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Dietary Tools To Modulate Glycogen Storage in Gilthead Seabream Muscle: Glycerol Supplementation

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S *Supporting Information*

ABSTRACT: The quality and shelf life of fish meat products depend on the skeletal muscle's energetic state at slaughter, as meat decomposition processes can be exacerbated by energy depletion. In this study, we tested dietary glycerol as a way of replenishing muscle glycogen reserves of farmed gilthead seabream. Two diets were tested in duplicate (n = 42/tank). Results show 5% inclusion of crude glycerol in gilthead seabream diets induces increased muscle glycogen, ATP levels and firmness, with no deleterious effects in terms of growth, proximate composition, fatty acid profile, oxidative state, and organoleptic properties (aroma and color). Proteomic analysis showed a low impact of glycerol-supplementation on muscle metabolism, with most changes probably reflecting increased stress coping capacity in glycerol-fed fish. This suggests inclusion of crude glycerol in gilthead seabream diets (particularly in the finishing phase) seems like a viable strategy to increase glycogen deposition in muscle without negatively impacting fish welfare and quality.

KEYWORDS: *crude glycerol, muscle glycogen, gilthead seabream, flesh quality, fish proteomics, aquaculture*

INTRODUCTION

When fish are slaughtered, skeletal muscle and other peripheral tissues experience anoxia and energy depletion, since the circulatory system fails to provide an adequate supply of oxygen and fuel. As skeletal muscle becomes anaerobic and energy production efficiency drops, glycogen is quickly metabolized to replace ATP reserves and pH drops due to the onset of lactic fermentation.[1,2]

This leads to a cascade of tightly related processes and events (like Ca^{2+} leakage, oxidative stress, protein and lipid oxidation, protein denaturation, rigor onset, mitochondrial damage, breakdown of primary metabolism, proteolysis, rigor resolution, cell death processes) that characterize the postmortem transition of fish muscle to fish meat and which, depending on how extensive and intensive they are, can ultimately lead to detrimental changes in the meat quality criteria.[3]

The dynamics of these processes are affected by contextual factors (such as prior muscle energetic and oxidative status, temperature, pH) and therefore, it is important to take into account that certain management/logistic procedures can exacerbate (e.g., preslaughter stress) or delay (e.g., refrigeration) muscle decomposition and therefore affect the final product quality traits (like texture) and shelf life.[4,5] Specifically for gilthead seabream, there are indications that increased premortem starvation periods (and, therefore, impaired premortem energetic states) contribute to reduced shelf life.[6]

Given this, delaying energy depletion in muscle through dietary manipulation (by increasing its premortem glycogen reserves) could contribute to the preservation of the muscle's organoleptic traits (particularly texture). Similar types of strategies were already tried for cod[7] and pig,[8−10] where they attempted to delay these postmortem dynamics either by trying to slow down the glycolytic rate (with magnesium, sodium oxalate, ascorbate and quercetin) or through the use of muscle buffering substances such as citrate or histidine.

In this trial, we chose to study the effect of crude glycerol derived from biodiesel production in gilthead seabream muscle. There is evidence that different species of fish use glycerol in different ways.[11−13] In some cases, glycerol is not used itself as a

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gluconeogenic precursor, but still provides energy, sparing other gluconeogenic precursors (like lactate) and therefore indirectly contributing toward glucose production and glycogen replenishing, which seems to be the case for rainbow trout.[14]

Regarding land-based animals, like cows,[15] pigs,[16−20] and chicken,[21] there is a great wealth of studies regarding glycerol-inclusion in feeds and, at least for inclusion levels below 10%, it was often found to increase growth rates, increase feed intake, improve meat yield, increase ultimate muscle pH, replenish muscle glycogen reserves, and improve water holding capacity. On the other hand, some of these studies have also shown that glycerol can have some impact on body composition, fatty acid profile and organoleptic properties.

In fish and other marine organisms, although there are already some studies within this subject, knowledge on the dietary use of crude glycerol is still scarce and limited to a few species, namely, Nile tilapia and channel catfish.[22,23] Furthermore, no information on the possible impact of dietary glycerol on organoleptic properties has been obtained in any of these works. For gilthead seabream specifically, the only relevant study we found relates to addition of pure glycerol to the rearing water of prelarvae and larvae, for the purpose of boosting hepatic glycogen reserves, in order to improve survival during early stages of development.[24] Results of this study confirmed dose-dependent hepatic glycogen deposition due to glycerol uptake.

Our study was therefore mainly focused on testing the putative ability of glycerol to help replenish skeletal muscle glycogen energy reserves, and therefore improve its premortem energy status, in farmed gilthead seabream. We also sought to verify if this dietary supplementation induced any deleterious effects in terms of fish health and quality. For this, an interdisciplinary approach was undertaken, having obtained complementary information on physiological and quality status through both unspecific (proteome expression data) and specific means (biochemical, histological, instrumental and sensory organoleptic data), providing a holistic and unbiased view on the effects of crude glycerol on farmed gilthead seabream.

■ MATERIALS AND METHODS

Experimental Diets. Table 1 shows the formulations of the diets used in this trial. A control diet (CTRL), similar to commercial seabream feed, was formulated with practical ingredients to contain 50% crude protein, 18% crude fat and 20 kJ·g^{-1} gross energy. Another diet was formulated, similar to the control diet, with 5% crude glycerol, incorporated at the expense of a crude source of pea starch. All diets were formulated to fulfill the known nutritional requirements of the species. Main ingredients were ground (below 250 μm) in a micropulverizer hammer mill Hosokawa, model #1. Powder ingredients were then mixed accordingly to the target formulation in a Double-helix Mixer TGC, model 500L to attain a basal mixture. No oils were incorporated at this stage. All diets were manufactured by extrusion (pellet size 5.0 mm) by means of a pilot-scale twin-screw extruder CLEXTRAL BC45 (Clextral, France) with a screw diameter of 55.5 mm and temperature ranging 105−110 °C. Upon extrusion, all batches of extruded feeds were dried in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 2 h at 60 °C. Following drying, pellets were allowed to cool at room temperature, and subsequently the oil fraction was added under vacuum coating conditions in a Pegasus vacuum mixer (PG-10VCLAB, DINNISEN, The Netherlands). Throughout the duration of the trial, experimental feeds were stored at room temperature. Samples of each diet were taken for proximate composition analysis (Table 1).

Table 1. Ingredients and Proximate Composition of the Experimental Diets

ingredients (%)	CTRL	GLY
Fishmeal LT[a]	15.0	15.0
Fishmeal 65[b]	20.0	20.0
Soy protein concentrate[c]	5.0	5.0
Wheat gluten[d]	5.0	7.0
Corn gluten[e]	8.0	8.0
Soybean meal 48[f]	10.0	10.0
Wheat meal	12.0	10.0
Aquatex G2000[g]	10.0	5.0
Fish oil	13.0	13.0
Vit & Min Premix[h]	1.0	1.0
Binder (diatomaceous earth)[i]	1.0	1.0
Crude glycerol[j]		5.0
Proximate Composition		
Dry matter (DM) (%)	94.6 ± 0.1	93.6 ± 0.1
Crude protein (% DM)	48.4 ± 0.2	47.8 ± 0.2
Lipid (% DM)	17.8 ± 0.2	18.1 ± 0.3
Ash (% DM)	6.7 ± 0.0	6.6 ± 0.0
Phosphorus (% DM)	1.11 ± 0.1	1.16 ± 0.0
Gross energy (kJ/g DM)	20.7 ± 0.2	20.6 ± 0.1

[a]Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF), EXALMAR, Peru. [b]Fair Average Quality (FAQ) fishmeal: 62% CP, 12%CF, COFACO, Portugal. [c]Soycomil P: 65% CP, 0.7% CF, ADM, The Netherlands. [d]VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France. [e]GLUTALYS: 61% CP, 8% CF, ROQUETTE, France. [f]Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL, Portugal. [g]Dehulled grinded pea grits: 24% CP, 0.4% CF, SOTEXPRO, France. [h]PVO40.01 SPAROS standard premix for marine fish, PREMIX Lda, Portugal. [I]Kielseguhr: LIGRANA GmbH, Germany. [j]Biodiesel derived crude glycerol: 82% glycerol, <0.03% methanol, IBEROL, Portugal.

Fish and Rearing Conditions. Four groups of 42 gilthead seabream with a mean initial body weight of 321 ± 45 g were reared in 1000 L tanks at CCMAR facilities and hand-fed twice a day until apparent satiation. Natural seawater was supplied (mean temperature 26 ± 2 °C; mean salinity 36.6 ± 0.7 ‰), by a flow-through system with artificial aeration (mean dissolved oxygen 82 ± 20%). The experimental diets were tested in duplicate over a period of 90 days. Prior to harvesting, fish were starved for 48 h (common practice in the industry).

Sampling. Fish from both treatments were slaughtered by immersion in ice-saltwater slurry until death (common practice in the industry). Fish were then stored with the ventral side upward, covered with plastic and flaked ice in insulated polystyrene boxes.

Fourteen fish from each treatment (seven from each dietary replicate) were weighed, scaled and filleted 24 h after death. One fillet from each of eight fish was separately packed and stored at 4 °C until sensory assessment, carried out approximately 48 h after slaughter. The remaining fillets were either used for proximate composition and fatty acid profiles (n = 10), or were frozen at −20 °C for biochemical assays. Additionally, from four fish of each treatment (right fillets), small (1.0 × 0.5 × 0.5 cm) white muscle samples were taken from the dorsal area, under the dorsal fin, and fixed in Carnoy's solution at 4 °C (for 24 h, with occasional shaking) for muscle morphology by histology.

Twelve fish from each treatment (six from each dietary replicate) were used for muscle pH measurements (n = 6) and rigor mortis measurements (n = 6) at 0, 3, 6, 9, 24, and 48 h after death.

Samples for biochemical analysis were removed from 8 fish per treatment (four from each dietary replicate) at the time of death (T0) and 6 h after death (T6), immediately snap frozen in liquid nitrogen and stored at −80 °C prior to analysis.

Eight fish from each treatment (four from each dietary replicate) were kept for 48 h in a temperature controlled room (4 °C). Afterward, the fish were weighed, filleted, fork length, fillet yield, liver and visceral weight were recorded and samples for biochemical analysis were snap frozen in liquid nitrogen and stored at −80 °C (T48). Unless otherwise specified, all analyses were run on individual fish (n = 8), in triplicate. Dorsal samples of white muscle for proteomic analysis were only collected at T0, being also snap-frozen in liquid nitrogen and stored at −80 °C until further processing.

Table 2 contains an overview of how the fish sampling and distribution between the different assays and techniques was performed.

Table 2. Distribution of Samples Taken from Each Individual Fish (per Experimental Treatment)

fish ID	left fillet	right fillet
1−4	Sensory analysis Fatty acid analysis Proximate composition	Instrumental texture (raw)
5−8	Instrumental texture (cooked) Color measurements	Sensory analisys Fatty acid analysis Proximate composition
9−14	Instrumental texture (cooked) Color measurements	Instrumental texture (raw)
15−20	muscle pH	muscle pH
21−26	Rigor mortis	Rigor mortis
27−30	Histological characterization *Time 0:* - proteomic analysis - volatile compound analysis - lipid oxidation - muscle glycogen - muscle ATP - specific enzymes activity	*Time 6:* - lipid oxidation - muscle glycogen - muscle ATP - specific enzymes activity
31−34	*Time 6:* - lipid oxidation - muscle glycogen - muscle ATP - specific enzymes activity	*Time 0:* - proteomic analysis - volatile compound analysis - lipid oxidation - muscle glycogen - muscle ATP - specific enzymes activity
35−42	*Time 48:* - lipid oxidation - muscle glycogen - muscle ATP - specific enzymes activity	

Proximate Composition. Ash (Method 942.05), moisture (Method 950.46), crude protein (Method 992.15) and fat (Method 991.36) contents were determined by the reference methods,[25] using minced fish muscle, without skin. Four pools of two fish per treatment were used.

Fatty Acid Analysis. Fatty acid composition was determined through the analysis of methyl esters, as previously described,[26] from homogenized and lyophilized skinless white muscle. Briefly, the analysis was executed using a Varian Star CP 3800 GC (Walnut Creek, CA), equipped with auto sampler and flame ionization detector. The separation of the different methyl esters was performed in a polyethylene glycol capillary column DB-WAX (Folsom, CA) (30 m × 0.25 mm i.d., film thickness: 0.25 μm). Identification of fatty acid methyl esters was achieved by comparison of their retention times with those of mixed chromatographic standards (Sigma Aldrich). Peak areas were determined using the Varian software. Results were expressed as relative percentage of total fatty acids. Four pools of two fish per treatment were used.

Instrumental Texture Analysis. The fish were kept packed in closed polystyrene boxes with ice, in a temperature-controlled room at 8−11 °C until the texture analyses (approximately 48 h after death). All the right fillets were used for raw texture measurement while the corresponding left fillets were cooked in a saturated steam oven (Rational Combi-Master CM6 Cross Kuchentechnik GmbH, Landsberg a. Lech) during 6 min at 100 °C. The fillets were allowed to cool down at room temperature and then were kept at 8−11 °C until the texture analysis (approximately 2 h). Fillet texture was measured instrumentally using a Texture Analyzer Model Instron 4301 (Instron Engineering, Canton, MA) equipped with a load cell of 1 kN and a spherical plunger (12.5 mm diameter). Compression was applied on raw and cooked fillets. The plunger was pressed into the fillets at a constant speed of 2 mm·s^{-1} and penetrated 6.0 and 3.0 mm into the raw and cooked fillets, respectively. This penetration depth was selected as the maximum distance which could be applied without breaking the muscle fibres and affecting the muscle structure. Measurements were performed on each fillet (n = 10 per treatment) in the position corresponding to maximum thickness. Thickness at this point displayed low variability between fillets (control, 16 ± 2 mm; glycerol, 17 ± 2 mm) and should not account for any observed texture differences between treatments.

Color Measurements. Instrumental color measurements were performed with a tristimulus colorimeter (Macbeth Color-Eye 3000), in a 10 mm diameter measuring area. A 10° standard observer and a D65 illuminant were used. Before each measurement session, the colorimeter was calibrated with a white porcelain plate. The coordinates L^*, a^*, and b^* (CIELAB color space) were used as they relate to the human eye response to color. The Chroma (C^*) of the fillets was calculated according to the formula:[27] $C^* = (a^{*2} + b^{*2})^{1/2}$. Color was measured on the white dorsal muscle, on ten cooked fillets per treatment. At each location a value resulting from five consecutive measurements was obtained and the mean value of three locations was used.

Sensory Analysis. Sensory evaluation was carried out in an acclimatized room equipped with individual booths. To reduce the variability within the fillets, the parts close to the head and the tail were removed. Each fillet was individually wrapped with perforated aluminum foil and cooked for 6 min at 100 °C in a saturated steam oven (Rational Combi-Master CM6 Cross Kuchentechnik CmbH, Landsberg a. Lech, Germany). Cooked fillets were presented to the assessors sequentially in coded white dishes under normal white lighting. Eight fillets per treatment were assessed by a panel composed of eight trained assessors, male and female, nonsmokers, with ages between 34 and 60 years. Each panellist evaluated one fillet per treatment, using a 12 cm unstructured line scale in which the left end of the scale corresponded to "none" or "absence" while the right end represented high intensity,[28] to evaluate the intensity of the odor (typical odor) and color (white/yellowish) of the fillets.

Volatile Compound Extraction and Analysis. The volatile profiling of the raw fish fillets was evaluated using automated HS-SPME coupled to GC−MS, as previously described.[29] The analysis was performed on samples taken at the time of death, snap frozen in liquid nitrogen and kept at −80 °C prior to analysis. Two pools of four fish per treatment were used.

Histological Characterization. After fixation in Carnoy's solution, the muscle samples were then routinely dehydrated in a graded ethanol series, cleared in xylol, and finally embedded in paraffin. Four 10 μm-thick section were cut per block, and then stained with hematoxylin-eosin for morphometric analysis, and with Periodic Acid Schiff (PAS) for glycogen content, before being coverslipped.

The morphometric study was made using an interactive image analysis system (Olympus Cell*Family), working with a live-image captured by CCD-video camera (ColorView Soft Imaging System, Olympus) and a light microscope (BX51, Olympus, Japan). Relative number (density) of white muscle fibres per unit area (μm^2), mean individual muscle fiber area and mean cross-sectional fiber diameter were estimated according to standard procedures.[30]

Assessment of muscle glycogen content followed a semiquantitative approach based solely in three degrees: 1 (low), when muscle fibres in

PAS sections present a weak coloration; 2 (mild), PAS sections presented a mild coloration; 3 (intense), PAS sections presented a strong coloration. Six fish per treatment were used for histological characterization.

Measurement of Muscle pH. Muscle pH was measured (n = 6 per treatment) directly on the muscle using a pH meter with an insertion electrode (model pH Spear, Eutech Instruments), on 3 replicate locations in the dorsal area of the left fillet.

Rigor Mortis. The *rigor* index was evaluated (n = 6 per treatment) as previously described.[5] Fish were handled carefully in order to prevent secondary effects on the development of the *rigor* state.

Lipid Oxidation. Oxidation of lipids was determined (n = 8 per treatment) by measuring the thiobarbituric acid reactive substances (TBARS), as previously described.[5]

Measurement of Glycogen Content. The amount of glycogen in the white muscle samples was determined (n = 8 per treatment) as previously described.[31] Glycogen content of the samples was expressed as μg per mg muscle (dry weight).

Measurement of ATP. Lyophilized muscle (around 10 mg) was suspended in 500 μL of a solution containing TCA (2% w/v) and EDTA (2 mM) and the mixture was vortexed vigorously. Samples were then centrifuged (10 000g, 4 °C, 40 min) and 500 μL of a buffer (pH 3.87) containing Tris-HCl (40 mM), magnesium chloride hexahydrate (20 mM) and EDTA (4 mM) was added to 100 μL of the supernatant. This extract was used to measure ATP content in the muscle (n = 8 per treatment) using the ATP determination kit (Biaffin GmbH & Co KG, Cat. #LBR-S010), and values were expressed as nmol ATP per g of muscle (dry weight).

Enzyme Activity Assays. The extraction of enzymes was performed as previously described.[32] Briefly, 2 g of muscle was homogenized twice for 30 s using an Ultra Turrax T25 Basic (IKA Labortechnik) in 6 mL of extraction buffer (50 mM Tris-HCl (pH 7.5), 10 mM β-mercaptoethanol, 1 mM EDTA). The supernatant was collected after centrifugation at 10 000g for 40 min at 4 °C and frozen at −80 °C prior to analysis.

Cathepsins B and H activity measurements were performed as previously described.[32] Cathepsin activities in the protein extracts were determined at 30 °C in a 298 μL reaction (6 μL 5% CHAPS, 1 μL of 1.40 M β-mercaptoethanol, 16 μL of 5% (w/v) Brij 35, 5 μL of synthetic fluorogenic substrate prepared in ultrapure water and 70 μL of 0.4 mM acetate/acetic acid (pH 4) buffer containing 10 mM β-mercaptoethanol and 1 mM EDTA). *Z*-Arginine-arginine-7-amido-4-methylcoumarin hydrochloride (10 mM) and L-arginine-7-amido-4-methylcoumarin hydrochloride (5 mM) were used as the fluorogenic substrates for Cathepsin B and Cathepsin H, respectively. The reaction was initiated by adding 200 μL of protein extract. Collagenase-like activity measurement was done as previously described.[33] After thawing, the extracts were centrifuged (7840g, 4 °C for 10 min), and the enzymatic activity was measured against a synthetic fluorogenic substrate, *N*-succinyl-glycine-proline-leucine-glycine-proline-7-amido-4-ethylcoumarin (SGP). Concentration of SGP (dissolved in DMSO) was 0.0625 mM in 100 mM bis-Tris, 5 mM $CaCl_2$, pH 7.0. Samples were run in triplicate and a standard curve was prepared with 7-amido-4-methylcoumarin (AMC), and a control with extraction buffer instead of enzyme extract was run in parallel. Increase in fluorescence was monitored using a fluorescence spectrophotometer (Synergy 4, BioTek, Winooski, VT), and the excitation (λ_{ex}) and emission (λ_{em}) wavelengths were set to 385 and 460 nm, respectively. One unit of enzyme activity was defined as the amount of enzyme that hydrolyses 1 nmol of substrate per minute, and the results were expressed as $U \cdot g^{-1}$(protein).

Glycogen phosphorylase activity (a + b) was measured spectrophotometrically (340 nm) by following the release of G-1-P from glycogen, using a method previously described.[34] The reaction mixture contained 50 mM Tris (pH 7.6), 5 mM EDTA, 10 mM KH_2PO_4/Na_2HPO_4 (pH 7.6), 10 mM L-cystein, 0.3 mM NADP, 0.05 mM glucose-1,6-diphosphate, 2 mM AMP, 1.68 U phosphoglucomutase, 28 U glucose-6-phosphate dehydrogenase and 0.2 $mg \cdot mL^{-1}$ glycogen. In a microplate, 200 μL of reaction mixture was added to 25 μL of sample. The slope of the increase in absorbance was determined and activity results were expressed as $\Delta abs \cdot mg^{-1}$(protein)$\cdot min^{-1}$.

Protein content in the enzyme extracts was determined using the DC Protein Assay (Bio-Rad, Cat. #500-0111), using bovine serum albumin as standard. Enzymatic assays were performed on 8 fish per treament.

Protein Extraction and Labeling for DIGE. Proteins from muscle samples were extracted and the sarcoplasmic fraction retained, following a method previously described.[35] All protein extracts were then cleared from salts and contaminants using a standard TCA/acetone-based 2DE sample precipitation kit (Bio-Rad), adjusted to pH 8.5 with NaOH and quantified using a standard Bradford colorimetric method (Bio-Rad). An internal standard was obtained by mixing equal amounts of protein from each sample (n = 6 per treatment) in a different container. Minimal CyDye labeling was performed on all samples (Cy3 and Cy5) and internal standard (Cy2) following the manufacturer's recommended procedures (GE Healthcare), ensuring proper dye balancing to prevent variable confounding.

Protein Separation by Two-Dimensional Gel Electrophoresis. Each IPG strip (24 cm, pH 4−7, Bio-Rad) was rehydrated by passively reswelling them overnight with a Cy3-labeled sample, a Cy5-labeled sample and the Cy2-labeled internal standard (50 μg of protein from each, for a total of 150 μg protein, pooled and diluted in standard IPG-IEF rehydration buffer to a final volume of 450 μL). Isoelectric focusing was performed using an Ettan IPGphor (GE Healthcare), at 20 °C, for a total of about 70 kV·h and with current below 75 μA per strip. Strips were then equilibrated using standard Bio-Rad 2DE reduction/alkylation buffers (15 min each step), loaded on to manually cast large-format 12.5% Tris-HCl SDS-PAGE gels and run at 50 mA/gel (after an initial 30 min period at 10 mA/gel), using a standard Tris-glycine-SDS running buffer, until the bromophenol blue front reached the end of the gels.

Gel Image Acquisition and Analysis. Labeled proteins in the gels were visualized using a Typhoon TRIO (GE Healthcare), using three different filters (520BP40, 580BP30 and 670BP30), at 100 μm resolution. Obtained images were analyzed using Progenesis SameSpots 2DE gel analysis software (Nonlinear Dynamics), which performs semiautomatic gel alignment, spot detection, background subtraction and abundance estimation. Student's t test was then applied on resulting protein abundance data (expressed in relation to the internal standard), having considered proteins with a $P < 0.05$ as being likely to be affected by the dietary supplementation. These protein spots were then manually excised from preparative gels poststained with colloidal CBB for digestion and identification.

Protein Identification by MS Analysis of Peptides and Database Search. After reduction and alkylation (using DTT and iodoacetamide, respectively), protein spots were digested with trypsin and the resulting peptides extracted with acetonitrile and trifluoroacetic acid. After a final desalting step, the peptides were then cocrystallized with the matrix (DHB) on a MALDI plate and analyzed with a MALDI-TOF-TOF mass spectrometer. The obtained MS and MS/MS mass lists were then used as input to MASCOT MS/MS Ion searches of the Actinopterygii subset of the NCBInr database, using the Matrix Science webserver (http://www.matrixscience.com/). 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 70 ppm for MS data and 0.5 Da for MS/MS data. Whenever no significant hits were obtained with the NCBInr database, searches were repeated against the Actinopterygii subset of the Vertebrates_EST database. Identity of transcripts was assessed using blastp (http://blast.ncbi.nlm.nih.gov/) against the NCBInr database. For each identified spot, we also attempted to determine the specific isoform (whenever isoform-specific peptides could be identified from the MS/MS data).

Statistical Analyses. All results are expressed as mean ± standard deviation. Unless otherwise specified, a two-way ANOVA was used to test the effects of time postmortem and inclusion of glycerol in the diets, followed by a Tukey HSD test if appropriate, except for analysis performed at only one time-point, where results were compared using

Student's t test. The comparison of histological degrees of muscle glycogen was performed using the Mann−Whitney test. Statistical significance was defined as $P < 0.05$. All tests were run with SPSS ver.17.0 (SPSS, Inc., Chicago, IL).

■ RESULTS

At the end of the feeding trial, seabream weight did not differ significantly between dietary treatments (Table 3). Similarly, no differences were found in terms of fish length, viscerosomatic and hepatosomatic indices and proximate composition of the fillets. However, seabream fed the GLY diet had a significantly higher fillet yield (34 ± 3% vs 31 ± 3% for CTRL; skinless fillets, $P = 0.010$).

Table 3. Weight, Length, Fillet Yield, Viscerosomatic Index, Hepatosomatic Index, and Proximate Composition of the Fillets of Gilthead Seabream Fed the Experimental Diets[a]

	CTRL	GLY
Weight (g)	623 ± 66	617 ± 61
Length (cm)	29 ± 1	30 ± 1
Fillet yield (%)	31 ± 3	34 ± 3*
Viscerosomatic Index (%)	5.4 ± 0.7	5.3 ± 0.9
Hepatosomatic index (%)	1.5 ± 0.2	1.5 ± 0.2
Proximate Composition (%)[b]		
Lipids	9 ± 3	9 ± 3
Protein	21 ± 1	22 ± 1
Moisture	69 ± 2	68 ± 3
Ash	1.4 ± 0.0	1.4 ± 0.0

[a]Values are means ± standard deviation ($n = 16$). Means with * are significantly different from the control ($P < 0.05$). [b]$n = 4$ pools of 2 fish.

Table 4 shows an overview of the results for the fatty acid profiles of seabream muscle from both diets. The individual fatty acids analyzed did not differ between diets tested (results not shown), and there were no significant differences between major lipid classes.

Table 4. Resumed Fatty Acids Profile of Seabream from the Two Dietary Treatments, Expressed as Percentage of Total Fatty Acids[a]

fatty acids	CTRL	GLY
C20:4n-6 [AA]	0.7 ± 0.2	0.7 ± 0.1
C20:5n-3 [EPA]	5.2 ± 1.2	5.1 ± 0.5
C22:6n-3 [DHA]	9 ± 4	8 ± 1
Σ saturated	32 ± 4	31 ± 2
Σ monounsaturated	36 ± 2	37 ± 1
Σ polyunsaturated	31 ± 7	30 ± 2
Σ n-3	21 ± 6	20 ± 2
Σ n-6	8.5 ± 0.5	8.7 ± 0.5
Ratio Σ n-3/Σ n-6	2.4 ± 0.6	2.2 ± 0.1

[a]Values are means ± standard deviations ($n = 4$ pools of 2 fish).

The instrumental texture of cooked fillets was not significantly affected by the inclusion of glycerol in the diet. However, glycerol significantly increased the hardness of raw fillets (control, 2.5 ± 0.9 N vs glycerol, 3.5 ± 1.6 N), which can be seen as a positive effect.

The microscopy analysis of the glycogen content of gilthead seabream muscle showed significant differences between treatments. Fish fed the glycerol diet showed a significantly higher glycogen deposition (grade 3) compared to the control (grade 2), as can be confirmed by the more intense coloration of muscle fibres (Figure 1).

Muscle cellularity parameters did not vary significantly between dietary treatments. Although fish fed the glycerol diet had a tendency toward larger white muscle fibres compared to the control (80 ± 4 μm vs 74 ± 6 μm for the CTRL), this difference was not significant ($P > 0.05$).

Muscle color was not affected by dietary treatments (Table 5). Likewise, there were no significant differences ($P > 0.05$) in volatiles analyzed by GC−MS (Table 6). If we apply a relaxed significance threshold of $P < 0.1$, to ensure a more conservative assessment of the impact of glycerol on aroma, we observe that glycerol-fed fish display slightly higher values for 2-nonanone, (*E*)-2-nonenol, (*E*)-2-decen-1-ol and undecanal compared to control, while these display slightly higher values of total aromatic hydrocarbons compared to glycerol-fed fish. Nonetheless, sensory panellists did not find differences in color and aroma of seabream fed either the CTRL or the GLY diet (Figure 2), which is consistent with the lack of major changes in terms of volatile composition.

Muscle pH (Figure 3) decreased markedly throughout storage time. Seabream fed the GLY diet displayed higher muscle pH at the time of death ($P = 0.005$), but this difference was no longer present 3 h after death.

Rigor mortis showed a typical evolution, with the seabream attaining full rigor between 9 and 24 h postmortem, and this pattern was similar for seabream fed any of the diets (results not shown).

Muscle thiobarbituric acid reactive substances ranged from 4.8 ± 0.4 to 6.4 ± 0.5 μg MDA·g^{-1} and were not affected by storage time nor dietary treatments (see Figure II of Supporting Information). However, at 6 h postmortem, glycerol fed seabream showed a slightly lower lipid oxidation, although not significantly different from the control ($P = 0.057$).

Figure 4A shows the muscle's glycogen content. Seabream fed the glycerol supplemented diet showed a higher muscle glycogen content at the time of death ($P = 0.044$), but this difference was no longer evident 6 h postmortem. At 48 h glycogen levels in the muscle had decreased below the detection threshold (results not shown).

ATP content of the muscle decreased throughout storage time (Figure 4B). At the time of death, seabream fed GLY had a higher ATP content in the muscle ($P = 0.024$), but no differences were evident at 6 and 48 h postmortem.

Cathepsin B activity increased throughout storage time, and average activity at 48 h postmortem (23 ± 10 U·g^{-1} protein) was significantly higher than activity at 0 and 6 h postmortem (8 ± 2 and 12 ± 3 U·g^{-1} protein, respectively, $P < 0.001$), but dietary treatments did not influence cathepsin B activity. Similarly, cathepsin H activity did not differ between dietary treatments, although the evolution throughout storage time showed a higher activity at 6 h postmortem (375 ± 90 U·g^{-1} protein) compared to 0 and 48 h postmortem (298 ± 103 and 253 ± 71 U·g^{-1} protein, respectively, $P = 0.002$). Collagenase activity was not significantly affected by either storage time or dietary treatments, and varied between 193 ± 70 and 282 ± 73 U·g^{-1} (protein).

Glycogen phosphorylase activity (Figure 4C) was not significantly affected by storage time ($P = 0.178$). Supplemen-

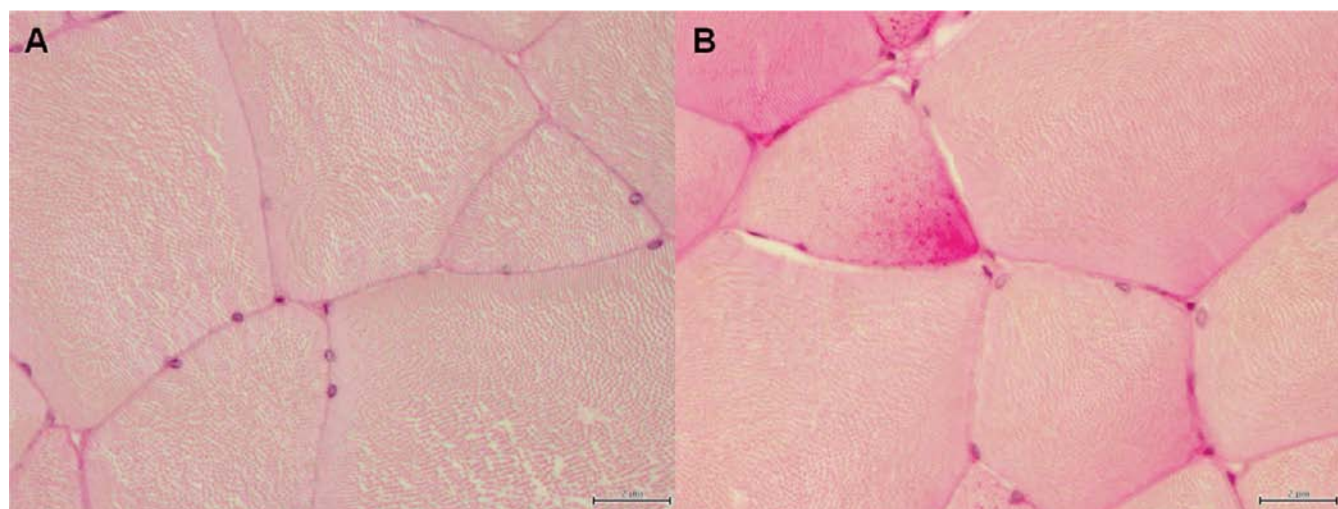

Figure 1. Transversal sections of white muscle fibres of gilthead seabream fed (A) the control and (B) glycerol supplemented diet after staining with PAS (100×). Scale size is 2 μm.

Table 5. Instrumental Color of Cooked Fillets from Seabream Fed the Two Experimental Diets[a]

	CTRL	GLY
*L**	85 ± 2	86 ± 2
*a**	−2.1 ± 0.3	−2.2 ± 0.3
*b**	9 ± 2	8 ± 2
*C**	9 ± 2	9 ± 1

[a]Values are means ± standard deviation (n = 10).

Table 6. Concentration of Volatile Compounds in Gilthead Seabream Fed the Two Experimental Diets[a]

compounds ($\mu g \cdot g^{-1}$)	CTRL	GLY
3-Methyl-butanal	1.34 ± 0.3	1.89 ± 0.3
1-Penten-3-ol	566 ± 44	498 ± 146
Hexanal	43 ± 17	47 ± 9
Ethylbenzene	12 ± 9	7 ± 1
(Z)-4-Heptenal	1.2 ± 1.0	1.4 ± 0.8
Heptanal	1.6 ± 0.4	1.9 ± 0.5
1-Octen-3-ol	6 ± 2	7 ± 2
Octanal	2.4 ± 0.3	2.6 ± 0.5
Eucalyptol	0.04 ± 0.04	0.07 ± 0.02
(*E*)-2-Octenal	0.02 ± 0.03	0.35 ± 0.27
2-Nonanone	0.28 ± 0.06	0.51 ± 0.08
(*E*)-2-Nonenol	7.0 ± 1.7	12.4 ± 0.3
(*E*.*Z*)-2.6-Nonadienal	0.02 ± 0.03	0.87 ± 1.10
4-Ethyl-benzaldehyde	0.6 ± 0.1	0.6 ± 0.2
(*E*)-2-Decen-1-ol	0.5 ± 0.1	1.0 ± 0.2
Undecanal	7 ± 2	14 ± 4
Σ aldehydes	13.62	23.55
Σ alcohols	13.97	20.14
Σ aromatic hydrocarbons	11.55	6.68
Σ ketones	0.28	0.51

[a]Values are means ± standard deviation (n = 2 pools of 4 fish each).

tation with glycerol in the diets resulted in a higher glycogen phosphorylase activity (P = 0.029), specifically at 6 h postmortem, when seabream fed CTRL diet showed a marked decrease in activity (0.056 ± 0.008 $\Delta abs \cdot mg^{-1}$ (protein)·min^{-1}) while GLY fed seabream glycogen phosphorylase activity remained similar in all time points (average 0.092 ± 0.026 $\Delta abs \cdot mg^{-1}$ (protein)·min^{-1}).

In terms of protein expression, 2D-DIGE analysis revealed a relatively low impact of the glycerol-containing diet on muscle proteome expression, with only 17 (out of 387 quantified proteins) displaying significant differences between treatments ($P < 0.05$). Of these 17 proteins, 9 were reliably identified using mass spectrometry (Table 7). Abundance estimation for these 17 proteins can be seen in Figure 5.

DISCUSSION

Effects on Fish Growth and Performance. Results from this trial showed a generally beneficial effect regarding the inclusion of crude glycerol in gilthead seabream diets. Looking at measured zootechnical parameters, we observed good performance results for both diets, confirming that glycerol inclusion at 5% does not seem to induce any immediately apparent deleterious effects in adult gilthead seabream. Specifically, proximate composition and hepatosomatic index values obtained generally suggest the fish are able to metabolically cope in an adequate way with the partial replacement of typical carbohydrates/starch with glycerol as an energy yielding substance. This is consistent with results obtained for channel catfish, where it was shown that inclusion of up to 10% glycerol in diets induced no detrimental effects on typical zootechnical performance criteria.[22] In fact, unlike the results of this catfish study, our results showed no induced differences even in terms of body moisture content.

Interestingly, although no differences could be seen in terms of final weight, glycerol-fed fish displayed higher fillet yield, compared to control. A similar situation has already been described in broilers fed 2.5% and 5% glycerol diets, where a higher breast yield was obtained (compared to 0% glycerol diet), although no differences in final weight could be measured.[21] This effect was attributed to higher protein deposition due to the sparing effect glycerol can have on glucogenic amino acids like alanine and glutamate. Our data from a separate study, in which we assessed the effect of dietary glycerol on the growth performance and metabolic nutrient utilization in juvenile gilthead seabream, confirms that a 5% dietary glycerol level significantly improved dietary protein and fat retention (J. Dias, personal communication).

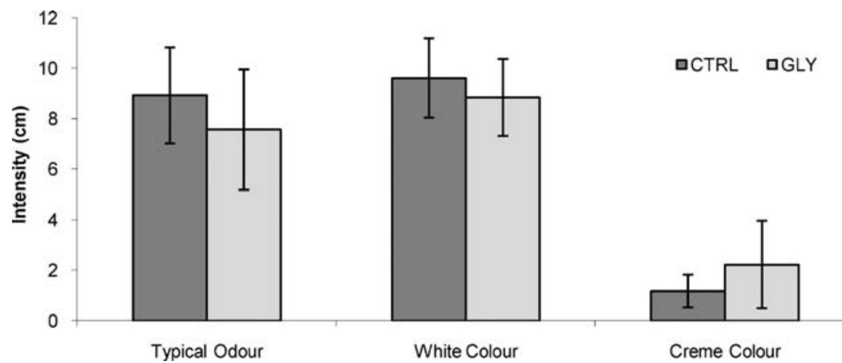

Figure 2. − Sensory scores of gilthead seabream fed the control and glycerol supplemented diet. Values are means ± standard deviation ($n = 8$).

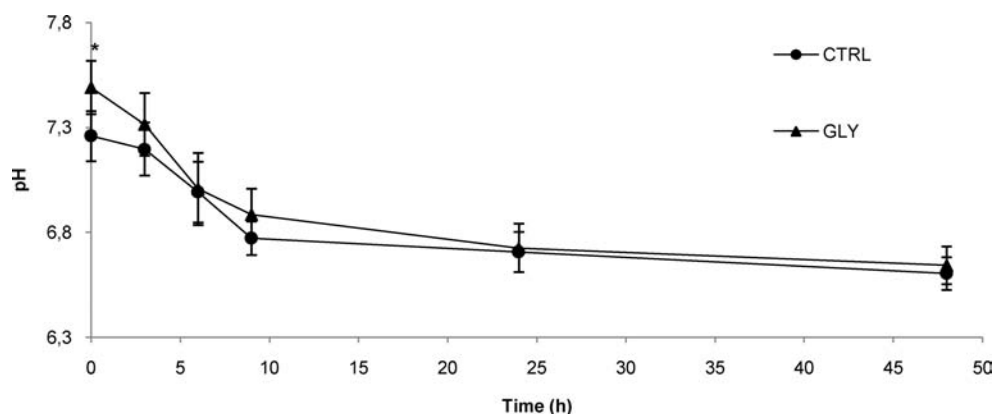

Figure 3. Postmortem muscle pH of gilthead seabream fed the control and glycerol supplemented diet, over 48 h in ice storage. Values are means ± standard deviation ($n = 6$). Means with asterisk (*) are significantly different from the control ($P < 0.05$).

Effects on Metabolism and Premortem Muscle Energy Status. In terms of the putative ability of dietary glycerol to improve the premortem energetic status of muscle, our results do seem to confirm it for gilthead seabream: both spectrophotometric and histological assays showed increased glycogen deposition in the muscle fibres of glycerol-fed fish, as well as increased levels of ATP (measured immediately postmortem). Downstream indicators of the muscle energetic status (muscle pH and rigor index) show delayed (or at least unaffected) progression of their usual temporal profiles for glycerol-fed fish, which is consistent with the glycogen and ATP measurements. These signals of improved muscle energy were particularly apparent immediately postmortem, while after 6 h postmortem little differences could be measured between the two experimental groups.

To contextualize these results, it is important to note that both control and experimental diet were formulated to ensure that all known nutritional requirements for adult gilthead seabream were adequately met, and that these were fed during a period of less demanding energetic requirements (finishing phase). Besides this, we attempted to replicate as accurately as possible standard aquaculture practices, namely enforcing a 48 h preslaughter starvation period, catching the fish with a net and slaughtering them using ice−water slurry. These practices unavoidably constitute challenges and therefore affect fish metabolism, in part due to the combined effect of glucagon, epinephrine and glucocorticoids (like cortisol), and also due to the induced hypoxia and energy depletion.

Metabolically, gilthead seabream is relatively flexible, having a high tolerance toward dietary carbohydrates, compared to other carnivores like rainbow trout.[36] Specifically, studies have shown that gilthead seabream does not display the same type of hyperglycaemia that rainbow trout does when fed carbohydrates, but only a postprandial transient peak (at least in part due to repression of hepatic gluconeogenesis).[37] On the other hand, during fasting or feed-restriction periods, gilthead seabream liver displays both higher gluconeogenic rates and lower glucose catabolic capacity (due to decreased expression of glucokinase).[38] Furthermore, several studies point out that cortisol and epinephrine are likely to have a cumulative effect with feed deprivation on hepatic and peripheral metabolism.[39,40] Finally, there are indications that, at least for gilthead seabream, 3-carbon compounds may have a higher importance than glucose in providing energy and carbon for short-term hepatic glycogen replenishment during feeding-fasting cycles,[41] as well as during stressful situations associated with feed deprivation,[40] possibly because triacylglycerides are an important secondary store of energy in gilthead seabream and their mobilization entails higher glycerol availability.

Regarding skeletal muscle metabolism, information on glycerol uptake and glycogenesis in gilthead seabream is scarce, but there are indications that, in rainbow trout, it can have (at least indirectly) a positive effect on glycogen deposition in muscle.[14] Our results, coupled with the knowledge that gilthead seabream is more adaptable to and tolerant toward carbohydrates than rainbow trout, clearly suggest glycerol does contribute significantly to increased glycogen deposition in muscle, either directly (possibly after being converted to glucose by hepatic gluconeogenesis) or indirectly (as it is the case for rainbow trout, through a sparing effect on lactate and other glucogenic metabolites). Besides this, we were aware that dietary glycerol, particularly during low energy demand periods,

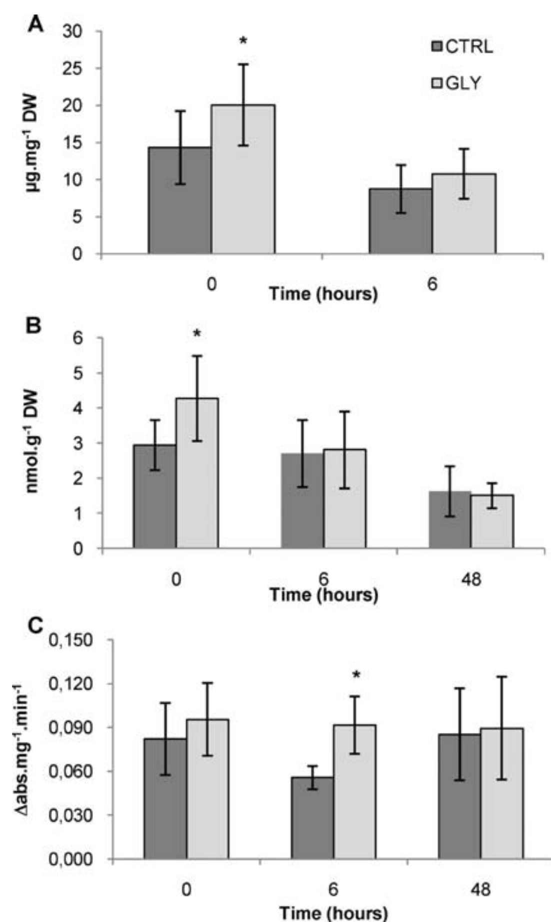

Figure 4. Muscle glycogen (A) and ATP (B) content of gilthead seabream fed the control and glycerol supplemented diet. Muscle glycogen phosphorylase activity (C) of gilthead seabream fed the control and glycerol supplemented diet. Values are means ± standard deviation (n = 8). Means with asterisk (*) are significantly different from the control (P < 0.05).

could induce increased lipogenesis in liver, muscle or adipose tissue, as well as induce changes in the fatty acid profiles (i.e., increased saturation levels), as it was demonstrated for other animal models.[16,17] As mentioned before, none of these issues occurred during this experiment, as no differences or abnormalities could be noted in terms of HSI, VSI, fatty acid profile or whole body composition. These results again reinforce the notion that gilthead seabream has a relatively high metabolic adaptability to dietary carbohydrates (particularly glycerol) for a carnivorous teleost.

Effects on Fish Meat Quality. When looking at the impact of glycerol on ultimate fish meat quality indicators, results were also encouraging, as dietary glycerol induced no deleterious effects in terms of aroma, color, proteolytic potential and texture parameters (instrumental and morphometric).

In fact, increased hardness of raw fillets is seen as a very positive result in terms of quality and freshness measures for gilthead seabream meat.[42] Although none of the studies on the effect of glycerol on marine species explored the possible impact on their organoleptic properties, a few experiments in pigs support the idea that glycerol might increase muscle hardness/firmness, particularly when included in a finishing diet.[18,20] This last study attempts to explain this through the effect glycerol supplementation seems to have in terms of fatty acid composition, namely, a general increase in the degree of fatty acid saturation. This impact in fatty acid profile was not observed in the current experiment though, suggesting that the underlying factor in seabream pertains to a different cellular trait. A likely possibility is that the slight texture differences observed are attributed to proteins of the extracellular matrix (like collagen), which would explain why no differences could be observed for the cooked fillets (as discussed elsewhere[43]). It is therefore plausible that glycerol supplementation might have induced a sparing effect on glutamate (required for proline synthesis, a limiting factor in collagen production), which would explain the instrumental texture observations for raw and cooked fillets, despite no significant differences in terms of cellularity parameters (i.e., fiber diameter and density).

Table 7. Protein Spots Identified by Mass Spectrometry (Using Peptide Fragment Fingerprinting), along with Relevant Details[a]

spot no.	protein identity	accession no.	Exp. M_w/pI	Theo. M_w/pI	coverage (%)	no. matches	best peptide match: sequence, E-value	Mowse score
124	Transferrin	ACN80997.1	77 kDa/6.0	74 kDa/5.9	0[b]	0[b]	VPAHAVVTR, 0.08	39
129	Transferrin	AEA41139.1	76 kDa/6.1	76 kDa/5.9	3	2	ASSIEQYYGYAGAFR, 1.1e-05	103
188	Heat shock cognate HSPA8-type	CAA72216.1	61 kDa/5.3	71 kDa/5.2	3	2	ARFEELNADLFR, 0.0079	84
189	Heat shock cognate HSPA8-type	AAF71255.1	61 kDa/5.3	72 kDa/5.3	5	3	ARFEELNADLFR, 9.2e-05	169
202	Aldehyde dehydrogenase ALDH7A1	AAX54912.1	57 kDa/5.9	56 kDa/5.9	6	3	IYVEGVGEVQEYVDVCDYAVGLSR, 0.016	53
204	Aldehyde dehydrogenase ALDH6A1	FM154143	56 kDa/6.0	55 kDa/6.7	7	4	EWLPELVER, 0.00021	174
211	Aldehyde dehydrogenase ALDH1A2	FM152135	56 kDa/5.9	57 kDa/5.9	5	4	DIDKANYVSSGLR, 7.2e-11	222
421	Peroxiredoxin 6	ADI78069.1	28 kDa/6.4	25 kDa/6.3	14	2	LSILYPATTGR, 1.6×10^{-6}	86
532	Phosphohistidine phosphatase	FM145195	16 kDa/6.3	16 kDa/8.8	16	2	IPDVEIDPEGTFK, 6.9×10^{-7}	181

[a]Experimental M_w and pI values were estimated from the spots' positions on the 2DE gel. Theoretical M_w and pI values were estimated using available sequence data. Only peptides with E-value below 0.05 (discarding oxidated versions and other duplicates) were counted for the calculation of the number of matched peptides per protein, estimated coverage and combined Mowse score. [b]Despite the fact that no peptides were found with an E-value below 0.05 for this protein spot, there were two borderline peptide matches, and since the spot's position in the 2DE gel is consistent with a transferrin isoform, we assumed it was valid to exceptionally consider this identification as reliable.

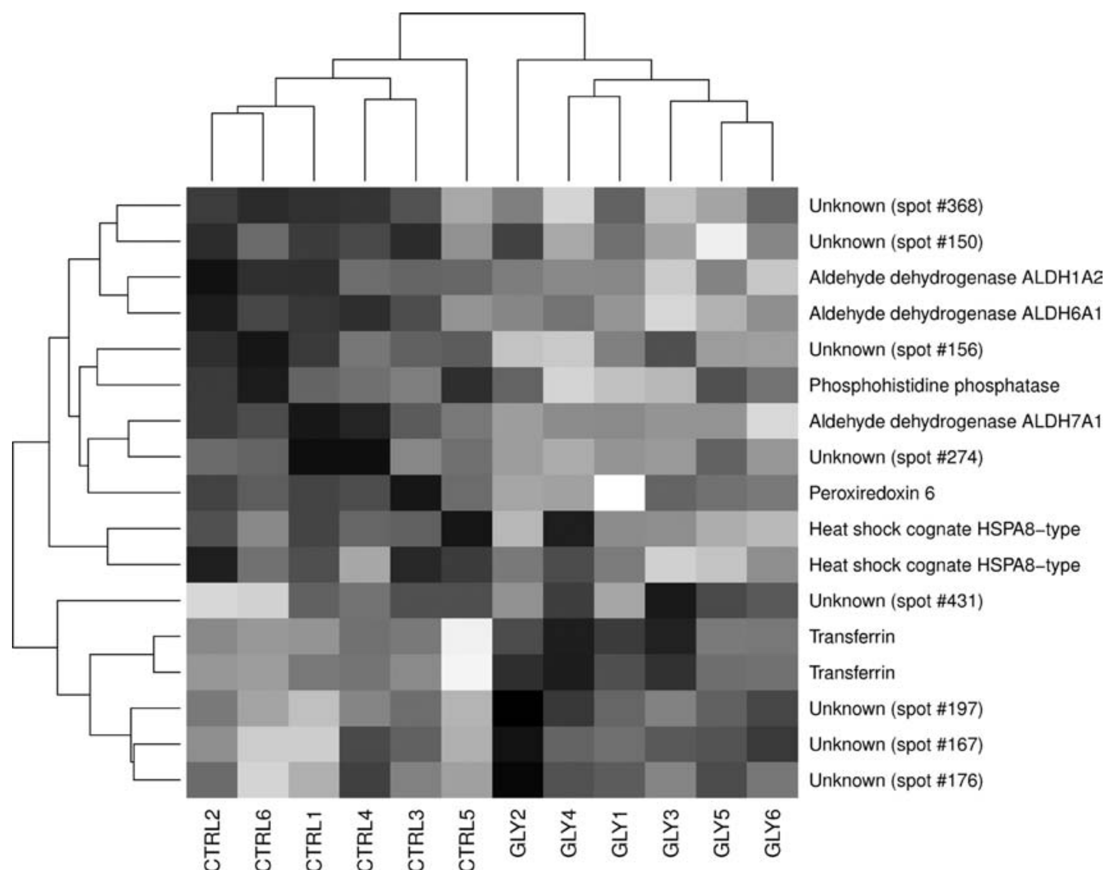

Figure 5. Heat-map detailing protein expression levels across all muscle samples at time-point 0 h, for all protein spots significantly different between control and glycerol-fed individuals ($P < 0.05$). Dark tones indicate "below average expression", while bright tones indicate "above average expression". Protein identity is present, whenever available.

Another relevant factor in fish meat quality is aroma: a complex feature that arises from the release of a mixture of volatile components mostly resulting from bacterial metabolism, lipid oxidation, enzymatic reactions and other decomposition processes. According to Kawai and Sakaguchi,[44] fresh saltwater fish give off a relatively low number of different volatiles, with 1-penten-3-ol as major component: this is generally consistent to what we observed. This particular fact is also evident in other analyses of fresh gilthead seabream.[45−47] Lipid oxidation usually generates different kinds of volatile aldehydes, such as pentanal, hexanal, octanal, and nonanal, but hexanal is often predominant. Aldehydes are particularly relevant volatiles since their presence is subjectively noticeable at comparatively low quantities. Of the four volatile components that could be considered slightly increased ($P < 0.1$) in glycerol-fed fish, two are pleasant-smelling alcohols (2-nonenol and 2-decenol), one is a pleasant-smelling ketone (2-nonanone), and one is a more pungent, although pleasant-smelling, aldehyde (undecanal). All of these are usually associated with organoleptic terms such as fresh, green, herbal, waxy, fruity, citric, sweet, and fatty, suggesting these substances are not likely to be responsible for any fishy or otherwise foul odors. This was corroborated by the sensorial panel analysis, which found no differences between the two fish groups.

Effects on Muscle Sarcoplasmic Proteome. The proteomic results mostly confirm the low impact of glycerol on muscle metabolism, as only a very reduced set of proteins (17 out of 387) were identified as being affected by the dietary treatment, even when taking a relatively relaxed threshold for statistical significance (Student's t test, $P < 0.05$, with no multiple comparison correction). Looking at affected pathways, we observe mostly changes at the level of proteins involved in detoxification processes (ALDH1A2, ALDH6A1, ALDH7A1), response to oxidative stress (peroxiredoxin, HSPA8 proteins, transferrins) and signaling (phosphohistidine phosphatase), with most signs pointing toward higher expression of stress response proteins in glycerol-fed fish.

Regarding proteins associated with detoxification processes, specifically in the case of ALDH7A1 (known to be involved in the detoxification of lipid peroxidation products), this enhanced expression seems to suggest that increased lipid peroxidation was occurring in glycerol-fed fish. On the other hand, this observation is inconsistent with the fact that we observed lower levels of TBARS in these fish. This could be an indication that increased expression of ALDH isoforms should probably be interpreted not as a sign of increased lipid oxidation, but as a sign of increasing muscle capacity to process and neutralize lipid peroxidation products.

Looking at proteins involved in the response to oxidative stress, we found that, in comparison to control fish, glycerol-fed fish showed increased expression of peroxiredoxin and heat shock proteins of the HSPA8 family in muscle. Peroxiredoxins are antioxidant proteins, while heat shock proteins prevent protein aggregation, having a role in protein folding. Regarding HSPA8 isoforms, they are usually constitutive and not stress-inducible, which makes the interpretation of the results difficult, especially if we take into account the nonproteomic observations. As mentioned before, we should be careful in

directly interpreting these results as increased signs of oxidative stress, since we have no other indicators that glycerol-fed fish were indeed more stressed. It is important to note that both peroxiredoxin and HSPs generally have a protective and antiapoptotic effect, so these results could also imply that these fish have a higher capacity to cope with oxidative stress-induced protein misfolding and aggregation. Still in reference to the pathways of cellular response to stress, we observed lower abundance of transferrin spots in glycerol-fed fish compared to control. Transferrin is mainly associated to iron transport to peripheral tissues. Also, due to the fact that transferrin helps to maintain low concentrations of free iron, it most likely also has a role in reducing the generation of ROS via Fenton reactions. Experiments in mammals and fish have shown transferrin expression in liver and release into the bloodstream is increased as part of the primary stress response.[48−50] It is also relevant to note we have previously seen increased transferrin abundance in gilthead seabream muscle in response to harvesting and slaughter stress.[35] Also, a recently published study on the effects of prolonged hypoxia on the sarcoplasmic proteome of rainbow trout muscle also demonstrates an immediate increase in transferrin isoforms after the onset of hypoxia.[51] These observations would again suggest that control fish were generally more stressed than those fed the glycerol-containing diet.

Finally, we also observed an increased expression of a phosphohistidine phosphatase (PHP) in the muscle of glycogen-fed fish, which is a protein involved in signaling, acting in opposition to nucleoside diphosphate kinase in phosphohistidine signaling. Although the topic of phosphohistidine signaling is still a young research field, there are already some studies in vertebrates which can help elucidate its relevance in this context (for a review see ref 52). Some possible targets are known for PHP, but two are of particular importance, since they have been shown to be specifically affected by PHP activity.[53,54] One of these is ATP-citrate lyase, a protein involved in the regulation of the cytosolic acetyl-CoA pool and therefore responsible for regulating lipid biosynthesis. A logic possibility is that increased dephosphorylation rate of ATP-citrate lyase (and, therefore, inactivation) might be a compensatory mechanism to prevent overly active lipogenesis due to increased availability of glycerol. A reduction of the metabolic flow of Krebs cycle carbon (probably originated by starvation-induced β-oxidation of fatty acids) toward lipogenesis should increase efficiency of energy-generating catabolic processes and gluconeogenesis. This suggests the muscle of glycerol-fed fish apparently displayed a more appropriate response toward the experimental challenges (starvation, slaughter stress, hypoxia and postmortem energy depletion) by effecting a change which confers higher metabolic efficiency. The other highly likely target of PHP is the G_β subunit of heterotrimeric G proteins, a component of the glucagon transduction pathway, among others. Phosphatase activity on this subunit apparently decreases the basal (i.e., ligand-independent) levels of signal transduction.[53] This suggests the decreased levels of PHP in fish fed the control diet might induce higher glucagon-independent stimulation of glycogenolysis during fed states, contributing toward lower glycogen deposition.

Effects on Enzymatic Activities and Lipid Oxidation in Muscle. Besides the proteomic results, directed biochemical assays suggest increased levels of active glycogen phosphorylase and slightly lower levels of lipid oxidation (as assessed by the amount of TBARS present in muscle) in glycerol-fed fish. This is consistent with information on glycogen metabolism in rainbow trout, where glycogen phosphorylase activity in the muscle was seen to increase in response to epinephrine and glucocorticoids, but only when glycogen stores were above 5 μmol/g.[55] Looking at all of these signals together, and taking into account that glycerol-fed fish showed otherwise clear signals of improved muscle energy status, we are led to interpret these observations as a sign of higher response magnitude and coping ability of glycerol-fed fish toward harvesting/slaughtering stress, since all mentioned proteins have a cytoprotective effect and glycerol-fed fish displayed signals of generally lower levels of oxidative stress. Analyzing these results within an allostatic framework,[56] we are therefore inclined to consider the observed proteomic changes as mostly adaptive, rather than maladaptive, specifically given the relatively low number of affected proteins.

Another interesting observation regards glycogen phosphorylase activity and TBARS abundance at 6 h postmortem: although control versus glycogen-fed differences for these parameters are negligible for most samples, a substantial difference in these parameters was seen for this time-point. Given that samples obtained at 6 h postmortem were collected from the same fish sampled at 0 h postmortem, we assume higher TBARS levels measured can be attributed to this repeated manipulation of the same fish for the first two samplings. What is relevant to note is that only fish from the control group displayed an induction of lipid oxidation due to postmortem manipulation, which again suggests that glycerol-fed fish appear to have improved stress coping abilities. In the same way, glycogen phosphorylase activity in control fish seems to have been suppressed for this time-point, which could be explained by inactivation due to increased oxidative stress. This seems particularly plausible, since we observed a relevant inverse correlation between glycogen phosphorylase activity and TBARS abundance, at this time-point ($\rho \approx 0.45$).

Impact of Possible Contaminants Present in Crude Glycerol. A major concern regarding the use of biodiesel-derived crude glycerol in animal feeds is the fact that it often contains significant amounts of salts, fatty acids and some methanol (or, less commonly, ethanol) residues, due to the transesterification process. While the impact of salts and fatty acids can be mitigated through adequate diet formulation, the presence of methanol in crude glycerol is of higher concern, as it is known to induce oxidative cellular stress in most animals. On the other hand, methanol is not as toxic toward fish as it is toward primates,[57] possibly due to a lower metabolic rate (and therefore lower endogenous production of formaldehyde and formate), since fish should be able to excrete methanol and its metabolites more efficiently than land animals. Additionally, due to the high temperature of the feed extrusion and drying process, it is very likely that any methanol present would evaporate. Experiments with inclusion of biodiesel derived crude glycerol in pig feeds confirm that the usual levels of methanol present do not induce any symptoms associated with methanol-induced acute metabolic acidosis and oxidative stress (e.g., optic nerve lesions).[17,19] Also, we have no reason to believe chronic exposure to low amounts of methanol is dangerous by itself, as its production by normal metabolic processes is unavoidable and its presence in the environment is ubiquitous; for this reason, it is also very unlikely that it bioaccumulates in any way. Given that no signs of oxidative stress or mitochondrial damage due to acute methanol

poisoning were observed in glycerol-fed fish, it seems safe to assume inclusion of crude glycerol with less than 0.03% methanol in gilthead seabream feeds has no observable deleterious effect. Nevertheless, and given that crude glycerol composition can vary between different sources, batches, and according to the type of fats used as raw material for biodiesel production, it might be relevant to enforce strict quality control criteria on feed-grade crude glycerol, to ensure it does not contain undesirable amounts of methanol.

Final Considerations. Concluding, glycerol seems like a promising choice for (at least partial) replacement of starch and other carbohydrate sources for gilthead seabream diets, both in terms of economic and sustainability criteria as in terms of fish nutrition, health, meat quality and nutritional value. Furthermore, the original hypothesis of using glycerol as a dietary supplement to improve the energy status of gilthead seabream muscle seems quite plausible, having seen in this experiment clear signals of increased glycogen and ATP abundance in muscle.

In the future, it would be interesting to explore how glycerol improves the gilthead seabream's stress coping capabilities, through experiments using explicit challenges or during situations when the energetic requirements of fish are particularly demanding (e.g., grow-out phase, sexual maturation, winter). Also, it would be relevant to assess the optimal inclusion levels for a given diet, depending on its purpose (e.g., grow-out vs finishing diets).

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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