Lipid metabolism was also affected, as shown both by plasma proteome and by the cholesterol plasma levels. Overall, the winter feed diet

tested seems to have positive effects in terms of fish condition and nutritional status, reducing the metabolic effects of thermal stress.

Keywords: Aquaculture, Gilthead seabream, Plasma, Winter disease, Winter syndrome, Thermal stress, Proteomics.

1. Introduction

Gilthead seabream (*Sparus aurata*) is one of the main species produced in Southern Europe, with its production having doubled in the last ten years up to 139.000 tons in 2010(FAO 2012). This species is a sparid teleost that lives in the Mediterranean Sea and the east coast of the Atlantic ocean, from the British isles to Cape Verde and rarely in the Black Sea(Sola 2005). Aquaculture of gilthead seabream has been improved by better knowledge of the requirements for optimal growth, although it is vulnerable to water temperature variations in the Mediterranean Sea(Ibarz et al. 2007). *Sparus aurata* is sensitive to low water temperatures, which may lead to a so-called “winter disease” or “winter syndrome”(Tort et al. 1998), that may lead in some cases to high mortalities(Ibarz et al. 2007; Tort et al. 2004). The disease has an average mortality of 7 to 10%(Sala-Rabanal et al. 2003) but some cases have been reported where mortality may be as high as 80%(Tort et al. 1998). A critical temperature of 12 ºC is suggested, below which fish stop feeding(Sala-Rabanal et al. 2003). Winter disease typically affects cultured seabream in the Mediterranean, since European sea bass and meagre do not seem to be affected in the same conditions(Ibarz et al. 2010b). In the wild, when surface water temperatures decrease in winter, gilthead seabream

seems to migrate to deeper warmer waters(Davis 1988). In winter farming conditions several physiologic, metabolic and immune disorders that affect gilthead seabream have been described(Ibarz et al. 2010b). These include an ionic imbalance caused by malfunctions of the gills and digestive system, altered blood composition and liver metabolism, often leading to fatty liver and steatosis, and an immune suppression that render fish more susceptible to infection. These seem to arise mainly from reduced feed intake and even periods of fasting, but also from reductions in the capacity to digest and absorb nutrients induced by cold stress(Ibarz et al. 2010b).

In gilthead seabream, Bavčević *et al.(Bavcevic et al. 2006)* studied different diets during winter-spring period, demonstrating that more lipids in the diet enhance growth during periods of cold temperature. Tort *et al*.(Tort et al. 2004) observed an improved seabream immune status when cold exposed fish were fed a diet with a high palatability, a high nutrient density, high in digestible proteins and lipids, rich in highly unsaturated fatty acids and phospholipids, and supplemented in vitamin C, vitamin E, choline, inositol, and minerals. These studies support the idea that the administration of specific diets at different temperature periods might help mitigate the effect of a thermal challenge on fish health and nutritional status, helping to prevent the winter disease outbreaks.

In order to improve, during winter months, the nutritional and metabolic status of *Sparus aurata* we formulated a fortified diet (called “winter feed” or WF), as reference for a high-quality feed. This diet contains a higher amount of marine-derived ingredients, marine phospholipids, taurine, soy lecithin, antioxidant vitamins and is supplemented with phagostimulants. It was

compared to a standard commercial formulation (called control diet or CTRL) containing low levels of fishmeal and whose fish oil was partially replaced by rapeseed oil. At the end of winter, we assessed the effects of these diets on the plasma proteome of the fish as well as on the concentration in metabolites involved in various metabolic pathways (individual amino acids, cholesterol, triacylglycerides) and metabolites involved in primary (cortisol) and secondary (lactate and glucose) stress response. In a companion study, Silva *et al.*(Silva et al. 2014) showed a positive effect of the WF diet in terms of nutritional and metabolic status, showing higher liver weights and hepatosomatic index associated with a hepatic accumulation of carbohydrates.

The present study aimed to verify to what extent the effects of winter thermal stress can be mitigated by improved nutrition, using plasma proteome and metabolome as proxies. Proteomics has been successfully applied in aquaculture and reported in several recent studies (Alves et al. 2010; Rodrigues et al. 2012; Silva et al. 2011; Silva et al. 2012a; Silva et al. 2012b). We choose to analyze plasma proteome which has previously been done (Piras et al. 2014) in order to improve knowledge of very early pathologic mechanisms and to support the scarce and complex diagnostic procedures currently available.

2. Material and methods

In Figure 1 a schematics representing the experimental design and assays is presented.

2.1 Experimental diets

Formulations of the diets used in this trial are represented in Table 1. A control diet (CTRL) was formulated with low fishmeal levels (15%), a significant amount of plant-protein sources and a blend of fish and rapeseed oils. An inorganic phosphorus source (di-calcium phosphate) and L-lysine (a crystalline essential amino acid) were supplemented to cover the nutritional requirements of the species. This control diet contained 48.3% crude protein, 19.6% crude fat and 22.8 MJ/kg gross energy. Comparatively, the experimental winter feed (WF) had a much higher proportion of marine-derived protein sources (45.8%), lower level of plant-proteins and the totality of the oil fraction associated to fish oil and krill phospholipids. As a phagostimulant and to facilitate fat emulsification during digestion, betaine and soy lecithin were supplemented respectively. To this diet, antioxidants like vitamin C, E and the non-essential amino acid taurine were added, knowing that taurine is also involved in bile acid conjugation. The WF diet contained 50.6% crude protein, 19.7% crude fat and 22.4 kJ/g gross energy.

Based on previous studies(Dias et al. 2009; Tibaldi et al. 2006) on digestibility of the ingredients used in the present study, it is easy to predict that the winter feed would have a higher digestibility for protein and fat (not measured in the present study). Moreover, the winter feed had a high quality fish meal, the generally accepted golden standard for fish feeds for carnivorous fish such as gilthead seabream, as a main ingredient, and within normal values for high quality fish feeds. Therefore, the WF is clearly a superior diet compared to the control formulation.

Main ingredients were ground (below 250 micron) in a micropulverizer hammer mill (Hosokawa Micron, SH1, The Netherlands). Powder ingredients and oil sources were then mixed accordingly to the target formulation in a paddle mixer (Mainca RM90, Spain). Diets were manufactured by temperature controlled-extrusion (pellet size: 5.0 mm) by means of a low shear extruder (Italplast P55, Italy). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 2 hours at 60ºC. Throughout the duration of the trial, experimental feeds were stored at room temperature, but in a cool and aerated environment. Samples of each diet were taken for analysis of proximate composition (Table 1).

2.2 Fish and rearing conditions

For this trial, four groups of 25 gilthead seabream were reared for 4 months (between November 2011 and March of the following year) in 1000 L circular plastic tanks, with natural flow-through seawater, at the Ramalhete experimental station of the University of Algarve, Faro, Portugal. Duplicates were used for each diet. Fish, with a mean initial body weight of 87 ± 5 g, were fed once a day by hand, *ad libitum* and kept with natural temperature (14.8 ± 2.1 ºC, with minimum and maximum values of 7.6 and 19.5 ºC, respectively) and artificial aeration (dissolved oxygen above 5 $mg.L^{-1}$), salinity (33 ± 2 ‰) and a rearing density of about 2.18 $kg\ m^{-3}$. Tanks were exposed to natural environmental and natural photoperiod conditions.

2.3 Sampling

When temperatures reached more than 15 ºC for over a week, winter period was considered over, and sampling occurred (116 days of trial). Five fish randomly selected from each tank were anesthetized with 200 ppm of 2-

phenoxyethanol (Sigma Aldrich, St. Louis, Missouri, USA), weighted and approximately 1 ml of blood was withdrawn using syringes rinsed with 1% EDTA solution. Blood was centrifuged at 2000 ×g for 20 minutes and plasma was collected and kept at -80 ºC for subsequent analysis. Fish were killed with a subsequent dose (1000 ppm) of 2-phenoxyethanol, measured and liver weight was taken for calculation of the hepatosomatic index (HSI). Prior to sampling, fish were starved for 48 hours, in order to guarantee that changes in proteome and metabolome are not just the result of postprandial metabolism; i.e., the objective was to measure effects in a post-absorptive metabolism (steady-state). The experiment described was conducted in accordance with the Guidelines of the European Union Council (Directive 2010/63/EU) and the Portuguese legislation for the use of laboratory animals, and under a "Group-1" license (permit number 0420/000/000-n.99-09/11/2009) from the Veterinary Medicine Directorate, the competent Portuguese authority for the protection of animals, Ministry of Agriculture, Rural Development and Fisheries, Portugal.

2.4 Protein labeling for DIGE (Difference gel electrophoresis)

Prior to protein separation, a quantification of the protein in plasma from each of five fish per tank (i.e. 10 fish per dietary treatment) was performed using the Bradford assay (Bio-Rad) and bovine serum albumin as standard. Samples were adjusted to pH 8.5 with 0.1 M NaOH and 50 µg of proteins were minimally labeled with 400 pmol of fluorescent amine reactive cyanine dyes freshly dissolved in anhydrous dimethylformamide (Alfa aesar, Ward Hill, Massachusetts, USA) following manufacturer's instructions (5 nmol labeling kit, GE Healthcare, Uppsala, Sweden). Labeling was performed on

ice for 30 minutes in the dark and quenched with 1 mM of lysine for 10 min. Five samples per dietary treatment were labeled with Cy3 and five with Cy5 to reduce impact of label differences, while an internal standard consisting of equal amounts of protein from all samples was labeled with Cy2.

2.5 Protein separation by 2D gel electrophoresis

Labeled proteins from each dietary treatment, plus 50 µg of internal standard were mixed together and rehydration buffer (6 M urea, 2 M thiourea, 4% CHAPS, 0.2 % (w/v) DTT, 0.002% bromophenol blue, 0.5% (v/v) IPG buffer pH 4-7, GE Healthcare, Uppsala, Sweden) was added to complete 450 µl. Rehydration was performed actively for 12 hours at 30 V using Ettan IPGphor at 20 ºC (GE Healthcare, Uppsala, Sweden) on 24 cm Immobiline™ DryStrips (GE Healthcare, Uppsala, Sweden) with linear pH 4-7, followed by isoelectric focusing (IEF) in 4 steps: 600 V gradient 2 hrs, 1000 V gradient 2 hrs, 8000 V gradient 2 hrs, and 8000 V step-n-hold for a total of 80.000 Vhr. Before second dimension, strips were reduced and alkylated using 10 ml of an equilibration buffer (1.5 M Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 0.007 M SDS, a few grains of bromophenol blue) with 1% (w/v) DTT or 2.5% (w/v) iodoacetamide respectively for 15 min each. Strips were loaded onto 12.5% Tris-HCl SDS-PAGE gels and run overnight in an Ettan DALT*twelve* Large Vertical System (GE Healthcare, Uppsala, Sweden) at 1 W/gel using a standard Tris-Glycine-SDS running buffer, until the bromophenol blue line reaches the end of the gel.

2.6 Gel image acquisition, analysis and statistics

Gels were scanned on a Typhoon scanner 9400 (GE Healthcare, Uppsala, Sweden) using three laser emission filters (526SP for Cy2, 580BP30 for Cy3 and 670BP30 for Cy5) at 100 µm resolution. Images were analyzed using the DeCyder 2D software version 7.0 (GE Healthcare, Uppsala, Sweden), which performs detection, quantitation, matching and analysis. Statistical significance was assessed using Student's t-test ($p<0.05$) and average fold-ratio (ratio>1.0). Spots displaying a statistically significant difference between groups were manually excised from preparative gels stained with colloidal Coomassie blue. For PCA (principal component analysis) representation, the software R v3.0.1 was used with mean-centered and autoscaled data and, for a heat map, PermutMatrix was used as described by Caraux and Pinloche in 2005 (Caraux and Pinloche 2005).

2.7 Protein identification by MS analysis of peptides and Database search

Protein spots were digested overnight with trypsin and the resulting peptides extracted with 2% acetonitrile and 0.1% trifluoroacetic acid. Tryptic peptides were separated on a Pepmap C18 reverse phase column (0.075 x 150 mm, LC Packings), using an Ultimate+ LC system (Famos / Swithcos / Ultimate, LC Packings, Thermo Scientific, Whaltham, Massachusetts, USA) with online analysis by electrospray ionization (ESI, Thermo Scientific, Whaltham, Massachusetts, USA) mass spectrometry on a LTQ Velos Orbitrap (Thermo Scientific, Whaltham, Massachusetts, USA). Peptide mixtures were eluted by a 5 – 85% v/v acetonitrile gradient (in 0.5% v/v formic acid) run over 45 min at a flow rate of 0.2 µl / min. The resulting MS/MS data were used as input to MASCOT MS/MS Ion search (Matrix Science,

http://www.matrixscience.com), on the *Actinopterygii* (ray-finned fish) subset of the NCBInr database (August, 2016). Due to limitations of this specific subset we were not able to identify all spots, as the genome of gilthead seabream isn't fully sequenced yet. These searches were performed assuming the formation of single-charged peptides, carbamidomethylation of cysteine residues, possible oxidation of methionine residues and up to 1 missed cleavage. Mass tolerance was 10 ppm for MS data and 0.5 Da for MS/MS data.

2.8 Plasma metabolites and cortisol

Plasma free amino acids concentrations were determined on two pools of plasma (one per tank) from five fish per dietary treatment (analyses performed in duplicates). Plasma samples were pre-column derivatised with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters Corporation, Milford, Massachusetts, USA). Analyses were done by ultra-high-performance liquid chromatography (UPLC) in a Waters reversed-Phase Amino Acid Analysis System, using norvaline as internal standard. The resultant peaks were analyzed with EMPOWER software (Waters Corporation, Milford, Massachusetts, USA). Concentrations of cholesterol, glucose, lactate, triacylglycerides and cortisol were individually determined in plasma of ten gilthead seabream per tank (measurements performed in duplicates and read in a microplate reader (synergy HT Model SIAFRTD, Biotek Instruments, Vermont, USA), using commercial kits (Cholesterol CHO-POD, Glucose-HK, Lactate LO-POD, triglycerides GPO-POD from Spinreact, Girona, Spain and Cortisol saliva ELISA from IBL International, Hamburg, Germany,

respectively). Cortisol saliva ELISA kit from IBL was previously validated for *Sparus aurata*(Lopez-Olmeda et al. 2009).

2.9 Statistical analysis

Results are given as mean ± standard deviation. All data were checked for normality and homoscedasticity of variance. Significant differences of zootechnical parameters and plasma metabolite concentrations were assessed by Student's t-test, using the p-value threshold of 0.05. Statistical analysis was performed using the R v3.0.1 software environment.

3. Results

Water temperature was monitored during the winter period (between 18-11-2011 until 7-3-1012) reaching values as low as 7.7 ºC in February, as shown in Figure 2. At the end of the winter period, seabream weight differed significantly between diets (Figure 3). No mortality was observed during this trial period.

Zootechnical results (see more details in Silva *et al*(Silva et al. 2014)) show a non-significant tendency for better relative growth and feed conversion rates, for fish fed the fortified diet. Significant (Student's t-test, $P=8.4 \times 10^{-10}$) higher hepatosomatic index was observed in fish fed the winter feed.

In terms of protein expression, DIGE analysis revealed an impact of the winter feed diet on plasma proteome with 92 protein spots significantly different ($p<0.05$) in abundance, out of a total of 2282 protein spots analyzed (due to technical reasons 1 gel was not considered in the analysis). We were able to identify 50 of these proteins by mass spectrometry, but only 18 are

shown as a consequence of repeated identifications of the same protein due to degradation, agglomerates, isoforms, etc. (Figure 4). Eight proteins were found to be down regulated and 10 up regulated in fish fed with the winter feed. Protein identifications are shown in table 2. Abundance estimations of proteins can be seen in the expression pattern showed as a heat map in Figure 5.

A principal component analysis (PCA) of protein spots expression data (only sequenced spots are shown) revealed a clear separation of samples into two clusters according to the type of diet administered (Figure 6). This clearly shows two different plasma protein profiles of gilthead seabream fed with a control and a winter feed diet. This is also evidenced in Figure 5 by the heat map generated from the relative abundance of the previously identified protein spots, in which there is a clear grouping in two clusters, over or under expressed in WF compared to CTRL. A simplified metabolism of three identified proteins, transferrin, apolipoprotein A-I and alpha-2-macroglobulin is shown in Figure 7.

Plasma free amino acid concentrations assessed at the end of winter in fish fed the two diets are presented in Table 3. Arginine, histidine, lysine, tryptophan, tyrosine, glycine, asparagine, serine and gamma-aminobutyric acid concentrations decreased significantly with WF diet.

The plasma lactate, triacylglycerides (TAG), glucose, cholesterol and cortisol concentration in gilthead seabream fed different experimental diets are shown in Figure 8. Fish fed the winter feed show a tendency for a higher concentration of plasma glucose, cortisol and lactate although not significantly

different (Student's t-test, values in graphics). There is a significantly higher concentration of plasma cholesterol (Student's t-test, P=0.044) in fish fed the winter feed, while triacylglycerides concentration is significantly (Student's t-test, P=0.024) lower in the same group.

After 116 days of trial, some altered livers showing possible first signs of winter disease were noticed, and this appeared to occur more frequently in control than in winter feed fish.

4. Discussion

4.1 Comparative proteomics

4.1.1 Lipid metabolism

Lipids have important roles in growth and fish health. An effect of WF on lipid metabolism has been identified by the way of up-regulation of apolipoproteins. High density lipoproteins (HDL) are the most abundant plasma lipoproteins in teleosts, and its principal constituent, apolipoprotein A-I (ApoA-I, spots 4 and 45), was shown to have antimicrobial activity in carp at submicromolar concentrations against *P. citreus* and at micromolar concentrations against *Pseudomonas* sp. and *Yersinia ruckeri*(Concha et al. 2003; Concha et al. 2004). Concha *et al.* (2003) demonstrated the presence of ApoA-I in fish skin, which stands for the first barrier against infections(Concha et al. 2003). Apolipoprotein C-I (spot 68), a constituent of HDL, controls plasma lipid metabolism and promotes cell growth(Nynca et al. 2010). Given the higher levels of ApoA-I observed in WF-fed fish, it seems the winter diet contributes towards an improved fish health status, compared to the CTRL diet.

4.1.2 Stress & immune response

The physiology and behavior of fish is affected by water temperature. A warm temperature acclimation-related 65 kDa protein (wap65, spot 6) was identified. An increase in the expression of this protein was observed as a response to stress, being up-regulated in fish fed the winter feed. Wap65 might be involved in the immune system as Kikuchi and colleagues demonstrated when exposing goldfish to lipopolysaccharide, showing a two-fold induction of wap65(Kikuchi et al. 1997).

An estrogen-regulated protein, also known as heat shock protein 27 (hsp27) (spot 43), was induced by the winter feed diet. Diverse functional roles have been proposed to hsp27. This protein plays a role against disease, injuries and homeostasis(Mao et al. 2005). Heat shock proteins (hsps) are up-regulated when a sudden temperature change occurs as protein stability is affected. In this trial the low temperatures may have destabilized hydrophobic interactions, and hsps act as chaperones to repair eventual damage caused by this destabilization(Feidantsis et al. 2013; Mao et al. 2005; Marvin et al. 2008). An up-regulation of hsps in gilthead seabream from February until May, has been previously reported(Feidantsis et al. 2013). This protein also regulates actin filament dynamics with its exact role depending on the phosphorylation state(Liang and MacRae 1997; Mounier and Arrigo 2002). As reported in our previous paper(Silva et al. 2014) fish fed the WF diet displayed higher relative growth rates. In order to cope with this, a proper regulation of actin dynamics in muscle is critical for assembly and maintenance of functional myofibrils, which might result in a higher expression level of hsp27 in this group.

An up-regulated protein in fish fed the WF diet (spot 30) was identified as a tissue inhibitor of metalloproteinase 2b (timp2b), which inhibits the matrix metalloproteinases (MMPs) in tissues. Similarly TIMP has been identified in common carp, and was suggested to be involved in immune response, as its expression raises with a bacterial infection in several tissues such as spleen, blood and head kidney(Xu et al. 2011). TIMPs regulate the activity of MMPs to maintain the balance in proteolysis. The ratio and the expression level between these proteins are quite important in the activation of the immune system and might be used as disease biomarkers(Castillo-Briceno et al. 2010). In gilthead seabream, TIMPs are induced when an inflammatory response occurs against pathogenic patterns(Castillo-Briceno et al. 2010).

One protein was identified as serpin (spot 41) with functional roles in growth, development, pathophysiology and injury(Umasuthan et al. 2011). This family might show down- and up-regulation as many isoforms are present in teleosts (e.g. different clades and members) resulting in slightly different functions(Gettins 2002; Kaiserman and Bird 2005). A BLAST alignment was performed against the NCBInr database (31-08-2016) and the alpha-1-antitrypsin was identified (SerpinA1 gene) for *Epinephelus coioides* with an E-value of 3e-122. This isoform shows up-regulation in fish fed the WF. The up-regulation of proteases at cold water temperatures is demonstrated in gilthead seabream that were exposed to 8 ºC, showing that proteolysis is reestablishing cells (Ibarz et al. 2010a).

A complement component C3 (thioester-containing family) was identified in spot 37. In gilthead seabream, five different forms of C3 were identified, each with specific binding(Watts et al. 2001). A BLAST alignment

was performed against the NCBInr database (31-08-2016) in order to identify the isoform of this C3, but no information about the morphism of this protein was obtained, although some polymorphisms have been identified on genes altering the functions of the protein (Watts et al. 2001). This protein, despite being down-regulated in fish fed the WF, participates in the classical and alternative pathway of the complement system which plays a key role in the innate immunity of fish (Boshra et al. 2006). The importance of this protein is also shown in human autoimmune diseases being involved in pathogenesis (Chen et al. 2010; Holers 2014). It has been described that at lower temperatures the innate parameters in fish are activated, with mucus production declining in Channel catfish when fish were exposed to a temperature shift from 22 to 10 ºC, decreasing skin protection against opportunistic pathogens(Magnadottir 2006).

Transferrin is down-regulated in fish fed the WF diet (spot 42). Belonging to the superfamily of iron-binding proteins, transferrins are involved in the control of iron levels in blood, as oxidative damage might occur with an excess of iron (Garcia-Fernandez et al. 2011). This protein has also been identified in a gilthead seabream study fed with different diets in, where a different expression of this protein was observed when fish where fed diets with hemoglobin supplementation (Ghisaura et al. 2014). Transferrins are also involved in the non-specific humoral defense mechanism (Dietrich et al. 2010), which uses factors to inhibit bacterial growth(Garcia-Fernandez et al. 2011). In rainbow trout, transferrin acts as a positive acute phase protein(Bayne and Gerwick 2001), 24-48 hours after induction of the immune system with lipopolysaccharide, free iron levels in plasma were lower (Bayne

et al. 2001). No iron has been specifically added to the diets but some heme and non-heme (complexed with transferrin and/or ferritin) iron is present in fishmeal. In the winter feed diet a vitamin and mineral premix, Lutavit C35 (vitamin C) and Lutavit E50 (vitamin E) have been added in a higher amount due to their antioxidant functions. Ascorbic acid, a form of vitamin C, has been used in a study with Atlantic salmon, and was proven to have a critical role in releasing iron from transferrin, while also preventing oxidative damage(Andersen et al. 1998; Ortuno et al. 1999).

Taken together, the observed changes in the expression of these proteins involved in the stress and immune responses suggest that fish fed the WF, seem to have a general improvement on their immune condition. However, further studies focusing on a set of specific and non-specific immune parameters would be needed to confirm this hypothesis.

4.2 Metabolites

Cortisol levels are, in general, 10-fold higher in this experiment than reported basal values(Laiz-Carrion et al. 2003). This may mean that the cold exposure conditions verified in the present study were inducing a chronic stress situation. Cortisol is commonly used as a parameter to assess stress response in fish species (Arends et al. 1999), and elevated cortisol levels were reported in chronic stressed seabream(Alves et al. 2010). WF fed fish show a tendency for a slightly higher value of cortisol but it is not significantly different compared to CTRL fish.

Glucose levels are 1.5-fold higher than reported basal values(Lopez-Olmeda et al. 2009), although acute stress due to crowding results in values

higher than those seen in the control fish in our experiment(Lopez-Olmeda et al. 2009; Ortuno et al. 2001). An increase in cortisol and glucose levels has also been shown when water temperatures dropped from 18 to 9 ºC in 24h in seabream while lactate showed no significant differences (Rotllant et al. 2000). In our trial basal lactate levels are lower and no significant differences were seen. Nevertheless, a tendency for a slightly higher concentration was detected in WF fed fish.

Taken together, cortisol and glucose levels suggest that both control and WF fish groups may have been under chronic stress induced by low temperatures.

Cholesterol is a precursor of cortisol and levels might be influenced by different ingredients in fish diets. Studies show that cholesterol levels are higher in individuals fed fish meal diets instead of plant protein diets(Sitja-Bobadilla et al. 2005). WF has a higher percentage of fish meal compared to the control diet (Table 1), which might explain the higher cholesterol levels measured in plasma. Higher plasma cholesterol levels observed in fish fed WF diet are consistent with the higher plasma levels of HDL suggested by the increase in apo A-I abundance measured in those fish.

Analysis of the amino acid (AA) composition in plasma shows a significant higher concentration in fish fed the control diet for 9 of them, namely Arg, His, Lys, Trp, Tyr, Gly, Asn, Ser and GABA. Studies performed with different diets and under different stressors in Senegalese sole show that levels of some plasma free AA are significantly affected by both diet AA composition and stress conditions(Conceicao et al. 2012; Costas et al. 2012). Moreover, it has been described for Senegalese sole that chronic stress tends

to decrease plasma free AA concentrations, but the individual AA affected and magnitude of these changes seem to depend on the type of chronic stress(Costas et al. 2012), and have been related to differences in amino acid requirements(Conceicao et al. 2012). Therefore, one possibility is that fish fed the WF had higher requirements for the nine amino acids whose concentration has decreased. These eventual additional requirements may be related to the improved immune condition already proposed for the WF fed fish. In any case, and once both groups in the present study seem to have been chronically stressed by low temperature, it seems that fish fed the two diets found different metabolic adaptions to cope with thermal stress.

5. Conclusions

Comparative plasma proteome analysis revealed that the winter feed induced an up-regulation of some proteins involved in the immune system and cell protection compared to control diet, suggesting that the addition of vitamins and higher-quality lipids and proteins to the diet benefits fish health condition. Diet WF also affected lipid metabolism as shown by the reduction in plasma triglycerides levels, associated with an increase in cholesterol which seemed consistent with the higher abundance of HDL-Apo A-I observed with the comparative plasma proteome analysis. High cortisol and glucose levels compared to literature data suggest that fish from both treatments were under chronic thermal stress. However, no significant effects on stress related plasma metabolites were observed in fish fed the two diets. Overall, this study suggests that winter feed diet had a positive effect on fish condition during

winter and might help preventing metabolic dysfunctions associated with cold thermal stress. Proteomics has also proven once again to have a positive impact in animal science particularly in fish biology, presenting itself as an important tool to be included in future investigation of fish health, welfare and production (Almeida et al. 2015).

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8. Figure legends

Figure 1 – Schematic representation of the experimental design.

Figure 2 – Temperature shift during the winter period (from 18-11-2011 until 7-3-2012). The light grey line denotes the critical 12 ºC temperature threshold.

Figure 3 – Wet body weight (g) of the fish fed with diet CTRL and diet WF, at the start of the trial (November) and at the sampling of the end of winter period (March). Values are means (n=20), error bars represent standard deviation. Mean values are significantly different between groups at the end of winter (Student's t-test, p=0.0165).

Figure 4 – Representative 2D-DIGE gel of plasma of *Sparus aurata* on a 12.5% polyacrylamide gel, in which spots marked with a circle are up regulated and spots marked with a square are down regulated in fish fed with winter feed.

Figure 5 – Heat map representation of identified proteins (white/grey means "below average expression", while dark grey/black means "above average expression"). Dendograms attached to the columns and rows display hierarchical agglomerative clustering of samples and variables based on their Euclidian distance. Cx – fish fed the control diet, Wx – fish fed the winter feed.

Figure 6 – PCA biplot representation of the control fish and the study group along with sequenced spots. CTRL represents control samples and WF represents winter feed samples. Numbers with an x represent spot numbers.

Figure 7 – Simplified metabolism version of three identified proteins in plasma of gilthead seabream, namely transferrin (TF), apolipoprotein A-I (ApoA-I) and alpha-2-macroglobulin (a2M). Legend: TFR – transferrin receptor, HDL – high density lipoprotein, SR-B1 – scavenger receptor class B member 1 (HDL receptor).

Figure 8 – Plasma lactate (mM), TAG (mM), glucose (mM), cholesterol (mM) and cortisol (nM) concentration of gilthead seabream after 116 days of trial (end of winter period). Values are means and error bars represent standard deviation. Different letters (a and b) represent significant differences (Student's t-test, values in graphics).

9. Table legends

Table 1 - Ingredient formulation and proximate composition of the experimental diets.

[a] Peruvian fishmeal LT: 670 g kg^{-1} crude protein (CP), 90 g kg^{-1} crude fat (CF), EXALMAR, Peru.
[b] Fish by-products meal: 540 g kg^{-1} CP, 80 g kg^{-1} CF, COFACO, Portugal.
[c] Krill protein hydrolysate: >700 g kg^{-1} CP, <30 g kg^{-1} CF, OLYMPIC SEAFOOD AS, Norway.
[d] Soycomil PC: 630 g kg^{-1} CP, <10 g kg^{-1} CF, ADM, The Netherlands.
[e] Lysamine GP: 780 g kg^{-1} CP, 80 g kg^{-1} CF, ROQUETTE, France.
[f] GLUTALYS: 610 g kg^{-1} CP, 80 g kg^{-1} CF, ROQUETTE, France.
[g] VITEN: 857 g kg^{-1} CP, 13 g kg^{-1} CF, ROQUETTE, France.
[h] Solvent extracted dehulled soybean meal: 470 g kg^{-1} CP, 26 g kg^{-1} CF, SORGAL SA, Portugal.
[i] Dehulled grinded pea grits: 240 g kg^{-1}, <10 g kg^{-1} CF, SOTEXPRO, France.
[j] Henry Lamotte Oils GmbH, Germany.
[k] Krill PPC (25-30% phospholipids): 450 g kg^{-1} CP, 500 g kg^{-1} CF, OLYMPIC SEAFOOD AS, Norway.
[l] Yelkinol AC (65% phospholipids): 750 g kg^{-1} CF, ADM, The Netherlands.
[m] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): sodium menadione bisulphate, 10 mg; retinyl acetate, 8000 IU; DL-cholecalciferol, 1700 IU; thiamin, 8 mg; riboflavin, 20 mg; pyridoxine, 10 mg; cyanocobalamin, 0.02 mg; nicotinic acid, 30 mg; folic acid, 6 mg; inositol, 300 mg; biotin, 0.7 mg; calcium panthotenate, 70 mg; betaine, 400 mg. Minerals (mg/kg diet): cobalt carbonate, 0.1 mg; copper sulphate, 5 mg; ferric sulphate, 60 mg; potassium iodide, 1.5 mg; manganese oxide, 20 mg; sodium selenite, 0.25 mg; zinc oxide, 30 mg; sodium chloride, 80 mg; excipient: wheat middlings.
[n] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): sodium menadione bisulphate, 15 mg; retinyl acetate, 12000 IU; DL-cholecalciferol, 2250 IU; thiamin, 12 mg; riboflavin, 30 mg; pyridoxine, 15 mg; cyanocobalamin, 0.03 mg; nicotinic acid, 45 mg; folic acid, 9 mg; inositol, 450 mg; biotin, 1.05 mg; calcium panthotenate, 105 mg; betaine, 600 mg. Minerals (mg/kg diet): cobalt carbonate, 0.15 mg; copper sulphate, 7.5 mg; ferric sulphate, 90 mg; potassium iodide, 2.25 mg; manganese oxide, 30 mg; sodium selenite, 0.38 mg; zinc oxide, 45 mg; sodium chloride, 120 mg; excipient: wheat middlings.
[o] Dicalcium phosphate: 18% phosphorus, 23% calcium, Fosfitalia, Italy.
[p] Vitamin C: >35% sodium and calcium salts of ascorbyl-2-phosphate, BASF, Germany.
[q] Vitamin E: >50% DL-alpha-tocopheryl acetate, BASF, Germany.
[r] L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France.
[s] L-Taurine 99%: Ajinomoto Eurolysine SAS, France.
[t] Betafin S1 (>96% betaine): DANISCO, Denmark.

Table 2 - Protein identification of plasma of gilthead seabream by ESI-orbitrap mass spectrometry, using the MASCOT search engine, showing accession number, the theoretical (T) and calculated (C) molecular weight (Mw) and isoelectric point (pI), percentage of sequence coverage, number of peptide matches with sequence, mowse score, ANOVA p-value, FDR – false discovery rate and fold change expression (positive value stands for an up-regulation in fish fed the winter feed).

Table 3 – Plasma free amino acid concentration (µM) in gilthead seabream at the end of winter period, fed control (CTRL) and winter feed (WF) diets.

Values are means (n=2 pools of 5 fish per dietary treatment) in duplicate ± standard deviation. Values not sharing a common letter in the same line are significantly different (Student's t-test).

Figure 1

Click here to download Figure Fig1.docx

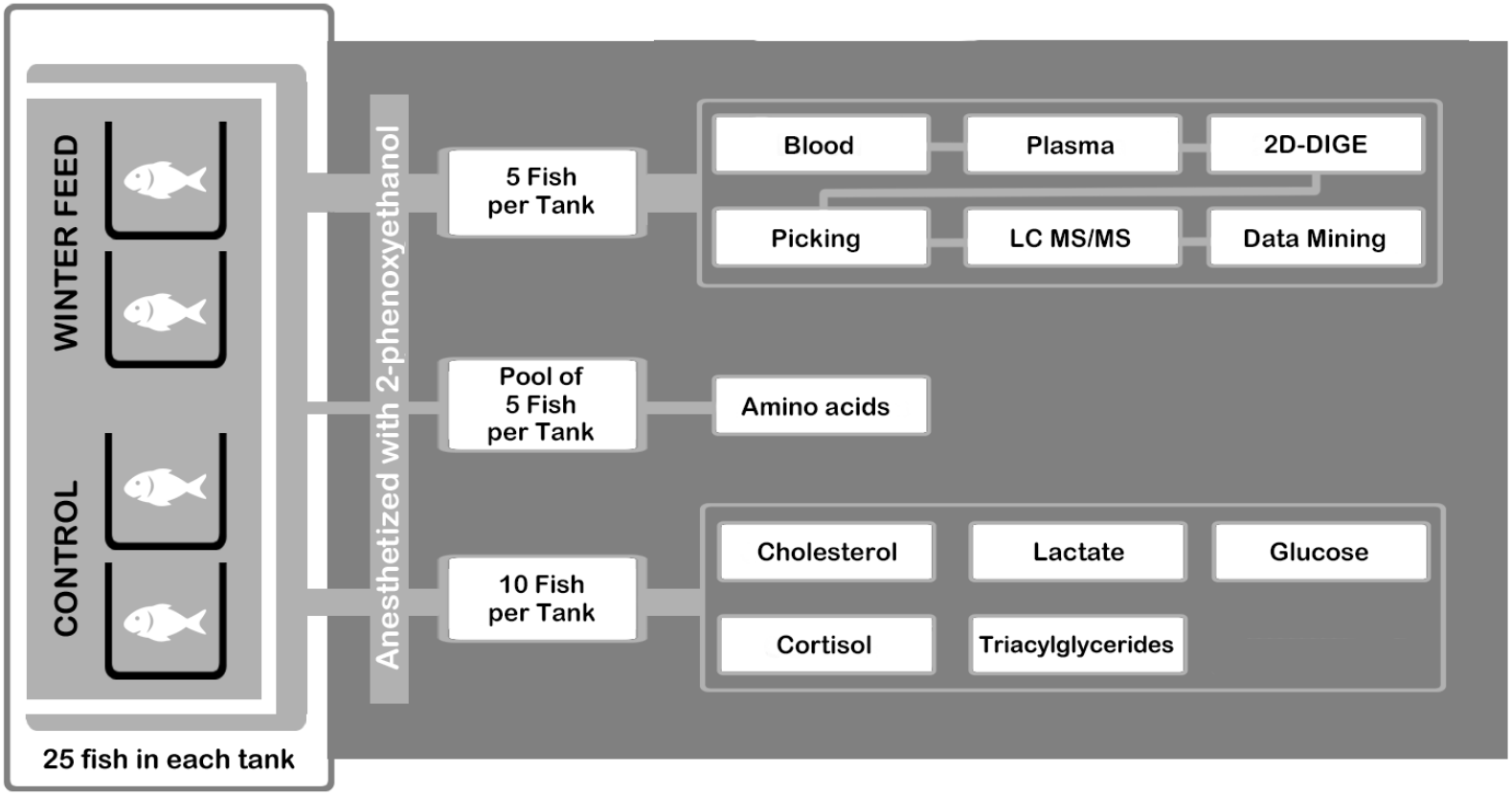

Figure 2

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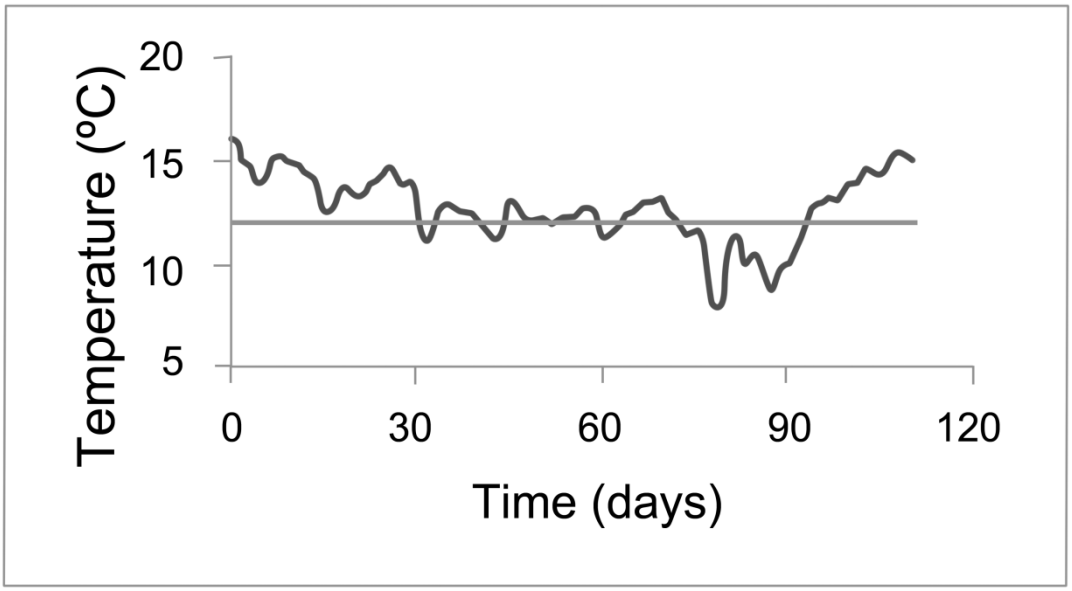

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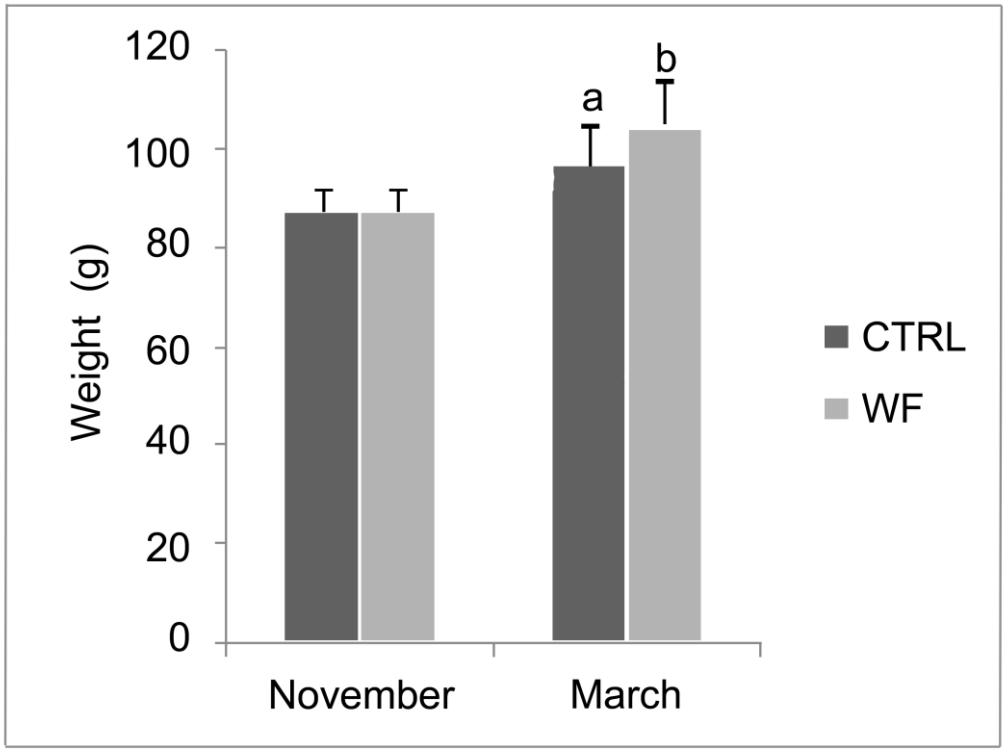

Figure 4

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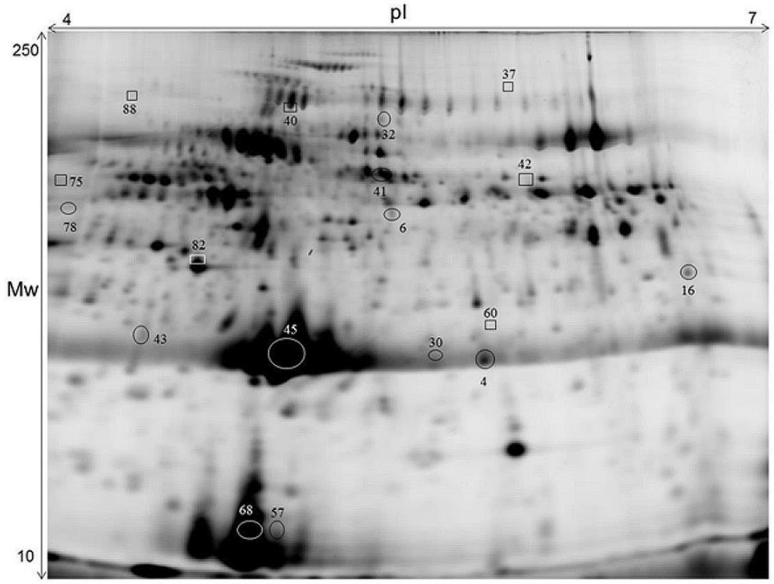

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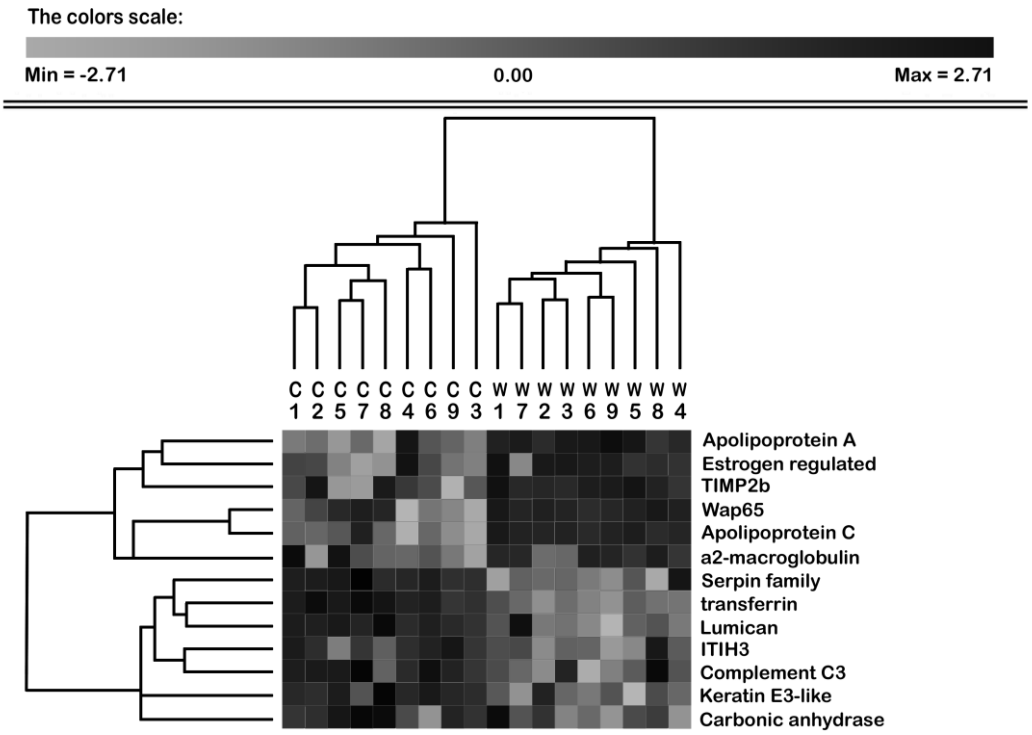

Figure 6

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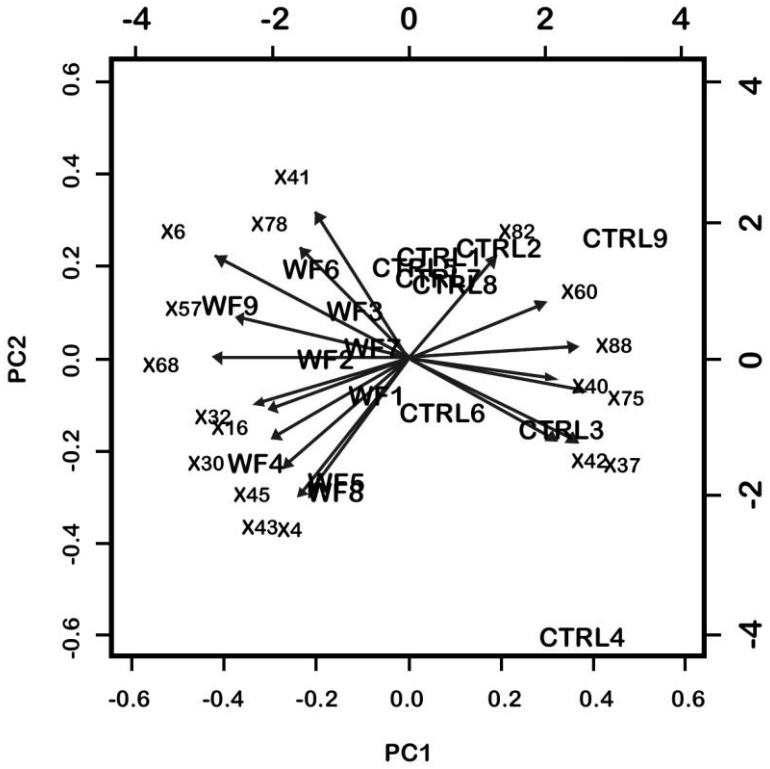

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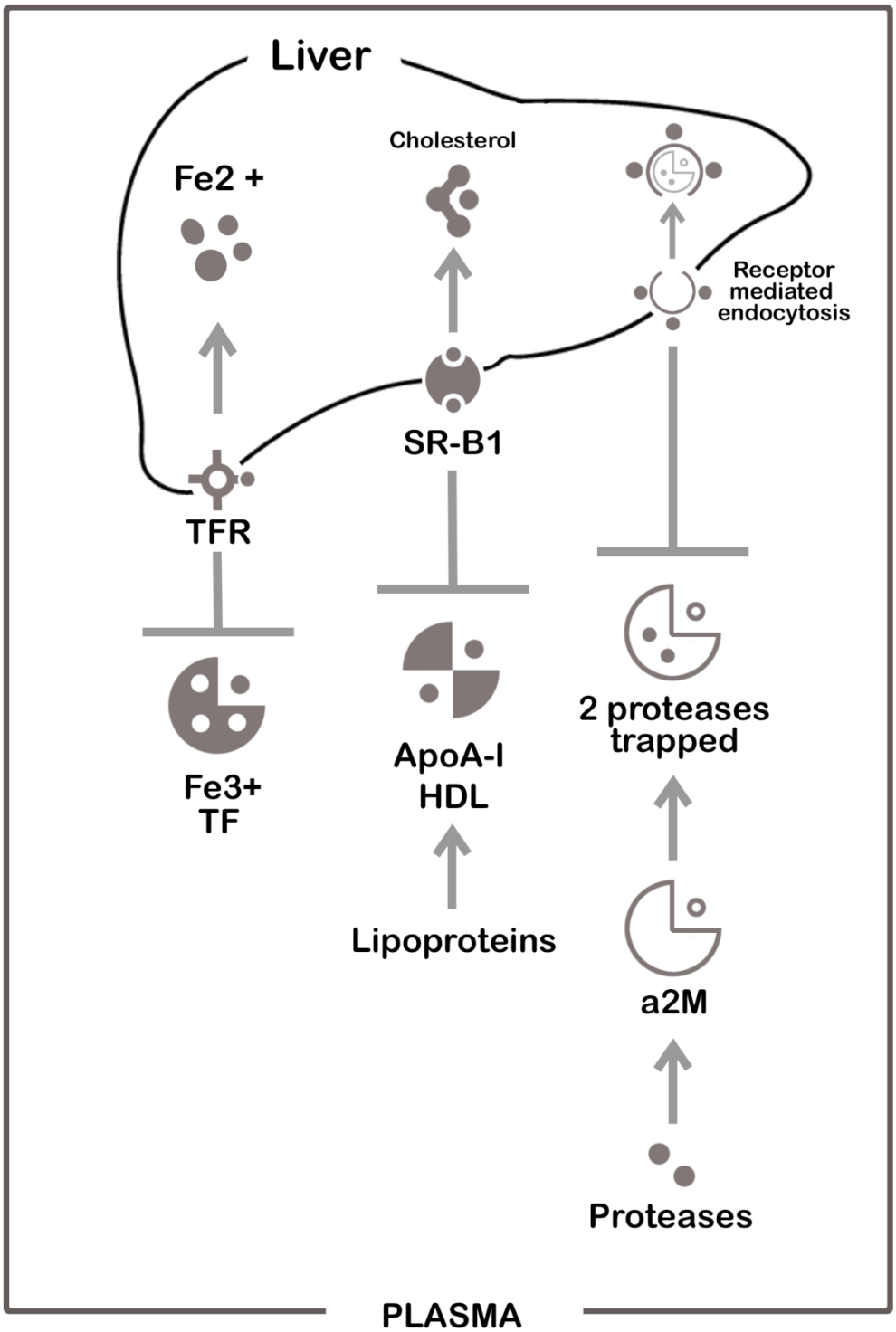

table I

	CTRL	WF
Ingredients (% w/w)		
Fishmeal 70 LT[a]	10.0	30.0
Fishmeal 65[b]	5.0	10.8
Krill protein hydrolysate[c]	0.0	5.0
Soy protein concentrate[d]	8.0	0.0
Pea protein concentrate[e]	4.0	0.0
Corn gluten[f]	16.0	5.5
Wheat gluten[g]	8.4	0.0
Soybean meal 48[h]	16.5	7.0
Wheat meal	5.0	12.5
Rapeseed meal	4.0	0.0
Aquatex G2000 (bran)[i]	2.0	3.0
Fish oil[j]	10.0	8.0
Rapeseed oil[j]	5.7	0.0
Krill PPC[k]	0.0	12.5
Soy lecithin[l]	0.0	1.0
Guar gum (binder)	0.5	0.5
Vit & Min Premix PVO 40/02	0.2[m]	0.3[n]
DCP[o]	4.0	1.0
Lutavit C35[p]	0.0	0.3
Lutavit E50[q]	0.1	0.5
L-Lysine[r]	0.5	0.0
L-Taurine[s]	0.0	1.0
Choline chloride	0.1	0.1
Betaine[t]	0.0	1.0

	CTRL	WF
Proximate composition		
Dry matter (DM), %	97.5	94.3
Crude protein, % DM	48.3	50.6
Crude fat, % DM	19.6	19.7
Ash, % DM	8.2	10.9
Gross Energy, MJ/kg	22.8	22.4
Phosphorus, % DM	1.5	1.7

table II

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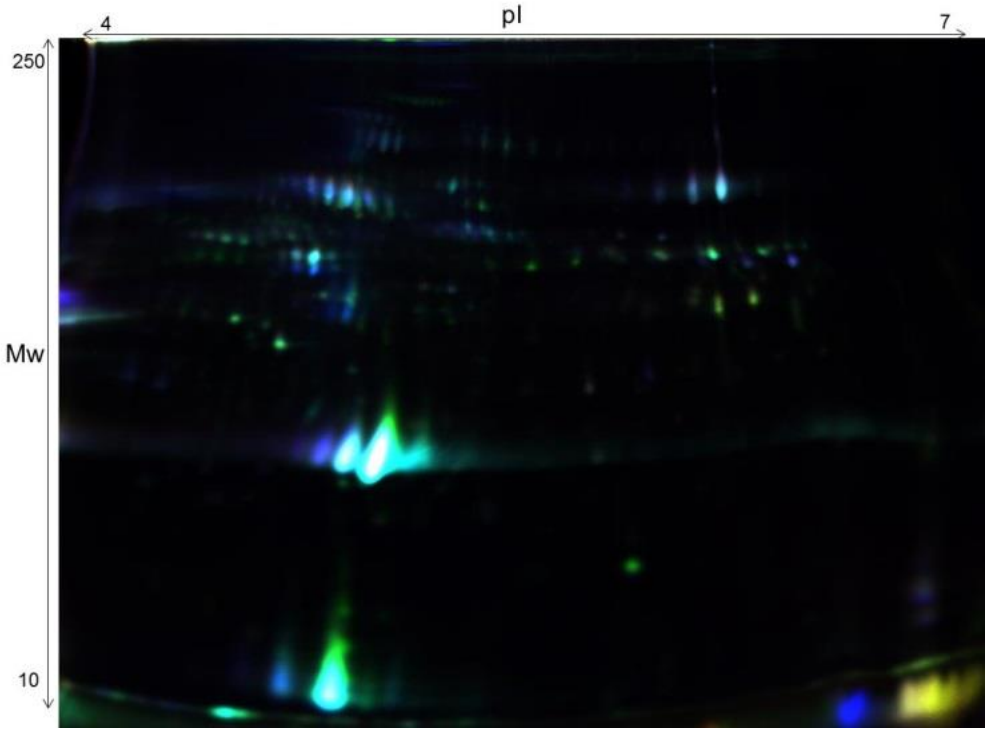
Functional category or description	Spot nº	protein name (species)	GI number	Mw T/C	pI T/C	sequence coverage %	No. of peptide matches	best peptide match: sequence; E-value	Mowse score	ANOVA	FDR	Fold change
Lipid metabolism	4	Apolipoprotein A-I (Sparus aurata)	gi|6686379	29615/19860	5.21/5.5	60	15	MNAIFEIIAASVTK; 2.2e-08	1490	0.0008	0.41	1.85
	45	Apolipoprotein A-I (*Sparus aurata*)	gi|6686379	29615/22223	5.21/4.9	76	29	MNAIFEIIAASVTK; 3.3e-09	15627	0.023	0.89	1.43
	57	Unknown (14 kDa apolipoprotein) (*Sparus aurata*)	gi|48526090	15914/5928	5.26/4.85	50	8	VATALGEEASPLVDK; 4.1e-08	2004	0.029	0.89	2.32
	68	Unknown (Apolipoprotein C-I family) (*Sparus aurata*)	gi|48526102	9483/5516	4.89/4.8	21	2	VTQVGQDLAEK; 0.00019	66	0.036	0.89	1.76
Protein metabolism	41	Unknown partial (Serpin family) (Epinephelus coioides)	gi|48526097	29658/62493	5/5.15	79	17	EMGITDAFGDTADFSGMSSEVK; 7.7e-10	3736	0.022	0.89	1.73
	88	Inter-Alpha-trypsin inhibitor heavy chain H3 (*Dicentrarchus labrax*)	gi|317419026	94919/107178	5.23/4.4	3	3	AVSSGQTAGLVK; 4.7e-05	195	0.048	0.89	-1.58
	30	Tissue inhibitor of metalloproteinase 2b (*Sparus aurata*)	gi|187606700	23940/19861	5,44/5,4	9	1	LVGEQEVEVGNDIYGNPIK; 0.031	42	0.016	0.89	1.8
	32	Alpha-2-macroglobulin partial (*Sparus aurata*)	gi|42415863	53038/80022	6.24/5.15	12	6	STNYLTSGYQR; 0.00046	138	0.018	0.89	1.46
	40	Alpha-2-macroglobulin-P-like (*Larimichthys crocea*)	gi|164454243	69117/95785	5.51/4.9	6	5	GAIVMQGLK; 0.0018	107	0.022	0.89	-1.26
	78	Hypothetical protein (Separin) (*Cyprinus carpio*)	gi|966637826	245728/61275	6.59/4.1	1	2	TLNNDNFLLK; 0.11	40	0.045	0.89	2.41
Immune system	37	Complement component C3 (*Sparus aurata*)	gi|303305915	186908/120548	8.08/5.55	4	6	EGSYDVGPQNQVR; 0.0023	50	0.021	0.89	-2.33
	42	Transferrin (*Sparus aurata*)	gi|327243042	76116/59746	5.93/5.6	72	48	HTIVDENSNGNGPAWASGVNK; 7.6e-10	9366	0.022	0.89	-1.4
Cellular process	75	Lumican (*Oreochromis niloticus*)	gi|348514924	38722/66852	6.45/4.1	8	3	YLYLQNNLIEEIK; 1.3e-05	97	0.042	0.89	-1.63
	82	Type II keratin E3-like protein (*Sparus aurata*)	gi|48476437	38600/40773	4.89/4.6	9	2	YEDEINKR; 0.015	47	0.046	0.89	-1.49
Stress response	6	Warm temperature acclimation-related 65 kDa protein (*Sparus aurata*)	gi|224551742	49754/53753	5.41/5.2	35	13	VHLDAITSDNAGNMYAFR; 3.5e-08	316	0.0015	0.52	2.09
	43	Estrogen-regulated protein (*Sparus aurata*)	gi|261825909	26274/24314	4.68/4.45	43	8	DDPSGTGDWEPLSNLR; 3.6e-06	817	0.022	0.89	1.74
Energy homeostasis	16	Muscle-type creatine kinase CKM1 (*Sebastes inermis*)	gi|359390897	43130/37089	6.22/6.2	27	8	GTGGVDTASVGGVFDISNADR; 4e-07	272	0.0078	0.89	2.71
	60	Carbonic anhydrase 1 (*Dicentrarchus labrax*)	gi|317420103	28610/27925	5.18/5.5	13	2	VLDSFDAIK; 0.0022	82	0.032	0.89	-2.15

table III

Aminoacid	CTRL	WF	P-value
Arginine	66.1 ± 3.2[a]	48.5 ± 2.9[b]	0.005
Histidine	23.3 ± 2.4[a]	18.8 ± 2.9[b]	0.041
Lysine	66.3 ± 1.1[a]	51.4 ± 9.0[b]	0.003
Threonine	136 ± 47.3	126.7 ± 28.2	0.95
Isoleucine	25.4 ± 0.8	23.3 ± 4.3	0.54
Leucine	26.8 ± 4.4	28.0 ± 6.2	0.96
Valine	43.9 ± 10.1	33.7 ± 15.1	0.41
Methionine	18.9 ± 1.5	15.7 ± 1.7	0.051
Phenylalanine	25.5 ± 0.4	24.1 ± 4.1	0.82
Tryptophan	2.7 ± 0.2[a]	1.8 ± 0.1[b]	0.0000077
Sum IAA	435 ± 58.6	372.1 ± 74.4	0.27
Cysteine	0 ± 0	0.2 ± 0.1	0.070
Tyrosine	17.6 ± 1.0[a]	11.4 ± 1.1[b]	0.0000031
Aspartic acid	4.7 ± 1.5	3.8 ± 1.6	0.74
Glutamic acid	14.6 ± 2.6	12.3 ± 2.7	0.65
Alanine	38.8 ± 16.1	33.8 ± 14.6	0.87
Glycine	24.1 ± 0.7[a]	14.6 ± 5.8[b]	0.0045
Proline	14.5 ± 3.6	10.2 ± 0.0	0.095
Asparagine	67.7 ± 0.7[a]	53.6 ± 12.3[b]	0.032
Glutamine	87.2 ± 26.2	81.0 ± 16.7	0.91
Serine	37.7 ± 4.1[a]	28.7 ± 1.6[b]	0.0011
Taurine	95.7 ± 63.2	98.7 ± 29.2	0.99
Hydroxyproline	1.8 ± 0.0	2.3 ± 0.9	0.44
Beta-alanine	0.4 ± 0.3	0.2 ± 0.2	0.17
Gamma-aminobutyric acid	5.9 ± 0.3[a]	4.3 ± 0.9[b]	0.014
Alpha-aminobutyric acid	3.8 ± 1.2	2.7 ± 0.2	0.13
Ornithine	11.8 ± 0.3	9.4 ± 1.8	0.052
Sum PAA	741.9 ± 105.5	621.6 ± 127.5	0.19
Sum DAA	426.4 ± 111.6	367.1 ± 86.2	0.53



Supplemental Figure – Color overlay of the 2D-DIGE gel of plasma of *Sparus aurata*.