Feed restriction up-regulates uncoupling protein 3 (UCP3) mRNA levels in heart and red muscle tissues of gilthead sea bream (*Sparus aurata* L.). New insights in substrate oxidation and energy expenditure

Azucena Bermejo-Nogales, Laura Benedito-Palos, Josep Alvar Calduch-Giner and Jaume Pérez-Sánchez

Fish Nutrition and Growth Endocrinology Group, Department of Biology, Culture and Pathology of Marine Fish Species, Instituto de Acuicultura Torre de la Sal, IATS-CSIC, 12595 Ribera de Cabanes, Castellón, Spain

Running title: Respiration uncoupling in gilthead sea bream

Corresponding author: Jaume Pérez-Sánchez
E-mail: jperez@iats.csic.es
Tel.: +34 964319500
Fax: +34 964319509

Abstract. The physiological regulation of the mitochondrial uncoupling protein 3 (UCP3) remains practically unexplored in fish and the aim of this study was to examine the effects of ration size on the regulation of UCP3 in heart, red skeletal muscle and white skeletal muscle of gilthead sea bream (*Sparus aurata* L.). Juvenile fish were fed at three different levels for 11 weeks: i) full ration until visual satiety (R_{100} group), ii) 70% of satiation (R_{70} group) and iii) 70% of satiation with two finishing weeks at the maintenance ration (20% of the satiation level) (R_{70-20} group). The thirty percent feed restriction improved fish performance, increasing feed conversion efficiency and circulating levels of insulin-like growth factor-I (IGF-I). In contrast, fish of the R_{70-20} group showed low circulating levels of IGF-I and increased circulating concentrations of growth hormone and free fatty acids. Dietary treatment did not alter UCP3 transcript levels in white skeletal muscle, but improved this tissue's oxidative capacity as assessed by changes in glycolytic and oxidative mitochondrial enzyme activities. In contrast, in cardiac and red skeletal muscle tissues, UCP3 mRNA expression was markedly increased with the level of feed restriction in coincidence with a reduced sensitivity to nutritionally-mediated changes in oxidative capacities. The respiratory control ratio of freshly isolated heart mitochondria was higher in R_{70-20} fish as compared with R_{100} fish, which suggests that there was an increase in mitochondrial uncoupling concomitant with the enhanced UCP3 expression. Altogether, these findings highlight different adaptive mechanisms for the rapidly adjusting of muscle metabolism to varying feed intake.

Key words: free fatty acids, growth hormone, heart, insulin-like growth factor-I, lipotoxicity, mitochondrial respiration, oxidative capacity, skeletal muscle.

1. Introduction

Uncoupling proteins (UCPs) are mitochondrial transporters that uncouple oxidative phosphorylation by the net discharge of proton gradient (Krauss et al., 2005). This protein family is widely distributed in animal phyla with a core group of three UCP genes that are expressed in a tissue-specific manner. In particular, UCP3 is preferentially expressed in skeletal muscle and to a lesser extent in heart (Boss et al., 1997; Vidal-Puig et al., 1997). The physiological role of this UCP paralogue is still under debate, but it may share a role as redox-sensor to match both energy demand and antioxidant defense in a wide range of physiological conditions. Thus, mammalian UCP3 counterparts are inversely related to fat oxidative capacity (Hoeks et al., 2003), are low in type 1 muscle fibers (Hesselink et al., 2001), are reduced by endurance training (Russell et al., 2003; Schrauwen et al., 2005) and predisposing factors for developing type 2 diabetes mellitus (Schrauwen et al., 2006b). Conversely, UCP3 is up-regulated by high fat diets (Hesselink et al., 2001) and elevated circulating levels of free fatty acids (Weigle et al., 1998).

In fish species, major changes in UCP3 mRNA expression are also associated to switches in energy demand or oxidative capacities. Thus, fasting up-regulates transcript levels of UCP3 in the skeletal muscle of carp (Jastroch et al., 2005). Likewise, UCP3 mRNA expression in on-growing gilthead sea bream is markedly higher in glycolytic muscle (white skeletal muscle) than in highly oxidative muscle tissues (heart and red skeletal muscle) (Bermejo-Nogales et al., 2010). In the same study, it is highlighted a close association between UCP3 and fatty acid metabolism with aging and seasonal changes on muscle fat deposition. Fish with signs of essential fatty acid deficiencies also share an altered pattern of UCP3 mRNA expression. In contrast, high stocking densities do not significantly alter UCP3 transcript levels, which is indicative that the regulation of UCP3 depends on the intensity, duration and nature of the stressor. In this sense, the aim of the present study was to underline the specific muscle-type regulation of UCP3 by ration size. To achieve this goal, juvenile gilthead sea bream were fed at varying feeding levels: i) full ration, ii) 70% of satiation; and iii) 70% of satiation with a finishing period at the maintenance ration. Changes in UCP3 transcript levels and tissue oxidative capacity were assessed in heart, white skeletal muscle and red skeletal muscle. The study also included measurements of fish performance and oxygen consumption on freshly isolated heart mitochondria.

2. Materials and methods

2.1 Animal care, experimental setup and sampling

Juvenile gilthead sea bream (*Sparus aurata* L.) of Atlantic origin (Ferme Marine de Douhet, Ile d'Oléron, France) were acclimatized to laboratory conditions for 25 days before the start of the trial in the indoor experimental facilities of Institute Aquaculture Torre de la Sal (IATS). After this initial period, fish of 17 g initial mean body mass were randomly distributed into 500 L-tanks in triplicate groups of 50 fish each. Fish were fed from May to August (11 weeks) with a commercial diet (D-2 Excel 1P, Skretting, Stavanger, Norway) twice per day at three different feeding levels: i) full ration until visual satiety (R_{100} group), ii) 70% of satiation (R_{70} group) and iii) 70% of satiation with the last two weeks at the maintenance ration (20% of the satiation level), (R_{70-20} group). The trial was conducted under natural photoperiod and temperature conditions at IATS latitude (40°5N; 0°10E). Water flow was 20 L/min, oxygen content of water effluents was always higher than 85% saturation, and unionized ammonia remained below toxic levels (<0.02 mg/L).

At the end of trial (following overnight fasting), nine randomly selected fish per dietary treatment were anaesthetized with 3-aminobenzoic acid ethyl ester (MS-222, 100 μg/mL). Blood was taken with heparinized syringes from caudal vessels, centrifuged at 3000 *g* (20 min at 4ºC) and plasma aliquots stored at -80ºC until hormone and metabolite assays. Heart ventricles and tissue samples of white and red skeletal muscle (right-hand site) were rapidly excised, frozen in liquid nitrogen and stored at -80°C until RNA extraction and enzyme activity analyses. From the same animals, whole fillets from the left-hand side were taken for proximate composition analyses of entire skeletal muscle. Mesenteric adipose tissue was excised from the ventral cavity and weighted to calculate the mesenteric fat index.

Additional fish from the two extreme groups (R_{100},R_{70-20}) were sampled in consecutive days for measurements of oxygen consumption on freshy isolated heart mitochondria. Each day, a maximum of three-four fish per dietary treatment were procesed to preserved mitochondrial integrity.

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

2.2 Muscle proximate composition

The proximate composition of entire skeletal muscle (whole fillet of left-hand site) was analyzed by standard procedures (AOAC, 1990). Moisture content was determined by drying in an oven at 105ºC for 24h. The remaining samples were freeze-dried and blended for lipid determinations using a Soxhlet 4001046 Auto extraction apparatus (Selecta, Barcelona, Spain) according to manufacturer's instructions.

2.3 Circulating hormone and lipid profiles

Plasma GH levels were determined by a homologous gilthead sea bream radioimmunoassay (RIA) as reported elsewhere (Martínez-Barberá et al., 1995). The sensitivity and midrange (ED_{50}) of the assay were 0.15 and 1.5-2 ng/mL, respectively. Plasma IGFs were extracted by acid–ethanol cryoprecipitation (Shimizu et al., 2000), and the concentration of IGF-I was measured by means of a generic fish IGF-I RIA validated for Mediterranean perciform fish (Vega-Rubín de Celis et al., 2004). The assay is based on the use of red sea bream (*Pagrus major*) IGF-I (GroPep, Adelaide, Australia) as tracer and standard, and anti-barramundi (*Lates calcarifer*) IGF-I serum (GroPep) (1:8000) as a first antibody. The sensitivity and midrange of the assay were 0.05 and 0.7–0.8 ng/mL, respectively.

Plasma levels of triglycerides were measured by means of the Infinity triglycerides liquid stable reagent kit (Sigma, catalog no. 337B). Plasma cholesterol levels were measured using the Infinity cholesterol reagent kit (Sigma, catalog no. 401-25P). The Wako NEFA C test kit (Wako Chemicals GmbH, Neuss, Germany, catalog no. 999-75406) was utilized as an *in vitro* enzymatic colorimetric method for the quantization of non-esterified fatty acids in plasma.

2.4 Gene expression

Total RNA was extracted from heart and skeletal muscles using the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA). Reverse transcription (RT) with random decamers was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems). Measurements of muscle UCP3 transcript levels were made using an iCycler IQ Realtime Detection System (Bio-Rad,

Hercules, CA, USA) as described elsewhere (Bermejo-Nogales et al., 2010). Briefly, diluted RT reactions were used for PCR reactions in 25-μl volume. Each PCR-well contained a SYBR Green Master Mix (Bio-Rad), and specific gilthead sea bream primers for UCP3 (forward primer: 5'-AGG TGC GAC TGG CTG ACG; reverse primer: 5'-TTC GGC ATA CAA CCT CTC CAA AG) were used at a final concentration of 0.9 μM. In parallel, cytochrome c oxidase subunit IV (COX IV) was PCR amplified with specific primers (forward primer: 5'-ACC CTG AGT CCA GAG CAG AAG TCC; reverse primer: 5'-AGC CAG TGA AGC CGA TGA GAA AGA) and used as housekeeping gene. The efficiency of PCR reactions for the target and the reference gene varied between 95% and 98%. Reactions were performed in triplicate and the fluorescence data acquired during the extension phase were normalized to COX IV by the delta–delta method (Livak and Schmittgen, 2001). No changes in COX IV expression were found in response to ration level.

2.5 Enzyme activity assays

The tissue oxidative capacity of heart (pooled tissue samples) and skeletal muscle (individual fish samples) was assessed by specific activity measurements of pyruvate kinase (PK; E.C.2.7.40), lactate dehydrogenase (LDH; E.C.1.1.1.27), citrate synthase (CS; E.C.4.1.3.7.) and 3-hydroxyacyl CoA dehydrogenase (HOAD; 1.1.1.35) as described elsewhere (Michaelidis et al., 2007) with minor modifications. Briefly, tissue samples were homogenized in 20 volumes of a cold CelLytic MT Cell Lysis Reagent (Sigma, catalog no. C3228), centrifuged at 14,000 *g* for 10 min at 4ºC and supernatant aliquots stored at -80ºC until analyses. PK, LDH and HOAD activities were measured following the oxidation of NADH at 340 nm (mM extinction coefficient 6.22). CS activity was determined based on the reaction of free coenzyme A with DTNB (5.5 V dithio-bis (2-nitrobenzoic acid) at 412 nm (mM extinction coefficient 13.6). Enzyme activities were expressed as International milliunits per milligram of protein. One unit of enzyme activity was defined as the amount of enzyme required to transform 1 µmol of substrate per min at 25ºC. Protein content was determined with the Bio-Rad protein assay using bovine serum albumin (BSA) as standard.

2.6 Mitochondria isolation and respiration

Heart mitochondria from the two extreme groups (R100, R_{70-20}) were isolated by differential centrifugation. Briefly, tissue samples were washed, fine minced, disrupted with a Dounce homogenizer and digested with trypsin (2.5 mg/mL) in 10 volumes of an ice-cold isolation medium (200 mM mannitol, 50 mM sucrose, 1 mM EGTA, 10 mM HEPES pH 7.5). Defatted BSA was added to a final concentration of 10 mg/mL to quench the proteolytic reaction. After two spin cycles, digested samples were homogenized with 8 volumes of isolation medium and centrifuged at 900 *g* for 10 min at 4ºC. The supernatant was then centrifuged at 9,000 *g* for 10 min at 4ºC and the resulting pellet was the final mitochondria-enriched fraction. Prior to determination of protein concentration (Bio-Rad protein assay), isolated mitochondria were treated with 2% CHAPS in Tris buffered saline (150 mM NaCl, 25 mM Tris pH 7.2).

Intactness of the mitochondria inner membrane was assessed by means of the CS activity assay. The assay relies on the exclusiveness of CS as a marker of the mitochondrial matrix. Briefly, isolated mitochondria were suspended in a non-detergent Bicine buffer (1 M pH 5.2) and centrifuged at 11,000 *g* for 10 min. Pellets were then resuspended with either a Cell Lytic M Cell Lysis reagent (Sigma, catalog no. C2978) or Bicine buffer. The percentage of mitochondria intactness was calculated as the CS activity ratio in the two mitochondria samples.

Oxygen consumption of isolated mitochondria was monitored polarographically using a Clark type electrode (Hansatech, King's Lynn, Norfolk, U.K.). Briefly, mitochondria (0.5 mg protein/mL) were incubated at 25º C in a final volume of 1 mL of incubation medium (125 mM KCl, 2 mM KH_2PO_4, 1 mM EGTA, 5 Mm $MgCl_2$, 0.5% defatted BSA (w/v), 10 mM HEPES pH 7.2) containing 8 μM rotenone. Measurements were carried out with succinate (6 mM) as substrate. The active state of respiration (state 3, S3) was defined as the rate of oxygen consumption in the presence of substrate and ADP (200 μM). State 4 (leak-dependent respiration, S4) was measured in the presence of the ATP synthase inhibitor oligomycin (4 µg/mL) and the respiratory control ratio (RCR) was calculated as the S3/S4 quotient. Lastly, mitochondria were uncoupled by adding 0.5 μM of the uncoupler carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (FCCP).

2.7 Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.8 Statistical analyses

Data on mitochondrial respiration was analyzed by Student t-test. Data on growth performance, tissue composition, gene expression and circulating levels of lipids and growth factors were analyzed by one-way ANOVA followed by Student Newman-Keuls (SNK) test ($P < 0.05$). Data on tissue enzyme activities were analyzed by two-way ANOVA followed by SNK test ($P < 0.05$). Pearson Product Moment correlations was used for correlation analysis of circulating levels of free fatty acids and UCP3 mRNA levels of heart and red skeletal muscle. All analyses were performed with the SPSS 17.0 program (SPSS, Inc. Chicago, IL, USA).

3. Results

3.1 Fish performance and metabolic condition

Effects of ration size on fish performance and circulating hormone and lipid profiles are shown in Table 1. Fish of the R_{100} group grew from 17 to 72 g at specific growth rates (1.95 ± 0.01 % mass day^{-1}) in the upper range for this fish species and class size. The R_{70} group had lower specific growth rates (1.68 ± 0.01 % mass day^{-1}) and final body mass (59.9 ± 0.016 g), but a significant higher (by10%) feeding efficiency. In the R_{70-20} group, feed intake was adjusted in the last two weeks to maintenance ration and the final body weight (48.7 ± 0.13 g) was reduced accordingly.

Viscerosomatic and hepatosomatic indices were progressively and significantly lowered with the reduction of feed intake. The same trend, but not statistically significant was found for the mesenteric fat index. Muscle fat content was statistically significantly reduced by 30% and 50% in R_{70} and R_{70-20} groups, respectively. This lower muscle lipid deposition was concurrent with a 3% increase of muscle water content in the R_{70-20} group.

Plasma levels of GH increased progressively and significantly with the reduction of feed intake from 5.2 ± 1.1 ng/mL to 11.4 ± 1.3 ng/mL in R_{100} and R_{70-20} fish, respectively. The R_{70-20} group had 40-50% lower circulating levels of IGF-I (36.9 ± 2.2 ng/mL) when compared with that measured in the R_{100} (56.03 ± 2.7) and R_{70} (67.7 ± 4.8 ng/mL) groups, respectively. Circulating levels of triglycerides and total cholesterol were also significantly reduced in the R_{70-20} group. In contrast, plasma concentration of free fatty acids in fish with active growing (R_{100}, R_{70}) was 0.09 mM, while in fish at the maintenance group was markedly increased up to 0.17 ± 0.02 mM.

3.2 Tissue oxidative capacity

The activities of glycolytic (PK, LDH) and oxidative mitochondrial (CS, HOAD) enzymes were dependent on tissue, and a significant interaction between muscle-type and feeding regime was also found (Table 2). Thus, the overall activity of PK and LDH was lowest in heart and highest in the white skeletal muscle. Conversely, CS and HOAD activities were lowest in the white skeletal muscle and highest in heart or red skeletal muscle. Relative to the feeding level, all enzyme activities in the white skeletal muscle of R_{70-20} fish were approximately double those reported for fish fed to satiation. In the red skeletal muscle, a significant but lower increase (by 15-25%) of glycolytic enzyme activities was found in the R_{70-20} group. In contrast, CS and HOAD activities were progressively and significantly decreased with the reduction of feed intake. The cardiac muscle also appeared poorly sensitive to nutritionally-mediated changes in oxidative capacities, and no statistically significant changes were found for any of the analyzed enzymes.

3.3 UCP3 expression

The tissue-specific regulation of UCP3 by ration size is shown in Fig. 1. In the white skeletal muscle, ration size did not affect UCP3 mRNA expression when it was normalized to COX IV, although UCP3 transcript levels were 10-20 times higher in the whole muscle than in heart or red muscle. In contrast, a marked up-regulation in UCP3 transcript levels was seen in both heart and red skeletal muscle as feed intake was decreased. These nutritionally-mediated changes in UCP3 expression were positively correlated ($P < 0.05$) with circulating levels of free fatty acids.

3.4 Respiration uncoupling

Measurements of oxygen consumption were taken on mitochondria isolated from R_{100} and R_{70-20} fish. The integrity of isolated mitochondria, measured as CS activity ratio, was higher than 80%. The RCR for fish heart coupled mitochondria respiring on succinate were above two in both ration levels (R_{100} 2.84±0.54; R_{70-20} 2.18±0.19) and the maximal chemical uncoupling after FCCP induction remained unaltered, which denotes good quality of mitochondrial preparations regardless of the fish nutritional condition. A typical polarographic oxygen electrode trace is shown in Fig. 2. Interestingly, the RCR was 15-20% decreased in the R_{70-20} group over the reference values of the R_{100} group.

4. Discussion

The regulation of feed intake is an evolutionary adaptation in vertebrate species that integrates signals of appetite, satiation and energy balance (Bernier and Peter, 2001 Volkoff et al., 2010). Nevertheless, the fine regulation of feed intake is still a matter of intense debate since unrestricted feeding do not necessarily improve growth or feed efficiency in a wide range of fish, including sturgeon (Hung and Lutes, 1987), rainbow trout (Storebakken and Austreng, 1987), tilapia (Papoutsoglou and Voutsinos, 1988), striped bass (Hung et al., 1993), grass carp (Du et al., 2006) and mangrove red snapper (Abbas and Siddiqui, 2009). This is consistent with the observation that effective control of feed intake to avoid unwanted liver GH-desensitization is absent and/or deficient in gilthead sea bream fed high lipid-enriched diets (Pérez-Sánchez et al., 1995). Importantly, also in this fish species, high fat diets (Sitjà-Bobadilla and Pérez-Sánchez, 1999) and long-term unrestricted feeding (Sitjà-Bobadilla et al., 2003) increase the prevalence and progression of fish parasite infections, which agrees with the notion that dietary requirements for optimal growth do not necessarily coincide with those for optimal functioning of the immune system.

Overfeeding is also perceived as an increased risk of oxidative stress (Evans et al., 2004; Faine et al., 2002), and the inhibition of fatty acid oxidation and mitochondrial oxidative phosphorylation in peripheral tissues is viewed in rats fed high fat diets as an adaptive response to limit the production of reactive oxygen species

(ROS) with nutrient excess (Obici and Rossetti, 2003; Obici et al., 2002). Fish also adopt multiple strategies to cope with periods of nutrient excess or enhanced energy demand. For instance, the shift from glycolytic towards lipid and protein catabolism is a well-known metabolic adaptation to face long fasting spawning migration in salmonids (Miller et al., 2009; Mommsen, 2004). High intensity training also shifts the proportion of energy derived from fat oxidation to carbohydrate-derived energy in swimming muscles of sea trout (Anttila et al., 2010). In particular, in the present study, feed restriction reduced body fat stores leading to two different energy steady states. Thus, the R_{70} group had improved fish performance and increased circulating levels of IGF-I, which can be viewed as a healthy metabolic condition in comparison to fish fed to satiation. In contrast, the R_{70-20} fish showed low circulating levels of IGF-I in combination with a hypersomatotropic state (increased circulating levels of GH) that is indicative of a liver GH-desensitization (loss of hepatic GH receptors) and impaired growth performance as reported previously in a wide range of gilthead sea bream malnutrion models, arising from changes in diet composition and feeding levels (Benedito-Palos et al., 2007; Gómez-Requeni et al., 2004; Pérez-Sánchez et al., 2002; Pérez-Sánchez and Le Bail, 1999; Pérez-Sánchez et al., 1995).

In the present study, UCP3 transcript levels were 10-20 times higher in white than in red skeletal muscle regardless of ration size. Muscle-type differences in UCP3 mRNA expression were even more evident (up to 100 fold-lower) when comparisons are made with heart. This agrees with the global concept that UCP3 is preferentially expressed in the glycolytic skeletal muscle and to a lower extent in the highly oxidative cardiac muscle (Hesselink et al., 2001; Schrauwen et al., 2001). Moreover, it is generally accepted that UCP3 transcripts are up-regulated when fatty acid supply to the mitochondria exceeds the oxidative capacity (Schrauwen et al., 2006a; Nabben and Hoeks, 2008). In other words, UCP3 would act as a safety valve that activates a futile cycle of energy that becomes rapidly inactive when the oxidative capacity of glycolytic muscles is improved. However, in aerobic muscles, the compensatory mechanisms that enhance oxidative capacity are theoretically less operative, and respiration uncoupling would match the antioxidant defense system to protect the mitochondria against lipid-induced oxidative stress. This model is supported by the different muscle-type regulation of UCP3 in gilthead sea bream. Importantly, UCP3 protein content in rat glycolytic-fiber muscles is increased either by short- or long-term caloric restriction that facilitates the increased rates of substrate oxidation over energy storage during reduced

nutrient availability (Bevilacqua et al., 2004, 2005). In addition, the chicken UCP3 counterpart (avian-UCP) is quickly up-regulated in the glycolytic *pectoralis* muscle by fasting (Abe et al., 2006). A simple explanation for these apparently contradictory results remains unclear, although species differences in muscle fiber-type composition, thermoregulation, oxidative capacity and energy status can be hypothesized.

Attempts to correlate energy status and UCP3 transcript levels are sometimes confusing but in rodents the overall literature supports the up-regulation of UCP3 by high fat diets (Hoeks et al., 2003; Matsuda et al., 1997) and lipid emulsions (Weigle et al., 1998). Consistent with these findings, recombinant human UCP3 reconstituted into liposomes and assayed for fatty acids shares an enhanced proton efflux inhibited by purine nucleotides (Jabůrek et al., 1999; Žáčková et al., 2003). Moreover, uncoupling UCP3 activity in permeabilized muscle-fibers of 18 h exercised rats becomes evident upon the addition of palmitate or under substrate conditions eliciting substantial rates of H_2O_2 emission (Anderson et al., 2007). Changes in circulating free fatty acids also appear to mediate the nutritional and developmental regulation of UCP3 gene expression in humans and rodents (Boss et al., 1998a; Boss et al., 1998b; Brun et al., 1999; Peterson et al., 2008). Less is known in fish, but as in humans and rodents, the mRNA expression of UCP3 is increased in the skeletal muscle of carp by fasting (Jastroch et al., 2005). This fasting response would be mediated by the increase of circulating free fatty acids, and interestingly we found for the first time in fish a close positive association between circulating levels of free fatty acids and UCP3 mRNA expression in heart and red skeletal muscle. We should, however, point out the lack of response of white skeletal muscle, which in gilthead sea bream highlighted a different muscle-type regulation of UCP3 by ration size when UCP3 transcripts are normalized to COX IV, one of the most common biomarkers of mitochondrial biogenesis and oxidative phosphorylation (Kadenbach et al., 2000; Mitchell et al., 2002).

In the end, there is no doubt about the significant role of UCP3 in muscle metabolism, but the question that arises is whether the increases in UCP3 mRNA expression are accompanied by an increased mitochondrial proton leak. As reviewed by Nabben and Hoeks (2008), isolated mitochondria from mice lacking UCP3 show a significant decrease in S4 respiration (Vidal-Puig et al., 2000) and an increased proton motive force (Gong et al., 2000). However, many studies fail to observe changes in S4 respiration, proton conductance, total oxygen consumption or body mass gain in UCP3 knockout animals. More limited are data about the role of UCP3 in cardiac energy

metabolism, but again contradictory results are found when the role of UCP3 in respiration uncoupling (S3/S4 respiration) is considered (Nabben and Hoeks, 2008). Hence, a common feature is an increased concentration of circulating fatty acids. In that context, Murray et al. (2004) reported that increased cardiac UCP3 levels in heart failure patients are positively correlated to the amount of circulating free fatty acids, which may suggest a primary role for UCP3 in fatty acid metabolism rather than in energy metabolism. In the same way, the compromise of the oxidative phosphorylation efficiency due to UCP2/3 up-regulation would serve in heart (Bo et al., 2008) and skeletal muscle (Jiang et al., 2009) as an antioxidant function to protect the mitochondria during exercise-induced oxidative stress. The increase of uncoupling respiration has been associated to thermal resistance in various species of triplefin fish, and in the last term this might contribute to heart failure at ecologically high temperatures (Hilton et al., 2010). Significantly, the present study pointed out changes in mitochondrial respiration uncoupling in response to varying feed intake. These findings are far from conclusive since the study was reduced to mitochondria from the two extreme groups, but interestingly for the first time in fish is reported an increase in UCP3 transcripts levels, concomitant with enhanced circulating levels of free fatty acids and mitochondrial uncoupling respiration.

In summary, the three muscle types use different compensatory changes in substrate oxidation and energy expenditure to adjust to varying feeding levels. The UCP3 gene is preferentially expressed in the white skeletal muscle and UCP3 mRNA levels in highly oxidative muscles show a significant positive correlation with circulating free fatty acids. In fish glycolytic muscle, metabolic changes in oxidative capacity rather than UCP3 mRNA expression would represent a metabolic advantage in terms of energy preservation. In contrast, in oxidative muscles, the sharply increase in transcript levels of UCP3 would operate as the most immediate response. In a practical sense, UCP3 is emerging as a highly promising muscle biomarker of lipotoxicity and oxidative stress in ectothermic fish. This is of special relevance in aquaculture practice due to the increased use of lipid-energized diets and high feeding schedules to maximize fish growth.

Acknowledgements

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Legends

Figure 1. Effect of ration size on transcript levels of UCP3 in white skeletal muscle (A), red skeletal muscle (B) and heart (C). Data are the mean ± SEM (n = 9). COX IV is used as housekeeping gene and gene expression values in the white skeletal muscle of R_{100} fish were used as reference values in the normalization procedure (RU relative units). Different letters above each bar indicate statistical significant differences among groups (P < 0.05; Student Newman-Keuls test).

Figure 2. (A) Polarographic oxygen electrode trace of freshly isolated heart mitochondria after succinate, ADP, oligomycin and FCCP induction. (B) Oxygen consumption (S3, S4, FCCP uncoupling) and respiratory control ratio (RCR) in fish at the maintenance ratio (R_{70-20} group) are expressed as a percentage over control values (R_{100}, full ration until visual satiety). Data are the mean ± SEM (n = 3-4) of fish processed at the same time. Statistical significant differences between groups are indicated (*P < 0.05; Student's t test).

Table 1. Growth performance, biometric indices, muscle composition and plasma levels of lipids and growth factors in gilthead sea bream fed at three different feeding levels. Full ration until visual satiety (R_{100} group), 70% of satiation (R_{70} group) and 70% of satiation with the last two weeks at 20% of satiation level (R_{70-20} group). Data on fish growth performance are the mean ± SEM of triplicate tanks. Data on biometric indices, muscle composition and circulating parameters are the mean ± SEM of 9 individuals.

	R_{100}	R_{70}	R_{70-20}	P [1]
Initial body mass (g)	17.06 ± 0.07	17.28 ± 0.07	17.10 ± 0.07	*0.138*
Final body mass (g)	72.55 ± 0.20 [a]	59.99 ± 0.16 [b]	48.67 ±0.13 [c]	*<0.001*
DM intake (g/fish)	62.79 ± 2.5 [a]	44.09 ± 1.78 [b]	33.5 ± 1.35 [c]	*0.002*
SGR [2]	1.95 ± 0.01 [a]	1.68 ± 0.0 [b]	1.41 ± 0.01 [c]	*<0.001*
FE [3]	0.89 ± 0.04 [a]	0.97 ± 0.03 [b]	0.11 ± 0.01 [c]	*<0.001*
Viscera (g)	7.10 ± 0.43 [a]	4.78 ± 0.29 [b]	3.27 ± 0.18 [c]	*<0.001*
Mesenteric fat (g)	1.05 ± 0.10 [a]	0.76 ± 0.14 [ab]	0.58 ± 0.06 [b]	*<0.001*
Liver (g)	1.29 ± 0.07 [a]	0.93 ± 0.06 [b]	0.46 ± 0.02 [c]	*<0.001*
VSI (%) [4]	9.49 ± 0.54 [a]	8.02 ± 0.26 [b]	6.69 ± 0.21 [c]	*<0.001*
MFI (%) [5]	1.40 ± 0.13	1.26 ± 0.22	1.19 ± 0.13	*0.677*
HSI (%) [6]	1.72 ± 0.08 [a]	1.55 ± 0.05 [a]	0.94 ± 0.03 [b]	*<0.001*
Muscle moisture (%)	72.44 ± 0.36 [a]	74.75 ± 0.44 [b]	75.51 ± 0.53 [b]	*<0.001*
Muscle fat (%)	6.50 ± 0.40 [a]	4.49 ± 0.36 [b]	3.36 ± 0.24 [c]	*<0.001*
GH (ng/mL)	5.25 ± 1.15 [a]	7.44 ± 1.49 [ab]	11.40 ± 1.30 [b]	*0.003*
IGF-I (ng/mL)	56.03 ± 2.75 [a]	67.75 ± 4.83 [b]	36.95 ± 2.20 [c]	*<0.001*
Triglycerides (mmol/l)	0.89 ± 0.17 [ab]	1.05 ± 0.10 [a]	0.58 ± 0.06 [b]	*0.041*
Cholesterol (mg/dl)	123.13 ± 7.70 [a]	121.13 ± 10.30 [a]	62.81 ± 2.70 [b]	*<0.001*
Free fatty acids (mmol/l)	0.09 ± 0.01 [a]	0.09 ± 0.01 [a]	0.17 ± 0.02 [b]	*0.004*

[1]P values result from one-way analysis of variance. Different superscript letters in each row indicate significant differences among ration size (Student Newman-Keuls test, P<0.05).
[2]Specific growth rate = [100 × (ln final fish mass – ln initial fish mass)]/days.
[3]Feed efficiency = wet mass gain/dry feed intake. Values in the R_{70-20} group were referred to the last two weeks close to maintenance ratio.
[4]Viscerosomatix index = (100 × viscera mass)/fish mass.
[5]Hepatosomatic index = (100 × liver mass)/fish mass.
[6]Mesenteric fat index = (100 × mesenteric fat mass.) /fish mass.

Table 2. Muscle enzymes activities (mUI/mg protein) in gilthead sea bream fed at three different feeding levels. Full ration until visual satiety (R_{100} group), 70% of satiation (R_{70} group) and 70% of satiation with the last two weeks at 20% of satiation level (R_{70-20} group). Data on white and red skeletal muscle tissues are the mean ± SEM of 7-9 individuals. Data on heart are the mean ± SEM (n = 3) of pooled tissue samples from each tank experimental unit (three fish for each pool).

		Glycolysis		Citric acid cycle	B-oxidation
		PK[1]	LDH[2]	CS[3]	HOAD[4]
White Skeletal Muscle	R_{100}	1308.7 ± 71.2^a	4923.5 ± 375.5^a	20.9 ± 0.8^a	3.7 ± 0.3^a
	R_{70}	1493.1 ± 84.7^a	7368.7 ± 417.7^b	22.5 ± 1.1^a	4.1 ± 0.4^a
	R_{70-20}	2870.7 ± 66.4^b	13902.7 ± 530.7^c	39.9 ± 0.8^b	8.0 ± 0.4^b
Red Skeletal Muscle	R_{100}	1826.1 ± 50.4^a	3923.6 ± 82.6^a	145.0 ± 3.0^a	150.6 ± 3.2^a
	R_{70}	1650.0 ± 123.8^a	3874.6 ± 164.5^a	112.1 ± 7.6^b	127.9 ± 4.3^b
	R_{70-20}	2363.0 ± 156.2^b	4465.7 ± 168.7^b	116.7 ± 3.8^b	113.3 ± 3.2^c
Heart	R_{100}	809.9 ± 53.5	3010.3 ± 57.0	245.5 ± 12.6	74.5 ± 15.7
	R_{70}	720.1 ± 79.8	3014.2 ± 158.5	263.3 ± 8.7	72.5 ± 3.0
	R_{70-20}	758.2 ± 140.5	3830.3 ± 327.6	276.4 ± 25.1	104.3 ± 5.3
two-way ANOVA (*P* value)					
Tissue		<0.001	<0.001	<0.001	<0.001
Ration		<0.001	<0.001	0.001	<0.001
Tissue x Ration		<0.001	<0.001	<0.001	<0.001

[1]PK, pyruvate kinase; [2]LDH, lactate dehydrogenase; [3]CS, citrate synthase; [4]HOAD, 3-hydroxyacyl CoA dehydrogenase.
Different superscript letters in each row indicate significant differences among dietary treatments (Student Newman-Keuls test, P<0.05).
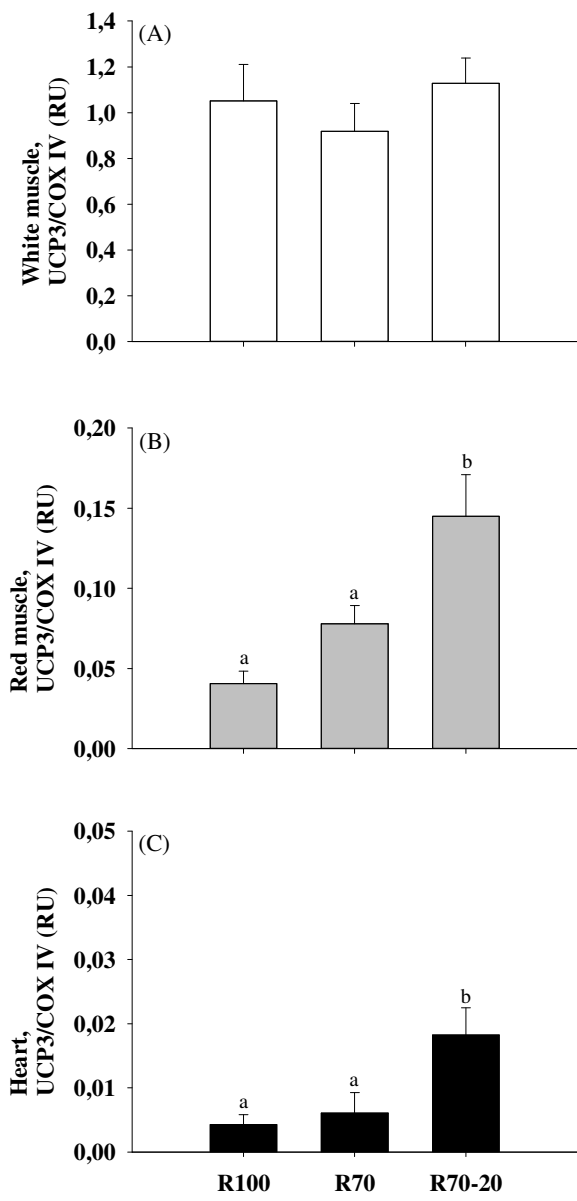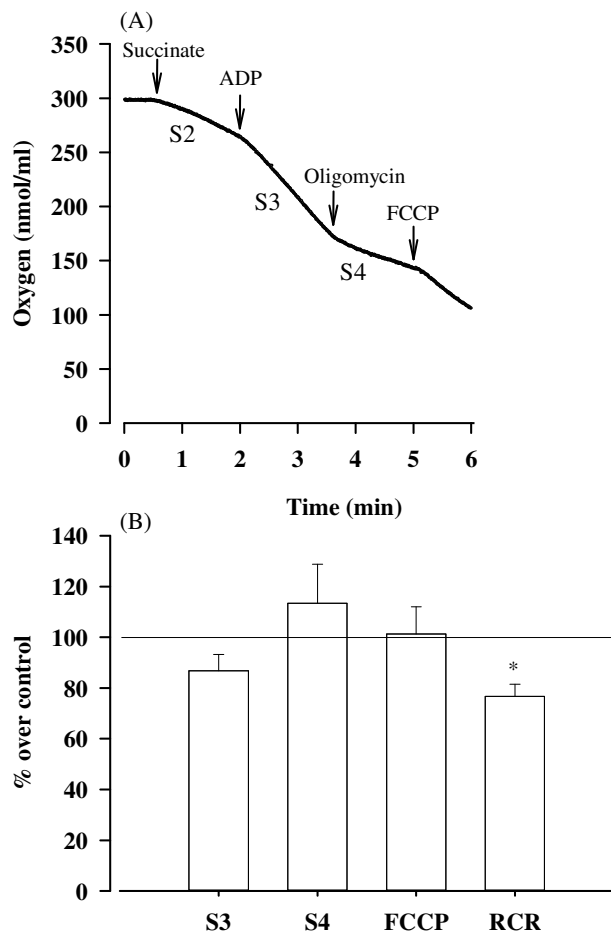
Fig. 1

Fig. 2