Tissue-specific gene expression and functional regulation of uncoupling protein 2 (UCP2) by hypoxia and nutrient availability in gilthead sea bream (*Sparus aurata*): Implications on the physiological significance of UCP1-3 variants

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Running title: UCP2 in gilthead sea bream

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Abstract

The aim of this study was to assess in an integrative manner the physiological regulation of uncoupling protein 2 (UCP2) in gilthead sea bream. A contig of 1325 nucleotides in length with an open reading frame of 307 amino acids was recognized as UCP2 after searches in our transcriptome reference database (http://www.nutrigroup-iats.org/seabreamdb). Gene expression mapping by quantitative real-time PCR revealed a ubiquitous profile that clearly differs from that of UCP1 and UCP3 variants with the greatest abundance in liver and white skeletal muscle, respectively. The greatest abundance of UCP2 transcripts was found in the heart, with a relatively high expression level in blood cells, where UCP1 and UCP3 transcripts were practically undetectable. Functional studies revealed that UCP2 mRNA expression remains either unaltered or up-regulated upon feed restriction in glycolytic (white skeletal muscle) and highly oxidative muscle tissues (heart and red skeletal muscle), respectively. In contrast, exposure to hypoxic conditions (18–19% oxygen saturation) markedly down-regulated the UCP2 mRNA expression in blood cells in a cellular environment with increased haematocrit, blood haemoglobin content, and circulating levels of glucose and lactate, and total plasma antioxidant activity. These findings demonstrated that UCP2 expression is highly regulated at the transcriptional level, arising this UCP variant as an important piece of the complex trade-off between metabolic and redox sensors. This feature would avoid the activation of futile cycles of energy wastage if changes in tissue oxidative and antioxidant metabolic capabilities are able to maintain the production of reactive oxygen species at a low, regulated level.

Keywords: Antioxidant activity, Blood chemistry, Feed restriction, Fish, Hypoxia, Mitochondria, Oxidative capacity, Respiration uncoupling

Introduction

Uncoupling proteins (UCP) are mitochondrial transporters that uncouple oxidative phosphorylation by the net discharge of a proton gradient (Krauss et al. 2005). This protein family is widely distributed in plants and animal phyla, with one UCP ortholog in avian species (avian UCP) and a core group of three mammalian UCP variants (UCP1-3) (Hughes and Criscuolo 2008). Mammalian UCP1 has recently evolved with the acquisition of a thermoregulatory role and a high abundance in the brown adipose tissue of rodents, hibernators, and newborns (Heaton et al. 1978; Nicholls and Locke 1984). Avian UCP (Dridi et al. 2004) and mammalian UCP3 (Nabben and Hoeks 2008) are abundant in heart or skeletal muscle, with enhanced expression when the energy supply exceeds the energy demand of the tissue. In contrast, UCP2 is expressed more ubiquitously and has either a metabolic or immunological role (Fleury et al. 1997; Rupprecht et al. 2012). Thus, the ancestral UCP protein, as well as the modern avian UCP and mammalian UCP2-3, would have a common role as redox sensors that might serve to attenuate the production of the dangerous reactive oxygen species (ROS) (Emre et al. 2007; Hughes and Criscuolo 2008; Rial and Zardoya 2009).

BLAST-searches in the fish genomic databases of European sea bass and fish model species (zebrafish, pufferfish, stickleback, and medaka) revealed that the teleost fish lineage has conserved the UCP1-3 core group with the incorporation of a novel member named UCP3-like (Tine et al. 2012; Jastroch et al. 2005; Tseng et al. 2011). To date, there is no information on the expression of UCP3-like, but a number of studies have addressed the physiological regulation of the UPC1-3 genes in a wide range of fish species. It should be noted that the expression of fish UCP1 is mostly restricted to liver being up-regulated in carp and gilthead sea bream with the increase of lipid influx or lipid deposition rates (Jastroch et al. 2005; Bermejo-Nogales et al. 2010). Likewise, fish UCP3 transcripts are abundantly expressed in skeletal muscle and, again in gilthead sea bream (Bermejo-Nogales et al. 2010; 2011) and carp (Jastroch et al. 2005) UCP3 is up-regulated when the lipid flux towards muscle exceeds the oxidative capacity of the tissue exposed to different environmental stresses. Additionally, studies in zebrafish (Tseng et al. 2011) and rainbow trout (Coulibaly et al. 2006) highlight a ubiquitous UCP2 expression and, interestingly, UCP2 transcripts are up-regulated by xenobiotics in zebrafish (Liu et al. 2008; Jin et al. 2010a; Jin et al. 2010b), marine medaka (Huang et al. 2012), and Chinese carp (Liao et al. 2006). Exposure to elevated water

temperatures also enhances mRNA and protein expression of UCP2 in Antartic fish (Mark et al. 2006), while UCP2 mRNA expression in skeletal muscle is reduced by fasting in rainbow trout (Coulibaly et al. 2006) or the full replacement of fish oil by vegetable oils in Atlantic salmon (Torstensen et al. 2009). Therefore, fish UCP expression and regulation is tissue species-specific, although some overlap and redundancy might occur among UCPs, especially in relation to UCP2.

The UCP2 isoform has yet to be molecularly characterized in gilthead sea bream, a highly cultured fish in all the Mediterranean area. Thus, the first aim of the present study was to identify the gilthead sea bream UCP2 by BLAST-searches in our recently updated transcriptome database (Calduch-Giner et al., 2013) hosted at www.nutrigroup-iats.org/seabreamdb. Secondly, we aimed to map, in an integrative manner, the tissue-specific regulation of UCP2, with emphasis on the functional transcriptional regulation of UCP2 by hypoxia and nutrient availability in heart, skeletal muscle, and blood. The subsequent objective was to determine how UCPs, and UCP2 in particular, contribute to regulate energy metabolism and nutrient utilization to improve feed conversion efficiency in gilthead sea bream aquaculture production.

Materials and methods

Animal care

Juvenile and adult gilthead sea bream were reared in the indoor experimental facilities of Institute Aquaculture Torre de la Sal (IATS) under the natural photoperiod and temperature conditions at IATS latitude (40º 5´ N; 0º 10´ E). Seawater was pumped from ashore (open system) and filtered through a 10 µm filter. The oxygen content of water effluents in standard conditions was always higher than 85% saturation, and unionized ammonia remained below toxic levels (< 0.02 mg/L). At the sampling time, fish were fasted overnight and decapitated under anaesthesia (3-aminobenzoic acid ethyl ester, 100 mg/L). Blood was taken from caudal vessels with heparinized syringes and sampled tissues were rapidly excised and frozen in liquid nitrogen in less than 10 min. All procedures were carried out according to the national (Consejo Superior de Investigaciones Científicas, Institute of Aquaculture Torre de la Sal Review Board) and the current EU legislation on the handling of experimental animals.

Experimental setup and sampling

Tissue screening of UCP2 gene expression was carried out in two-year-old fish fed commercial standard diets (Skretting, Stavanger, Norway). Randomly selected fish were sampled in October and target tissues (liver, white skeletal muscle, red skeletal muscle, heart, intestine, head kidney, spleen, gills, brain, eye, mesenteric adipose tissue, and testis) were stored at -80 ºC until RNA isolation. Blood samples (150 µl) were immediately put into a microtube containing 500 µl of stabilizing lysis solution (REAL TOTAL RNA spin blood kit, Durviz, Valencia, Spain, catalogue no. RBMER12) and stored at -20 ºC until RNA isolation.

Tissue samples to assess the effect of nutritional condition (feeding level) on UCP2 mRNA expression come from a previously published study (Bermejo-Nogales et al. 2011). Briefly, juvenile fish with an initial mean body mass of 17 g 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 comercial diet (D-2 Excel 1P, Skretting) at two different feeding levels twice per day: i) full ration until visual satiety (R_{100} group) and ii) 70% of satiation ration ($R_{70\text{-}20}$ group), with the last two weeks at the maintenance ration (20% of the satiation level). At the end of the trial, nine fish per dietary treatment were sampled for heart ventricles and skeletal muscle tissue collections.

The effect of hypoxia on blood UCP2 mRNA expression was assessed in juvenile fish (250 g, 16 fish per 500 L-tank) allocated in a re-circulatory system equipped with physical and biological filters, and programmable temperature and oxygen devices. Water temperature was fixed at 20–21 ºC. The day of the hypoxia challenge test all fish remained unfed. The water conditions for the control fish (normoxic fish) were keep unchanged, whereas hypoxic fish experienced a gradual decrease in the water oxygen level (unionized ammonia <0.05 mg/L) until it reached 1.3 ppm (18–19% oxygen saturation) (**Fig. 1**). After 1 h of exposure to this hypoxic steady state, eight randomly selected fish per experimental condition (normoxic and hypoxic fish) were anaesthetized and blood was taken from the caudal vessels. One portion of blood was processed for haematocrit and haemoglobin determinations, and total RNA extraction. The remaining blood was centrifuged at 3000 x *g* for 20 min at 4 ºC, and plasma samples were frozen and stored at -20 ºC until biochemical analyses were performed.

Blood biochemistry

Haematocrit was measured using heparinised capillary tubes and centrifuged at 1500 x *g* for 30 min in a Sigma 1–14 centrifuge (Sigma, Germany). Haemoglobin was assessed using HemocueHb 201^+ (Hemocue, Sweden). Plasma glucose levels were measured using the glucose oxidese method (Thermo Electron, Louisville, CO, USA). Blood lactate was measured in deproteinized samples (perchloric acid, 8%) by an enzymatic method based on the use of lactate dehydrogenase (Instruchemie, Delfzijl, The Netherlands). Total antioxidant capacity in plasma samples was measured with a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) adapted to 96-well microplates. This assay relies on the ability of antioxidants in the samples to inhibit the oxidation of ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS radical cation by metamyoglobin, a derivatized form of myo-globin. The capacity of the sample to prevent ABTS oxidation is compared with that of Trolox (water-soluble tocopherol analogue), and is quantified as millimolar Trolox equivalents.

Sequence and phylogenetic analyses

Multiple sequence alignments were performed using the ClustalW software (Larkin et al. 2007), and a UCP phylogenetic tree was constructed on the basis of amino acid differences (p-distance) with the Neighbor Joining (NJ) algorithm (pairwise deletion) using MEGA version 5 (Tamura et al. 2011). A total of 36 sequences of UCP homologues for 22 species were used in the final analyses. Reliability of the tree was assessed by bootstrapping using 1000 bootstrap replications.

Gene expression analyses

Total RNA from tissue samples was extracted using the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA) with a DNase step. The RNA yield was 30–50 µg, with absorbance measures ($A_{260/280}$) of 1.9–2.1. Total RNA from blood was extracted using the REAL TOTAL RNA spin blood kit (Durviz) with a DNase step. The RNA yield was 6 µg, with absorbance measures ($A_{260/280}$) of 1.9–2.1. Reverse transcription (RT) of 500 ng total RNA was performed with random decamers using the High-Capacity cDNA Archive Kit (Applied Biosystems) following the

manufacturer's instructions. Negative control reactions were run without reverse transcriptase and real-time quantitative PCR (qPCR) was carried out with an iCycler IQ Real-time Detection System (Bio-Rad, Hercules, CA, USA) as previously described (Calduch-Giner et al. 2003). Briefly, RT reactions were conveniently diluted and the equivalent of 660 pg of total input RNA was used in a 25 µL volume for each PCR reaction. Each PCR-well contained SYBR Green Master Mix (Bio-Rad) and specific primers for UCP1-3 at a final concentration of 0.9 µM (**Table 1**). β-actin and cytochrome c oxidase subunit 4 isoform 1 (Cox4a) were used as housekeeping genes. The program used for PCR amplification included an initial denaturation step for 3 min at 95 ºC, followed by 40 cycles of denaturation for 15 s at 95 ºC and annealing/extension for 60 s at 60 ºC. The efficiency of PCR reactions for the target and reference genes varied between 95% and 98%. The specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5 ºC/10 s over a temperature range of 55–95 ºC, yielding a single peak for each sample and gene), linearity of serial dilutions of RT reactions, and electrophoresis and sequencing of the PCR amplified products. PCR reactions were performed in triplicate and the fluorescence data acquired during the extension phase were normalized by the delta-delta Ct method with the selected housekeeping gene (Livak and Schmittgen 2001).

Statistical analyses

Data on gene expression and blood biochemistry were analyzed by the Student's t-test. All analyses were performed with the SPSS 17.0 program (SPSS, Inc. Chicago, IL, USA).

Results

Molecular identity and phylogenetic analyses

BLAST-searches with public fish UCP sequences (zebrafish, NM_131176; rainbow trout, DQ295325; stickleback, ENSGACT00000026955) in the gilthead sea bream database (www.nutrigroup-iats.org/seabreamdb) recognized as UCP2 a contig (C2_60770) of four assembled reads. After manual curation, the resulting assembled sequence was 1325 bp in length with an open reading frame of 307 amino acids. The

reliability of the assembled sequence, uploaded to GenBank with the accession number JQ859959, was verified by sequencing a heart PCR amplified product (**Fig. 2**). The best BLASTX hits (E-value = 0) for the uploaded sequence were for UCP2 transcripts and/or UCP2 gene predictions of fugu (XP_003971156), zebra mbuna (XP_004573395), tilapia (XP_003456871), Japanese medaka (XP_004076544), mandarin fish (ACI32422), orange-spotted grouper (ADH04489), common eelpout (AAT99594), Antartic eelpout (AAT99593), rainbow smelt (ACO09773), Atlantic salmon (ACI66881), and rainbow trout (ABC00182).

Amino acid sequence analysis of avian UCP, human UCP1-3, and gilthead sea bream UCP1-3 revealed that they shared the characteristic features of the UCP family (**Fig. 3a**), including three tandem repeats of approximately 100 amino acids in length, each containing two transmembrane domains (α-helices, H1-6), and a long hydrophilic loop that includes the conserved sequence motif of the mitochondrial carrier super-family P[LTV]D[TV][AV]K[VT]R[LFY] (**Fig. 3b**). Sequence alignments also revealed a high degree of amino acid identity and similarity that range between 52–76% and 73–86%, respectively (**Fig. 3c**). Overall, the highest degree of conservation was found between UCP2 and UCP3, showing the consensus sequence a strict conservation of 129 amino acid residues with seven invariant amino acids in the three tandem UCP-repeats (**Fig. 3b**). Among these amino acid residues are included the putative GDP-binding site (Arg86, Arg184, and Arg278 of the gilthead sea bream UCP2 sequence) that is essential for nucleotide interaction.

Phylogenetic analysis of vertebrate UCPs, including gilthead sea bream UCP1-3 sequences, clearly separated the UCP2/UCP3 node from the more recently evolved UCP1 group that was distantly related through vertebrate taxons (**Fig. 4**). The UCP2 and UCP3 nodes are separated according to the present hierarchy of vertebrates, whereas the vertebrate UCP1 group did not form a monophyletic cluster, and fish and marsupial UCP1 sequences resulted in a closer relationship to the UCP2/UCP3 branch than to the eutherian UCP1 node. Conversely, UCP2 from all vertebrates are assigned to the same clade, and the UCP2 branch of nine fish species clearly reflects the hierarchy of the teleost lineage.

Tissue-specific UCP expression

The tissue gene expression of gilthead sea bream UCPs clearly shows that the UCP1 gene was mainly expressed in liver and secondly in the intestine regardless of the housekeeping gene used in the normalization procedure (**Fig. 5a** and **5d**). Detectable levels of UCP2 mRNA were found in almost all of the tissues analyzed, although the highest expression level was found in the highly oxidative cardiac muscle followed by the red skeletal muscle and then white skeletal muscle (**Fig. 5b**). These different gene expression rates tended to be equalized when Cox4a, instead of β-actin, was used as the housekeeping gene, which becomes especially evident for the measurements of UCP2 mRNA expression in the blood and skeletal muscle (**Fig. 5e**). Conversely, UCP3 mRNA expression was mostly restricted to white skeletal muscle followed by red skeletal muscle and heart, and the normalization with the Cox4a housekeeping gene amplified the differences in UCP3 mRNA expression found among muscle tissues (**Fig. 5c** and **5f**).

Feed restriction and UCP gene expression

The transcriptionally mediated effects of feed restriction on UCP2-3 mRNA expression using Cox4a as housekeeping gene are shown in **Fig. 6**. In the glycolytic white skeletal muscle, UCP2 expression was up to 150-fold less than that of UCP3, and feed restriction did not affect either UCP2 or UCP3 mRNA levels (**Fig. 6a**). In red skeletal muscle, the level of UCP2 mRNA was approximately 10-fold lower than that of UCP3 mRNA, and feed restriction decreased in a similar manner to the expression of either UCP2 or UCP3 (**Fig. 6b**). The same pattern was found in the heart, but in this tissue, the expression of UCP2 was consistently greater than that of UCP3 (**Fig. 6c**).

Hypoxic regulation of UCP2 gene expression

As shown in **Table 2**, hypoxia significantly increased haematocrit, blood haemoglobin content, and plasma levels of glucose and lactate. The total plasma antioxidant capacity, measured as Trolox activity, was also significantly increased in hypoxic fish. In parallel, UCP2 mRNA expression was markedly down-regulated in blood cells, with a seven-fold decrease when UCP expression was referred to Cox4a as a housekeeping gene.

Discussion

The core group of UCP1-3 genes is conserved in the teleost fish lineage and, importantly, the present study highlights that the UCP1-3 variants are actively transcribed in gilthead sea bream. Besides, this marine fish is now one of the few animals, including vertebrate tetrapods, for which UCP expression has been assessed completely for the UCP1-3 gene family. Indeed, previous studies in carp evaluated the fasting and thermal regulation of UCP1 and UCP3 expression, but not that of UCP2 (Jastroch et al. 2005). Functional regulation of UCP1 and UCP2 has also been studied after cold (Tseng et al. 2011) or xenobiotic (Liu et al. 2008; Jin et al. 2010a; Jin et al. 2010b) exposure. However, to our knowledge, there is little information on the regulation of UCP3 in these fish species. Likewise, studies on the physiological regulation of UCP transcripts in Chinese carp (Liao et al. 2006), Antartic fish (Mark et al. 2006), rainbow trout (Coulibaly et al. 2006), Atlantic salmon (Torstensen et al. 2009), and marine medaka (Huang et al. 2012) have primarily focused on UCP2. The present study contributes, thereby, to filling the gaps in the literature regarding the physiological regulation of UCPs in fish, clearly showing unique tissue expression patterns for each UCP.

As reported earlier for gilthead sea bream (Bermejo-Nogales et al., 2010) and carp (Jastroch et al. 2005), UCP1 and UCP3 are abundant in liver and skeletal muscle, respectively. In contrast, UCP2 is more ubiquitous, with the greatest abundance in heart and relatively high expression in blood cells, which is not surprising given that fish erythrocytes differ from those in mammals in that they retain both a nucleus and mitochondria after the reticulocyte stage (Tiano et al. 2000). This gene expression pattern of UCP2 is opposite of that observed for UCP3, which shows the greatest expression in the glycolytic white skeletal muscle, specifically in gilthead sea bream (Bermejo-Nogales et al., 2010; 2011). This expression pattern is not the same in other models of mammals (Alán et al. 2009) and fish, such as carp (Jastroch et al. 2005), rainbow trout (Coulibaly et al. 2006), and Atlantic salmon (Torstensen et al. 2009). However, in all cases the highest relative gene expression of UCP2 was found in non-skeletal muscle tissues (spleen, heart, blood cells, ovary, and adipose tissue). One might speculate that UCP2 expression (nuclear-encoded mitochondrial protein) is tissue and species-specific, but it is more likely that it is highly dependent on the metabolic condition and development stage, which in turn, are closely associated with

mitochondria function and biogenesis (Ljubicic et al. 2010). In this sense, we consider that the use of Cox4a, an enzyme subunit of the mitochondria respiratory chain that is commonly used as a mitochondrial loading control gene in proteomic studies (Kim et al. 2004; Soane et al. 2001), could be especially valuable to normalize and/or equalize differences in UCP mRNA expression within and among different animal models.

Less controversial is the molecular identity of the UCPs; the UCP1-3 sequences reported by us for gilthead sea bream in this and a previous study (Bermejo-Nogales et al., 2010) shared a strict conservation of all the structural UCP domains, including the putative GDP-binding site that is essential for nucleotide binding (Modrianský et al. 1997). At the same time, phylogenetic analyses confirmed that sequence divergences among vertebrate UCP1-3 are high enough to unequivocally annotate them through the vertebrate lineage. Thus, the phylogenetic tree constructed herein with representative sequences of eutherians, marsupials, avians, reptilians, and a vast representation of fish species (Cyprinidae, Salmonidae, Gasterosteidae, Serranidae, Percichthyidae, Zoarcidae, and Sparidae) clearly differentiates as separates branches the UCP2 and UCP3 clades of tretrapods and fish within the UCP2-3 node. By contrast, the UCP1 node does not form a monophyletic cluster and the marsupial sequences, annotated as UCP1 (fat-tailed dunnart, EF622232) or UCP2-like (gray short-tailed opossum, XM_001377518; Tasmanian devil, XM_003773182) are more closely related to fish UCP1 rather than to eutherian UCP1 sequences, as previously reported by several authors (Emre et al. 2007; Hughes and Criscuolo 2008; Jastroch et al. 2008). This observation is probably due to the recently acquired thermogenic role of UCP1 in eutherians, whereas the UCP1 of marsupial and fish lineages would be mostly related to the ancestral UCP function. On the other hand, our phylogenetic tree confirms some misleading names in the literature, including the UCP2 sequence of red sea bream (Liang et al. 2003) and Nile tilapia (Wang et al. 2006) that can be recognized as UCP1.

Fasting (Samec et al. 2002) and moderate calorie restriction (Barazzoni et al. 2005) up-regulates UCP2 and UCP3 gene expression in either the soleus or gastrocnemius skeletal muscle of rats. However, in gilthead sea bream, we previously reported (Bermejo-Nogales et al. 2011) that feed restriction up-regulates UCP3 mRNA expression in highly oxidative tissues (heart and skeletal red muscle) with a lack of consistent changes in metabolic enzyme activities. This is the opposite of what is observed in the glycolytic white skeletal muscle, in which UCP3 mRNA expression remained unchanged and enzyme activities of the citric acid cycle (citrate synthase),

fatty acid β-oxidation (3-hydroxyacyl CoA dehydrogenase), and glycolysis (pyruvate kinase and lactate dehydrogenase) were enhanced. These metabolic re-adjustments are considered adaptive in nature and might serve to prevent the lipotoxic effects resulting from an increased supply of metabolic fuels (increased circulating levels of free fatty acids), at the same time avoiding the activation of futile cycles of energy wastage when still exists the capacity to increase the oxidative capacity of the tissue. Intriguingly, the results presented herein indicate that the transcriptional regulation of UCP2 paralleled that of UCP3 in all muscle tissues, though the relative contributions of UCP2 and UCP3 on respiration uncoupling seem to be different in each muscle tissue type. Hence, based on the abundance of UCP2-3 transcripts, the claim by Bermejo-Nogales et al. (2011) that restricted feeding increases respiration uncoupling in isolated heart mitochondria could be attributed to UCP2 rather than to UCP3. This notion is supported by the observation in rodents that the cardiac UCP2 decreases cross-membrane potential and ROS production during exercise and heart failure (Bo et al. 2008; Cabrera et al. 2012). However, functional studies addressing proton conductance by inhibitors or activators of UCP2 remain to be established in fish, and gilthead sea bream in particular.

Hypoxia with a switch from oxidative phosphorylation (OXPHOS) to anaerobic glycolysis is another good model of metabolic re-adjustments, which results in reduced mitochondria oxygen consumption and enhanced NADH production from glycolysis (Frezza et al. 2011). As a result of this, intracellular NAD^+ and NADH levels are highly regulated in mammals, not only by nutrient deprivation or energy consumption, but also by hypoxia, which commonly decreases the NAD^+/NADH ratio (Fulco et al. 2008; Lim et al. 2010). Multiple adaptive responses also occur in fish since they are exposed to varying levels of oxygen concentrations in a seasonal or daily basis. They vary from the responses of cold fish exposed to high oxygen levels to those of cyprinids that can survive in anoxic conditions due to an improved antioxidant-detoxifying system (Lushchak and Bagnyukova 2006). Gilthead sea bream is a temperate fish with a relative high tolerance to anoxia, and its exposure to moderate low levels of water oxygen saturation (30%) enhances liver antioxidant enzymes activities, but does not increase plasma glucose or lactate levels (Pérez-Jiménez et al. 2012). In the present study, fish were exposed to a strong hypoxic condition (18–19% saturation) for a short period of time and, interestingly, most biochemical parameters, including haematocrit, haemoglobin content, circulating glucose, and lactate, as well as plasma antioxidant activity, were significantly increased. This is likely the result of metabolic changes

mediated by oxygen sensors that drive the shift of the redox cellular status of NADH to a more reduced form (Gao and Wolin 2008). One possible mediator is sirtuin 1 (SIRT1), which modulates the cellular responses to hypoxia by sensing the intracellular levels of NAD^+ and deacetylating/inactivating hypoxia-inducible factor 1α in mice (Lim et al. 2010). Given that SIRT1 also governs the expression of UCP2 in murine pancreatic β cells by binding directly to its promoter (Bordone et al. 2006), a close link between redox and oxygen sensors is also suspected in fish. Regardless of the mechanism, in our hypoxic model, it appears likely that the down-regulated expression of UCP2 in blood cells might serve to cope with oxygen deficiencies with a more efficient mitochondrial ATP production and fast recycling of NAD^+ to NADH, as evidenced by the increase in total plasma antioxidant activity.

In summary, a gilthead sea bream nucleotide sequence with a full open reading frame of 307 amino acids has been molecularly characterized and unequivocally annotated as UCP2. The UCP gene expression mapping clearly demonstrates a tissue-specific pattern for each member of the UCP1-3 core group. Additionally, we found that UCP2 is highly regulated at the transcriptional level, remaining unaltered, up-regulated, or down-regulated depending on the tissue and environmental stress stimuli. This reinforces the important role of UCP2 as an important piece of the complex trade-off between metabolic and redox sensors that might prevent the activation of futile cycles of energy wastage (respiration uncoupling) if other adaptive metabolic responses are able to maintain the production of reactive oxygen species at a low, regulated level.

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Table 1. Fordward and reverse primers for real-time PCR.

Gene name	Symbol		Primer sequence
β-actin	ACTB	F	TCC TGC GGA ATC CAT GAG A
		R	GAC GTC GCA CTT CAT GAT GCT
Cytochrome C oxidase subunit IV isoform 1	Cox4a	F	ACC CTG AGT CCA GAG CAG AAG TCC
		R	AGC CAG TGA AGC CGA TGA GAA AGA AC
Uncoupling protein 1	UCP1	F	GCA CAC TAC CCA ACA TCA CAA G
		R	CGC CGA ACG CAG AAA CAA AG
Uncoupling protein 2	UCP2	F	CGG CGG CGT CCT CAG TTG
		R	AAG CAA GTG GTC CCT CTT TGG TCA T
Uncoupling protein 3	UCP3	F	AGG TGC GAC TGG CTG ACG
		R	TTC GGC ATA CAA CCT CTC CAA AG

Table 2. Data on blood biochemistry and blood UCP2 expression in control (normoxic) and hypoxic fish (oxygen saturation = 18-19%). Data are the mean ± SEM of 7-8 animals.

	Normoxia	Hypoxia	*P* [1]
Haematocrit (%)	36.19 ± 1.79	48.81 ± 1.75	*<0.001*
Haemoglobin (g/dL)	10.25 ± 0.39	13.84 ± 0.29	*<0.001*
Plasma glucose (mg/dL)	76.81 ± 10.25	122.52 ± 18.83	*0.026*
Plasma lactate (mg/dL)	10.90 ± 0.66	12.65 ± 0.44	*0.024*
Antioxidant capacity (Trolox mM)	1.79 ± 0.09	2.17 ± 0.11	*0.009*
UCP2 mRNA[2]	1.00 ± 0.22	0.13 ± 0.11	*0.004*

[1]*P* values result from analysis of Student t-test.
[2]Cox4a is used as housekeeping gene and gene expression values in normoxic fish were used as a reference value of 1 in the normalization procedure.

Figure legends

Fig. 1 Water oxygen kinetics in fish exposed to a hypoxic condition with a steady state of 18-19% oxygen saturation (1.3 ppm).

Fig. 2 Schematic representation of the gilthead sea bream UCP2 contig assembly. Forward and reverse PCR primers used for the verification of sequence reliability are shown. Triplet codons encoding for initial methionine and stop codon are indicated.

Fig. 3 Schematic representation of uncoupling proteins (**a**). Amino acid sequence alignments of human UCPs (hUCP1-3), avian UCP (avUCP) and gilthead sea bream UCPs (gsbUCP1-3) (**b**). Tandem repeats contain two transmembrane α-helices (dark blue) and the conserved sequence motif of the mitochondrial carrier superfamily (light blue). Asterisks denote strict conservation, double dot indicates homology and simple dot majority. Lines between blocks help to identify conserved residues highlighted in bold. Highlighted asterisks indicate conserved amino acids of the GDP-binding site. Amino acid sequence identity and similarity (bracket values) among sequences (**c**).

Fig. 4 Phylogenetic tree of vertebrate UCP family constructed on the basis of amino acid differences (p-distance) with the Neighbor Joining algorithm (pairwise deletion) in MEGA version 5. The analysis involved 36 amino acid sequences. BOOTSTRAP support values are indicated at the nodes

Fig. 5 Tissue gene expression pattern of UCP1-3 using either β-actin (**a**, **b**, **c**) or Cox4a (**d**, **e**, **f**) as housekeeping genes. Data in red skeletal muscle UCP2 were used as a reference value of 1 in the normalization procedure.

Fig. 6 Effect of feed restriction on transcript levels of UCP2 (grey bars) and UCP3 (black bars) in white skeletal muscle (**a**), red skeletal muscle (**b**) and heart (**c**). Cox4a is used as housekeeping gene and gene expression values in the red skeletal muscle of R_{100} fish were used as a reference value of 1in the normalization procedure. Data of UCP3 mRNA expression come from a previously published study (Bermejo-Nogales et al. 2011). Data are the mean ± SEM (n=9) and statistically significant differences between R_{100} and $R_{70\text{-}20}$ groups are indicated (*, $P<0.05$; Student t-test).

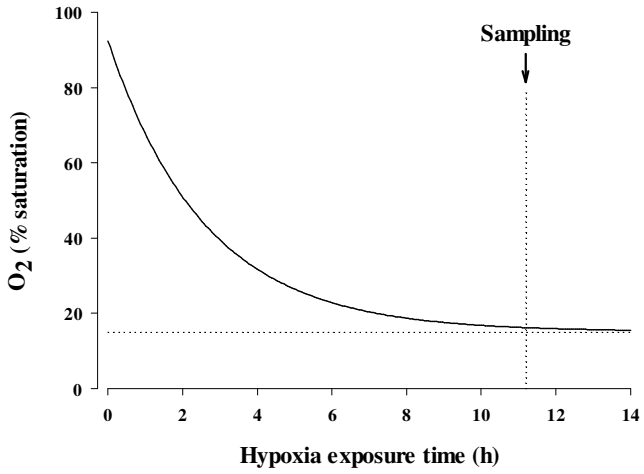
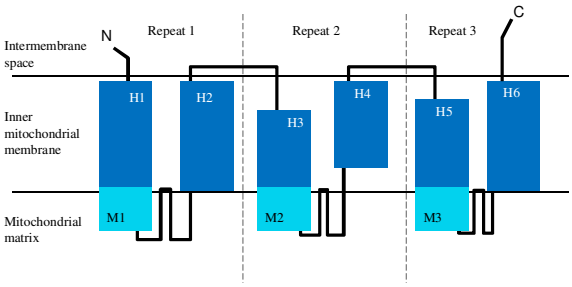

Sampling
100
80
60
40
20
0
O2 (% saturation)
0
2
4
6
8
10
12
14
Hypoxia exposure time (h)

Fig. 1


454 reads
Assembled contig
(C2_60770)
ATG
TAA
1
924
1325
TTG ATC AAA ATA TTT TGG GAA AAA A
AGC ACT TTA TAT AAA CAG TAC ACT TAA ACG

Fig. 2

A



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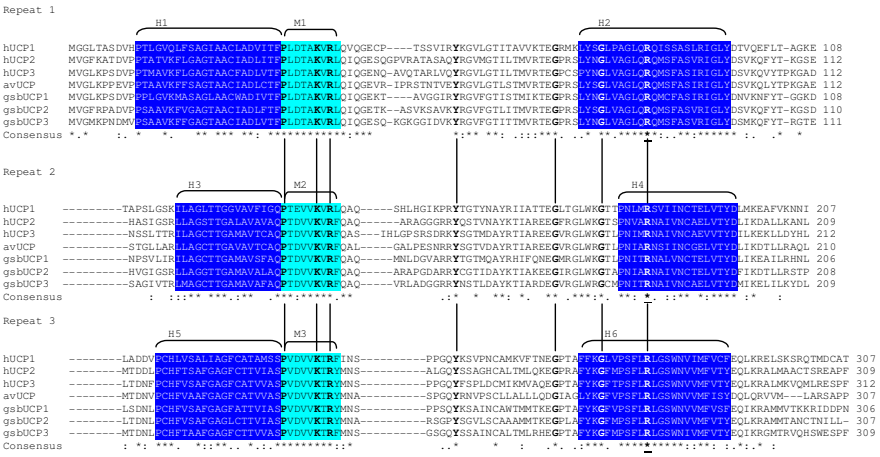

C

	gsbUCP1	gsbUCP2	gsbUCP3
hUCP1	61 (78)	54 (73)	52 (75)
hUCP2	68 (79)	75 (84)	76 (86)
hUCP3	68 (80)	69 (80)	70 (84)
avUCP	63 (78)	69 (82)	67 (83)
gsbUCP1		67 (79)	67 (81)
gsbUCP3		71 (81)	

Fig. 3

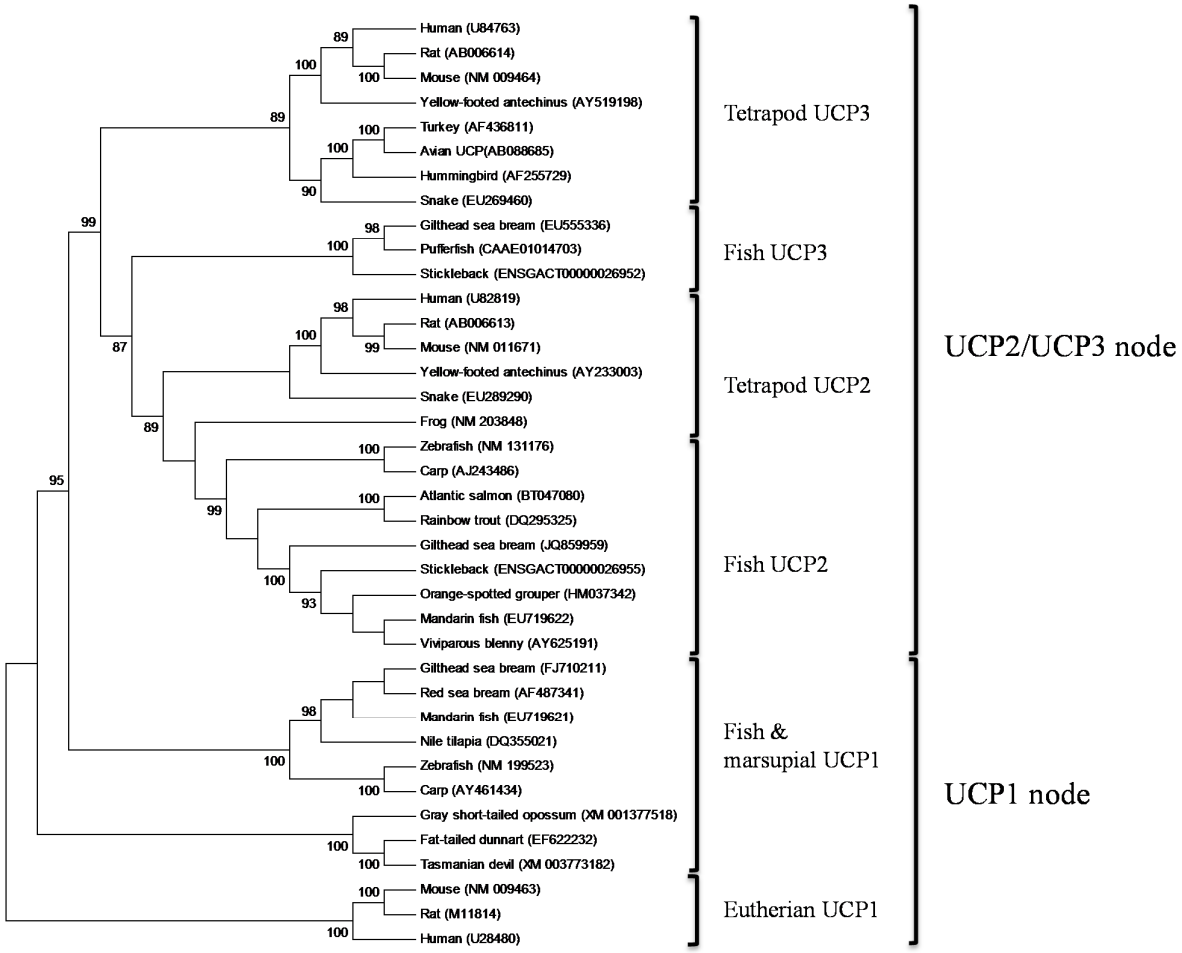
Human (U84763)
Rat (AB006614)
Mouse (NM 009464)
Yellow-footed antechinus (AY519198)
Turkey (AF436811)
Avian UCP(AB088685)
Hummingbird (AF255729)
Snake (EU269460)
Gilthead sea bream (EU555336)
Pufferfish (CAAE01014703)
Stickleback (ENSGACT00000026952)
Human (U82819)
Rat (AB006613)
Mouse (NM 011671)
Yellow-footed antechinus (AY233003)
Snake (EU289290)
Frog (NM 203848)
Zebrafish (NM 131176)
Carp (AJ243486)
Atlantic salmon (BT047080)
Rainbow trout (DQ295325)
Gilthead sea bream (JQ859959)
Stickleback (ENSGACT00000026955)
Orange-spotted grouper (HM037342)
Mandarin fish (EU719622)
Viviparous blenny (AY625191)
Gilthead sea bream (FJ710211)
Red sea bream (AF487341)
Mandarin fish (EU719621)
Nile tilapia (DQ355021)
Zebrafish (NM 199523)
Carp (AY461434)
Gray short-tailed opossum (XM 001377518)
Fat-tailed dunnart (EF622232)
Tasmanian devil (XM 003773182)
Mouse (NM 009463)
Rat (M11814)
Human (U28480)
Tetrapod UCP3
Fish UCP3
Tetrapod UCP2
Fish UCP2
Fish & marsupial UCP1
Eutherian UCP1
UCP2/UCP3 node
UCP1 node

Fig. 4

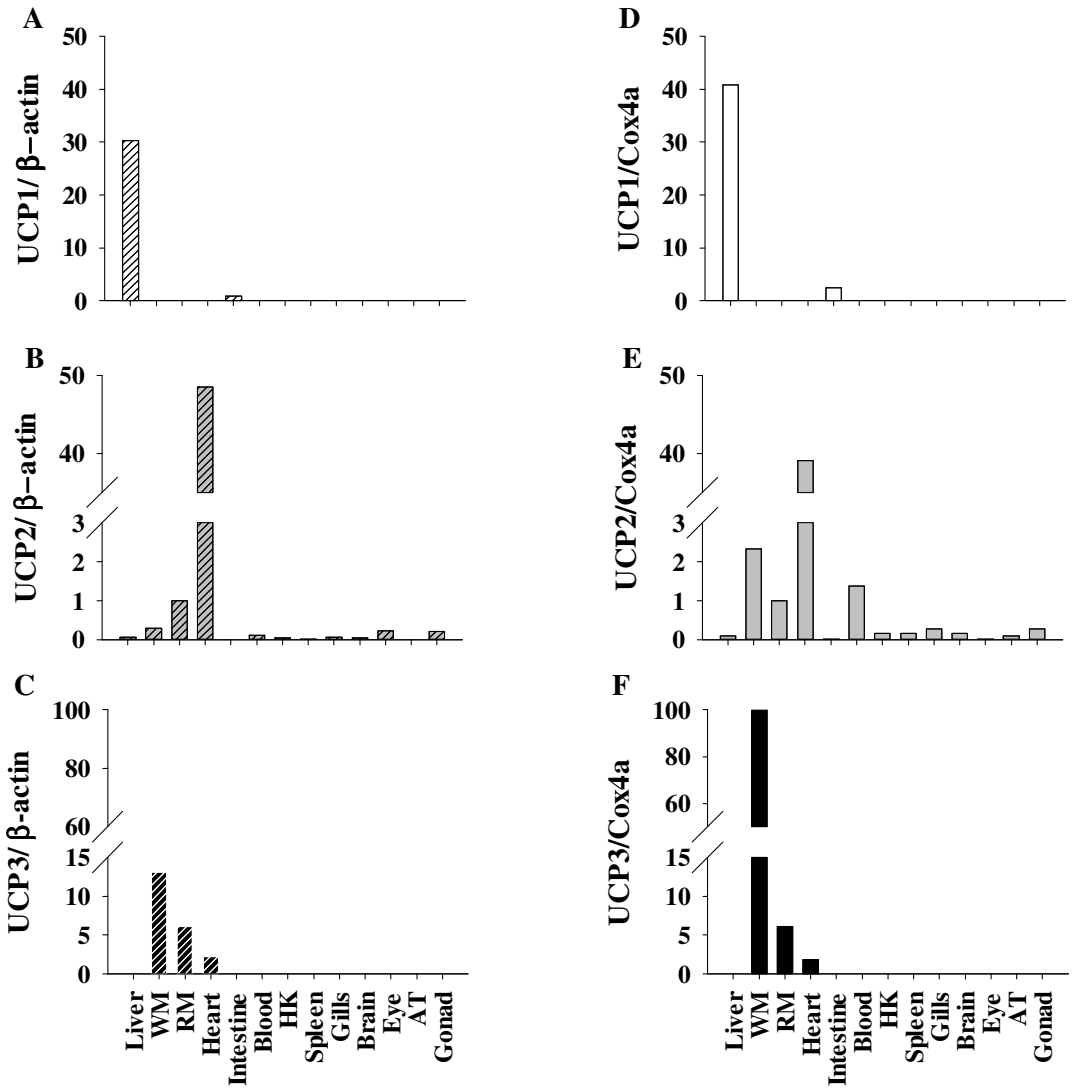
A
UCP1/ β–actin
50
40
30
20
10
0
D
UCP1/Cox4a
50
40
30
20
10
0
B
UCP2/ β–actin
50
40
3
2
1
0
E
UCP2/Cox4a
50
40
3
2
1
0
C
UCP3/ β-actin
100
80
60
15
10
5
0
F
UCP3/Cox4a
100
80
60
15
10
5
0
Liver
WM
RM
Heart
Intestine
Blood
HK
Spleen
Gills
Brain
Eye
AT
Gonad

Fig. 5

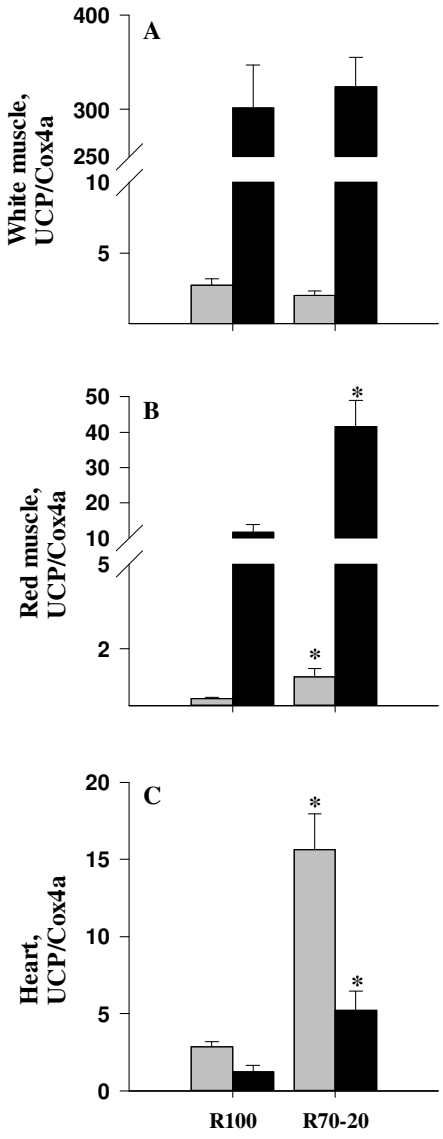
A
White muscle, UCP/Cox4a
400
300
250
10
5
B
Red muscle, UCP/Cox4a
50
40
30
20
10
5
2
*
*
C
Heart, UCP/Cox4a
20
15
10
5
0
*
*
R100
R70-20

Fig. 6