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ORIGINAL PAPER

Gene expression survey of mitochondrial uncoupling proteins (UCP1/UCP3) in gilthead sea bream (*Sparus aurata* L.)

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Abstract The aim of this work is to underline the biological significance of mitochondrial uncoupling proteins (UCPs) in ectothermic fish using the gilthead sea bream (*Sparus aurata* L.) as an experimental model. A contig of 1,990 bp in length was recognized as a UCP1 ortholog after initial searches in the gilthead sea bream AQUAFIRST database (http://www.sigenae.org/aquafirst). Additional searches were performed in skeletal muscle by RT-PCR, and the amplified PCR product was recognized as UCP3 after sequence completion by 5′- and 3′RACE. UCP1 expression was mostly detected in liver, whereas UCP3 transcripts were only found in skeletal and cardiac muscle fibres (white skeletal muscle > red skeletal muscle > heart). Specific gene regulation of UCP1 (liver) and UCP3 (white skeletal muscle) was addressed in physiological models of age, seasonal growth and energy-metabolic unbalances. Both the increase in energy demand (stress confinement) and the reduction in energy supply during adaptive cold response in winter down-regulated UCP1 expression. Conversely, transcript levels of UCP3 were higher with age, seasonal fattening and dietary deficiencies in essential fatty acids leading to the increase in fatty acid flux towards the muscle. This close association between UCP1 and UCP3 with the oxidative and metabolic tissue status is perhaps directly related to the ancestral protein UCP function, and allows the use of UCPs as lipotoxicity markers in ectothermic fish.

Keywords Age · Season · Fatty acids · Stress · Lipotoxicity

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Introduction

Uncoupling proteins (UCP) are mitochondrial transporters that uncouple oxidative phosphorylation by the net discharge of the proton gradient (Krauss et al. 2005). This protein family is widely distributed in plants and animal phyla, but cellular mechanisms and biological significance remain unclear and vary among the different UCP family members. Thus, a core group of three UCP genes has been recognized in mammals, but contribution to basal proton conductance (Parker et al. 2009) and a high nucleotide-sensitive proton conductance, strongly inhibited by purine nucleotides and activated by low concentrations of fatty acids (Locke et al. 1982; Rial et al. 1983), have only been reported for UCP1. Tissue distribution and relative abundance also vary among UCP family members. Thus, UCP1 is present in high concentrations (up to 10% of membrane protein) in the brown adipose tissue (BAT) of rodents, hibernators and newborns (Heaton et al. 1978), and has a well-documented role in non-shivering thermogenesis (Nicholls and Locke 1984). By contrast, closely related UCP paralogues are expressed ubiquitously (UCP2) or more specifically in heart and skeletal muscle (UCP3) at lower concentrations (Harper et al. 2002; Pecqueur et al. 2001). Hence, the physiological function of UCP2-3 remains currently under debate, but they share a common role as redox-sensors to attenuate the production of reactive oxygen species (ROS) (Krauss et al. 2005).

In chickens, cold exposure and feeding influence the gene expression level of avian UCP that shares 71, 70 and 55% amino acid identity with human UCP3, UCP2 and UCP1, respectively (Raimbault et al. 2001; Abe et al. 2006). In contrast to the pluripotent avian UCP, the three members of the core UCP family (UCP1-3) have been retained in ectothermic fish (Stuart et al. 1999; Jastroch et al. 2005). However, studies in this vertebrate lineage are not representative of all fish taxa and the primary goal of this work is to gain more understanding on the physiological regulation of UCPs in ectothermic and carnivorous fish, using gilthead sea bream (*Sparidae* family) widely cultured in all the Mediterranean area as experimental model. Initial searches in the gilthead sea bream cDNA database (http://www.sigenae.org/aquafirst) of the European AQUAFIRST project recognized a contig of 1,990 bp in length as a UCP1 ortholog. Additional UCP-searches were performed by means of RT-PCR, and amplified cDNA fragments in skeletal muscle were unequivocally recognized as UCP3. The study was then focused on the gene expression analysis of UCP1 and UCP3 in physiological models of age, seasonal growth and energy-metabolic unbalances induced either by crowding stress or nutritional deficiencies in essential fatty acids.

Materials and methods

Animal care and sampling

Juveniles and adults of gilthead sea bream (*Sparus aurata* L.) 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 (40°5′N; 0°10′E). Seawater was pumped from ashore (open system) and 10 μm filtered. The oxygen content of water effluents was always higher than 85% saturation, and unionized ammonia remained below toxic levels (<0.02 mg/l). Except where indicated, fish were fed on a commercial diet (Proaqua, Palencia, Spain) containing 47% protein and 21% lipid. At the sampling time, fish were fasted overnight and decapitated under anaesthesia (3-aminobenzoic acid ethyl ester, 100 mg/l). Targeted tissues were rapidly excised, frozen in liquid nitrogen and stored at −80°C until RNA extraction and analysis were performed. All procedures were carried out according to the national and institutional regulations on experimental animal handling (IATS-CSIC Review Board).

Experimental setup

Tissue screening of UCP1 and UCP3 gene expression was carried out in 2-year-old fish. Two randomly selected fish were sampled in October and targeted tissues (liver, intestine, white skeletal muscle, red skeletal muscle, heart, mesenteric adipose tissue, head kidney, gills, spleen, eye, testis, brain) were excised and nitrogen frozen in less than 10 min.

The age-related effects on UCP expression were monitored during the summer growth spurt in 1- and 3-year-old fish reared from fingerlings in the experimental facilities of IATS. At the sampling time (July), six growing fish from each age group were sampled for collection of liver and white skeletal muscle.

Tissue samples for analysis of the effect of nutritional background (dietary lipid source) on UCP expression come from a previously published dietary trial (Benedito-Palos et al. 2007). Briefly, juvenile fish of 16-g initial body weight were fed to visual satiety from May to mid-August with plant protein diets containing either fish oil (FO diet) or a blend of vegetable oils replacing fish-oils by 100% (VO diet). At the end of the trial period, eight randomly selected fish per dietary treatment were sampled for liver and white skeletal muscle collections.

Tissue samples for analysis of the effect of high stocking density (a common aquaculture stressor) on UCP expression come from a previously published pair-fed study (Bermejo-Nogales et al. 2007). Briefly, juvenile fish of 16–20-g initial body weight were randomly allocated into six 90 L-tanks. One triplicate group was reared at high density (45–50 kg/m^3; 80 fish per tank) and fed to visual satiety (HD group). The reference group (LD group) was reared at the optimum density (8–10 kg/m^3; 20 fish per tank) and fed with the same rations as the HD group. Three weeks later, nine fish per experimental condition were sampled for liver and white skeletal muscle collection.

The time course of changes in liver and muscle UCP expression was analysed in juvenile fish (1-year-old fish) fed from May to January to visual satiety. At the end of the growth trial (cold season), fish feed intake was naturally reduced to the maintenance ratio, and 8–9 fish were randomly selected for sampling of liver and white skeletal muscle. Additional tissue samples were taken in mid summer (July, growth period) and early autumn (October, fattening period).

RNA extraction and RT procedure

Total RNA extraction from target tissues was performed with the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA). Briefly, tissue samples were homogenized at a ratio of 25 mg/ml with a guanidine-detergent lysis reagent. The reaction mixture was treated with protease K, and RNA purification was achieved by passing the tissue lysate (0.4–0.5 ml) through a purification tray containing an application-specific membrane. Wash solutions containing DNase were applied, and

total RNA was eluted into a 96-well PCR plate. The RNA yield was 30–50 μg with absorbance measures ($A_{260/280}$) of 1.9–2.1.

Reverse transcription (RT) with random decamers was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems). For this purpose, 500 ng total RNA were reverse transcribed with a final volume of 100 μl. RT reactions were incubated for 10 min at 25°C and 2 h at 37°C. Negative control reactions were run without reverse transcriptase.

RT-PCR approach for UCP3 amplification

Degenerated primers for gilthead sea bream UCP3 were designed on the basis of available sequences in humans, pufferfish and carp. Forward primer (5′-CTG CAT AGC TGA CCT CVT CAC YTT YCC A) was located 68 nucleotides downstream from the initial methionine; reverse primer (5′-CTA GAC MAY RTT CCA SGA KCC CAG) was located 69 nucleotides upstream from the stop codon. PCR amplification was performed with 2 μl of RT reactions from white skeletal muscle in a total volume of 50 μl and two units of Platinum Taq DNA polymerase (Invitrogen, Gaithersburg, MD, USA). Thirty-five cycles were carried out with denaturation at 94°C for 30 s, annealing at 52°C for 60 s and extension at 72°C for 90 s.

Rapid amplification of the 3′-end (3′RACE) was performed according to the manufacturer's instructions (Clontech, Palo Alto, CA, USA). Briefly, 500 ng of total RNA (white skeletal muscle) were reverse transcribed with oligo $(dT)_{30}$ coupled to an anchor cDNA sequence. Amplification was performed with a specific forward primer (5′-GTG CGA CTG GCT GAC GGC GGG AGG AGG); the reverse primer corresponded to the anchor sequence. This PCR consisted of 30 cycles of 60 s at 94°C, 90 s at 62°C and 3 min at 72°C. A nested PCR (35 cycles of 60 s at 94°C, 90 s at 60°C and 3 min at 72°C) was performed with an inner forward primer (5′-AAA CAC GGT TCA TGA ACT CAG GGT CTG) and 1 μl of the first PCR reaction.

Rapid amplification of the 5′-end (5′RACE) was performed according to the manufacturer's instructions (Invitrogen). Briefly, 4 μg of total RNA (white skeletal muscle) were reverse transcribed with a specific reverse primer (5′-GTT CGG CAT ACA ACC TC). After RNAse H treatment and cDNA purification, an oligo (dC) tail was added at the 5′-end. The resulting product was PCR amplified with a universal primer containing a poly-dG sequence and specific oligonucleotides for the first and second PCR, respectively (5′-CCA GCG TGC TGT TGT ACC TCC TCC; 5′-ATC GAT GCC ACC TTT GCC TTT CTG A).

All amplified PCR products were gel-extracted and sequenced by the deoxy chain termination method (ABI PRISM dRhodamine terminator cycle sequencing kit, Perkin-Elmer, Wellesley, MA, USA). A BLAST-X search strategy and multiple sequence alignments were carried out with ClustalW to compare the identity of amplified products.

UCP expression analyses

Real-time PCR was performed using an iCycler IQ Real-time Detection System (Bio-Rad, Hercules, CA, USA) as previously described (Calduch-Giner et al. 2003). Briefly, diluted RT reactions were conveniently used for PCR reactions in 25-μl volume. Each PCR-well contained a SYBR Green Master Mix (Bio-Rad) and specific primers for UCP1 (forward, 5′-GCA CAC TAC CCA ACA TCA CAA G; reverse, 5′-CGC CGA ACG CAG AAA CAA AG) and UCP3 (forward, 5′-AGG TGC GAC TGG CTG ACG; reverse, 5′-TTC GGC ATA CAA CCT CTC CAA AG) were used at a final concentration of 0.9 μM to obtain an amplicon of 137 and 108 bp in length for UCP1 and UCP3, respectively. β-actin was used as the housekeeping gene and the efficiency of PCR reactions for the target and the reference genes varied between 95 and 98%. The dynamic range of standard curves (serial dilutions of RT-PCR reactions) spanned five orders of magnitude, and the amount of product in a particular sample was determined by interpolation of the cycle threshold (Ct) value. The specificity of reaction was verified by analysis of melting curves and by electrophoresis and sequencing of PCR amplified products. Reactions were performed in triplicate and the fluorescence data acquired during the extension phase was normalized to β-actin by the delta–delta method (Livak and Schmittgen 2001).

Statistics

Data on growth and UCP gene expression is represented as the mean ± SE. The effect of age, nutritional background and stocking density on UCP expression was analysed by the Student's t test. The in-season regulation of UCP expression was evaluated by ANOVA on ranks followed by the Tukey test for multiple comparisons. All statistical analyses were performed with the SPSS 13.0 program (SPSS, Inc, Chicago, IL, USA).

Results

Cloning of gilthead sea bream UCPs

The search for gilthead sea bream UCPs in the AQUAFIRST database (http://www.sigenae.org/aquafirst) unequivocally recognized a contig of five clones in depth and 1,990 bp in length as UCP1. Best BLAST-X hit with

Table 1 Identity and similarity (brackets values) of gilthead sea bream UCP1 (FJ10211) and UCP3 (EU555336) with fish UCP orthologues and paralogues available in public databases

	Accession number	Gilthead sea bream	
		UCP1	UCP3
UCP1			
Pufferfish	CR640550	93 (97)	70 (83)
Carp	AY461434	86 (93)	69 (83)
Zebrafish	NM_199523	86 (92)	69 (84)
Red sea bream[a]	AF487341	97 (99)	74 (87)
Stickleback[a]	ENSGACT00000022876	87 (93)	68 (82)
UCP3			
Pufferfish	CAAE01014703	69 (83)	92 (96)
Stickleback	ENSGACT00000026952	67 (82)	87 (93)
Carp[a]	AY505343	66 (85)	83 (94)

[a] Partial UCP sequences

E value of 1e-150 was for carp UCP1 (AY461434). The sequence encodes for an open reading frame of 306 amino acids introduced in GenBank with the accession number FJ710211. Additional search by RT-PCR for UCP3 yielded in the white skeletal muscle a PCR product of expected size (766 bp). The nucleotide sequence was completed by means of 3′- and 5′RACE and a cDNA fragment of 1,590 bp in length with an open reading frame of 309 amino acids, a 5′UTR of 245 bp and a 3′UTR of 415 bp were obtained and introduced in GenBank with the accession number EU555336. BLAST-X searches produced significant alignments (*E* values >7e-135) with lamprey, zebrafish and pufferfish UCP3. The best hits with *E* values for endothermic vertebrates were 1e-127 for dog (AB022020) and 3e-127 for the marsupial yellow-footed antechinus (AY519198).

Sequence alignments with fish UCP orthologues closely related gilthead sea bream UCP1 to carp and pufferfish UCP1 sequences (86–93% identity, 93–97% similarity). Likewise, gilthead sea bream UCP3 is closely related to stickleback and pufferfish UCP3 sequences with 87–92% identity and 93–96% similarity, respectively. These sequence identities decreased until 66–69% when comparisons were made among fish UCP paralogues already available in public databases as complete sequences (Table 1). Sequence alignments also revealed a high degree of amino acid identity (68%) and similarity (82%) between gilthead sea bream UCP1 and UCP3 (Fig. 1). Both the sequences shared the characteristic features of the UCP1-3 family with three tandem repeats of approximately 100 amino acids, with each domain containing two transmembrane domains and a long hydrophilic loop that includes the conserved sequence motif of the mitochondrial carrier superfamily P[LTV]D[TV][AV]K[VT]R[LFY]. The consensus sequence for human UCP1-3, avian UCP and gilthead sea bream UCPs showed a strict conservation of 132 amino acid residues with seven invariant amino acids in the three tandem UCP-repeats. The amino acids of the putative GDP-binding site essential for nucleotide interaction were strictly preserved in UCP1 (Arg83, Arg181, Arg276) and UCP3 (Arg86, Arg184, Arg279) gilthead sea bream sequences.

Gene expression analyses

Tissue-expression pattern of UCP1 and UCP3 is shown in Fig. 2. UCP1 was primarily expressed in liver and secondly in the intestine at tenfold lower levels (Fig. 2a). No detectable expression of UCP1 was found in any other tissue analysed (skeletal and cardiac muscles, mesenteric adipose tissue, head kidney, gills, spleen, eye, gonad and brain).The expression of UCP3 was also tissue-specific and was detected in skeletal and cardiac muscles (Fig. 2b). Comparisons of gene expression levels revealed that the abundance of UCP3 mRNA was highest in glycolytic muscle (white skeletal muscle), 2–3-fold lower in oxidative muscle (red skeletal muscle) and 5–6-fold lower in cardiac muscle. No detectable expression of UCP3 was found in any other tissue analysed (liver, intestine, mesenteric adipose tissue, head kidney, gills, spleen, eye, gonad and brain). Attention was then focused on gene expression analysis in liver and white skeletal muscle as the most important targets of UCP1 and UCP3 in gilthead sea bream. With this aim, we first analysed the expression of UCPs in the model of age. Interestingly, the abundance of hepatic UCP1 mRNA in 3-year-old fish (mature fish) was twofold higher than in 1-year-old fish (immature fish) (Fig. 3a). Similarly, the abundance of UCP3 mRNA was highest in the oldest fish and 4–5-fold lower in 1-year-old fish (Fig. 3b).

The tissue-specific expression of UCPs was altered by dietary manipulation and both hepatic UCP1 and white skeletal muscle UCP3 were up-regulated after the total replacement of fish oil with vegetable oils (Fig. 4a, b). However, the magnitude of change was higher for UCP3 (fourfold change) than for UCP1 transcript levels, which presented a slight but not significant increase in fish fed on the VO diet. Fish-rearing density did not alter the expression of UCP3 in white skeletal muscle (Fig. 5b), whereas a reduced but significant decrease was found for hepatic transcript levels of UCP1 in the HD group (Fig. 5a).

The expression of UCP1 and UCP3 was regulated on a seasonal basis. Thus, over the course of the seasonal trial, feed intake increased continuously from spring to early summer (growth period), reached a plateau during the second half of the summer (growth and fattening period) and decreased progressively during the autumn and winter period (overwintering period) (Fig. 6a, b). In this scenario, the abundance of hepatic UCP1 mRNA was highest in

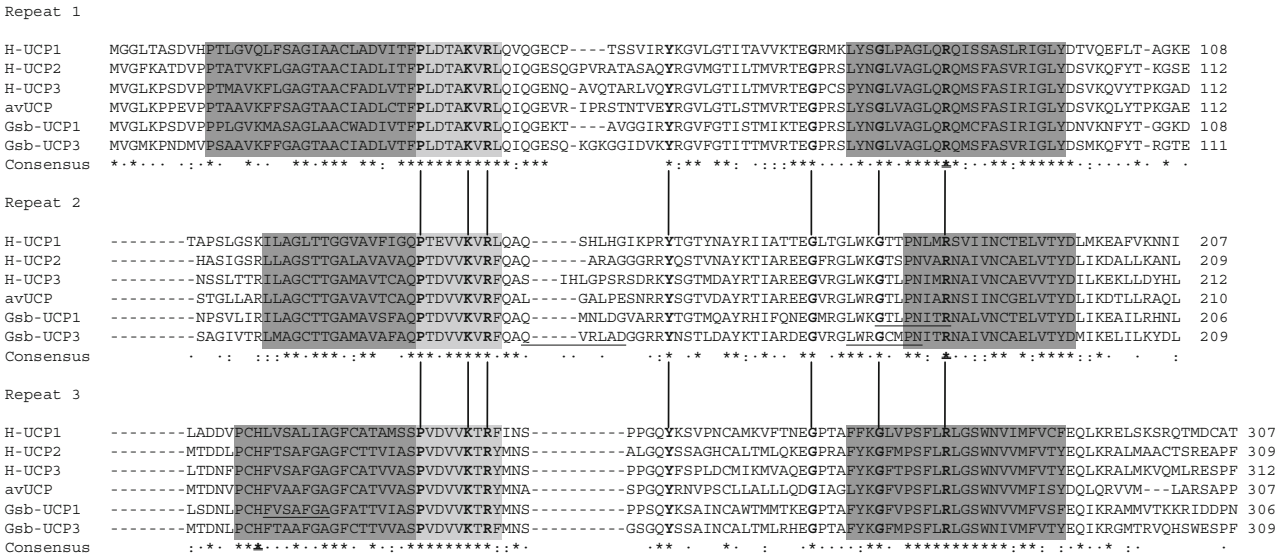

Fig. 1 Amino acid alignment of human UCP1-3 (H-UCP-1, H-UCP2, H-UCP3), avian UCP (avUCP), gilthead sea bream UCP1 (Gsb-UCP1) and gilthead sea bream UCP3 (Gsb-UCP3). Sequences are arranged to identify amino acid residues conserved in the three repeats and UCP homologues. Tandem repeats contain two transmembrane α-helices (*Dark grey*) and the conserved sequence motif of the mitochondrial carrier superfamily (*Light grey*). *Asterisks* denote strict conservation, while *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 essential for nucleotide interaction. Position of specific primers for real-time PCR is *underlined*

summer and early autumn and 2–3-fold lower in early winter (Fig. 6c). In the white skeletal muscle, the abundance of UCP3 mRNA was highest in autumn and lowest in summer in coincidence with fattening and growth periods, respectively. Intermediate values were reported in winter with the maintenance of feeding ratios (Fig. 6d).

Discussion

Gilthead sea bream belongs to the *Sparidae* family, *Perciformes* order, and this is the first report analysing the tissue-specific regulation of UCPs in this important group of teleosts. Mapping of UCP sequences in vertebrate taxa contributes to highlight invariant amino acids as well as the shared-derived amino acids of UCP family. Mutagenesis experiments have demonstrated that amino acids of the putative GDP-binding site are essential for nucleotide binding (Modriansky et al. 1997) and these amino acids are strictly preserved in gilthead sea bream sequences. Functional experimental evidence in cold acclimated carp indicates that purine nucleotides prevents fatty acid inducible proton conductance in isolated liver mitochondria (Jastroch et al. 2007). However, in both fish and higher vertebrates, the competition between inhibitors (GDP) and activators (fatty acids, pH, ROS, HNE) of UCP function is still unclear and more complex than initially envisaged (Krauss et al. 2005).

Phylogeny studies on available literature provide evidence for a long UCP1 branch that closely relates fish and marsupials to the large distanced eutherians (Hughes et al. 2009). Since adaptive non-shivering thermogenesis is not fully developed in marsupials, this divergent evolution can be interpreted as the acquisition of novel thermogenic functions in BAT (independently of shivering and locomotor activity) just after the split of eutherians from marsupial lineages ~150 million years ago (Jastroch et al. 2008). However, it seems that most UCP1 orthologues are specifically expressed in metabolically active tissues with high lipid contents, which can be converted into fat-burning machines during adaptive thermogenesis and excessive feed intake. This is inferred from obesity models in rodents, which are in most cases associated with low levels and activity of UCP1 in BAT (Kozak and Koza 1999). Moreover, UCP1 deficiencies increase the ageing susceptibility of mice to diet-induced obesity (Kontani et al. 2005), whereas ectopical expression of UCP1 in genetically obese mice decreases adiposity and increases temperature and metabolic rates (Gates et al. 2007). Less clear is the role of UCP1 in human obesity (Gonzalez-Barroso et al. 2000), but even in marsupials overexpression of UCP1 in the archetypal BAT can be of relevance for the whole-body energy balance (Jastroch et al. 2008). In fish, the number of analysed species is little representative of the phyla, but both this (gilthead sea bream study/superorder Acanthopterygii) and earlier studies in carp (superorder Ostariophysi) (Jastroch et al. 2005) support a high expression level of UCP1 in liver and notably not in cells of adipose tissue. Meanwhile, low but detectable UCP1 expression was found in the intestine,

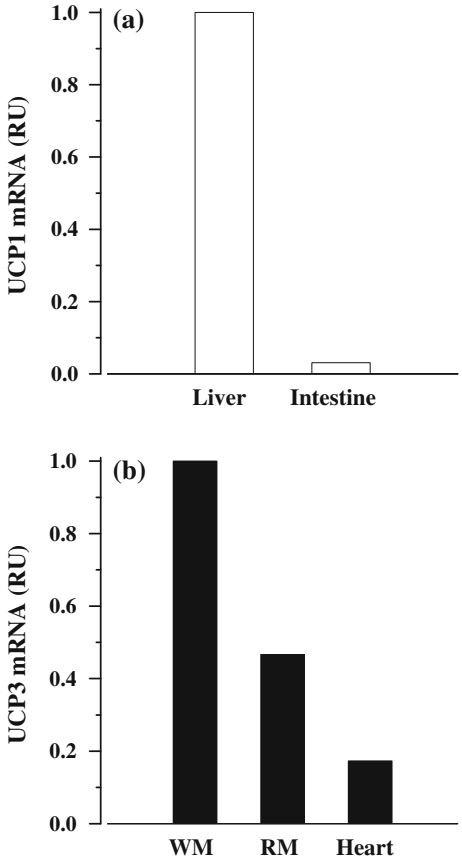

Fig. 2 Representative tissue-expression pattern of UCP1 (**a**) and UCP3 (**b**) in 2-year-old fish. White skeletal muscle (*WM*), red skeletal muscle (*RM*). UCP1 was not detectable in skeletal and cardiac muscles, mesenteric adipose tissue, head kidney, gills, spleen, eye, gonad and brain. UCP3 was not detectable in liver, intestine, mesenteric adipose tissue, head kidney, gills, spleen, eye, gonad and brain. Data in liver and WM were used as references values in the normalization procedure for UCP1 and UCP3, respectively (*RU* relative units)

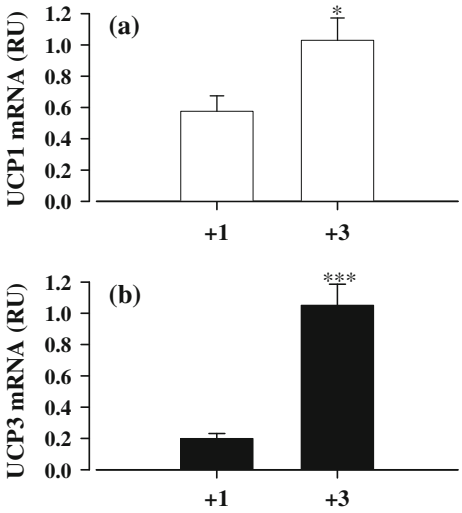

Fig. 3 Age-related changes in transcript levels of UCP1 in liver (**a**) and UCP3 in white skeletal muscle (**b**). Values are the mean ± SEM of six animals. UCP1 and UCP3 data in 3-year-old fish were used as reference values in the normalization procedure (*RU* relative units). Statistically significant differences between groups are indicated (*$P < 0.05$, ***$P < 0.001$; Student's *t* test)

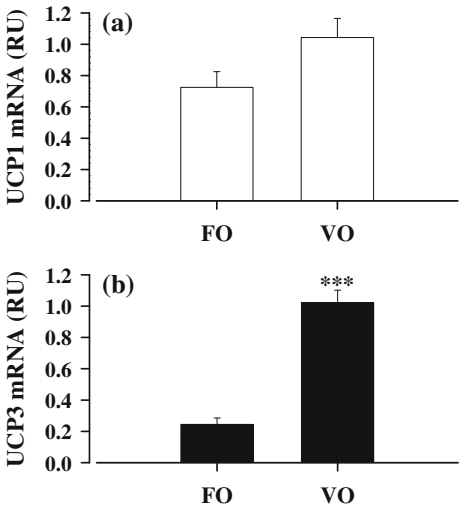

Fig. 4 Transcript levels of hepatic UCP1 (**a**) and white skeletal muscle UCP3 (**b**) in fish fed fish oil (FO diet) or a blend of vegetable oils replacing fish-oils by 100% (VO diet). Data are represented as mean ± SEM ($n = 8$). UCP1 and UCP3 data in fish fed the VO diet were used as reference values in the normalization procedure (*RU* relative units). Statistically significant differences between groups are indicated (***$P < 0.001$; Student's *t* test)

which is also highly vulnerable to oxidative stress due to constant exposure to transition metals, bacterial metabolites, bile acids and ROS generated by luminal contents.

Low amino acid substitution rates in UCP2 and UCP3 clades reflect the strong purifying selection in the UCP2/3 branch (Hughes and Criscuolo 2008; Hughes et al. 2009). Moreover, UCP2 and UCP3 genes are located adjacently in fish (Jastroch et al. 2005) and mammalian genomes (Pecqueur et al. 1999; Solanes et al. 1997), which suggests that the duplication of the ancestral UCP2/3 gene may have occurred before the divergence of higher and lower vertebrate lineages. Alternatively, different duplication events of the ancestral UCP2/3 gene may have arisen through fish and higher vertebrate evolution. However, UCP2 has not been yet characterized in the fish *Sparidae* lineage since a red sea bream UCP2 sequence, firstly identified as UCP2 by Liang et al. (2003), has been recognized as UCP1 after more exhaustive BLAST searches and phylogenetic analyses (Emre et al. 2007). Furthermore, extensive work by RT-PCR with degenerated primers failed to detect the expression of UCP2 in gilthead sea bream (authors' unpublished observations), but genome sequencing is needed to sustain the apparent loss or silencing of UCP2 in this fish lineage.

The present study highlights that gilthead sea bream UCP3 is primarily expressed in skeletal muscle and heart. This is not surprising given the expression in muscle fibres

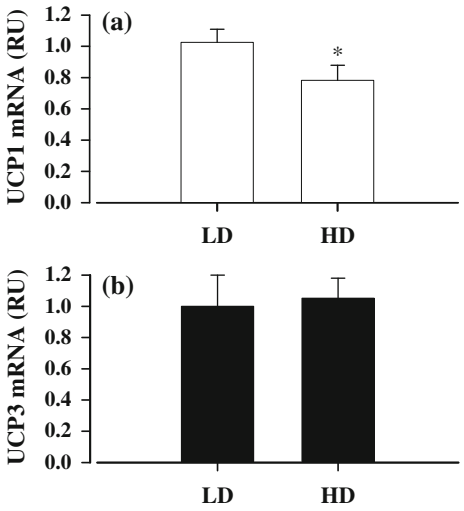

Fig. 5 Transcript levels of hepatic UCP1 (**a**) and white skeletal muscle UCP3 (**b**) in fish reared at high (*HD* 45–50 kg/m³) and low (*LD* 10 kg/m³) densities in a pair-fed study. Data are represented as mean ± SEM ($n = 9$). UCP1 and UCP3 data in LD fish were used as reference values in the normalization procedure (*RU* relative units). Statistically significant differences between groups are indicated (*$P < 0.05$; Student's *t* test)

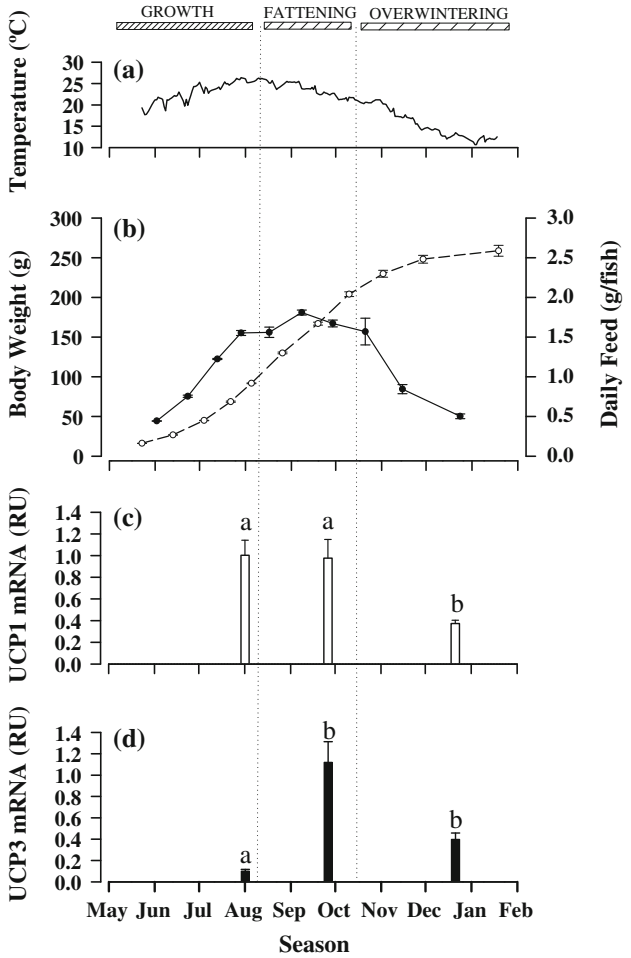

Fig. 6 **a** Seasonal changes in water temperature. **b** Body mass (*open circles*) and daily feed intake (*filled circles*) over the course of the experiment are represented as mean ± SEM ($n = 3$ tanks). *Boxes* at the top of the figure refer to the critical step windows over the course of the culture cycle. Transcript levels of UCP1 in liver (**c**) and UCP3 in white skeletal muscle (**d**) are the mean ± SEM of 8–9 animals. Data values of UCP1 and UCP3 in fish sampled in October were used as reference values in the normalization procedure (*RU* relative units). *Different letters* above each bar indicate statistically significant differences among step windows of the season ($P < 0.05$; Tukey test)

of carp UCP3 (Jastroch et al. 2005) and the closely related avian UCP (Raimbault et al. 2001) and mammalian UCP3 counterparts (Boss et al. 1997; Vidal-Puig et al. 1997). Moreover, the expression rates of the gilthead sea bream UCP3 was highest in glycolytic muscle (white skeletal muscle) and lower in oxidative (red skeletal muscle) and cardiac muscle fibres. In carp, however, UCP3 is preferentially expressed in red oxidative muscle, although fasting is able to strongly up-regulate UCP3 expression in white muscle fibre types (Jastroch et al. 2005). Samec et al. (2002) indicate that in rats there is not a muscle fibre-type-specific pattern for UCP3 mRNA levels in ad libitum fed animals, but other studies reveal a close negative association between UCP3 protein content and mRNA levels with fat oxidative capacity in white gastrocnemius, soleus and cardiac muscle (Hoeks et al. 2003). In the same line, protein expression of human UCP3 is highest in glycolytic muscle fibres and lower in oxidative ones (Hesselink et al. 2001). These findings does not easily match with a facilitative role for UCP3 in fatty acid oxidation, but available literature collectively indicates that UCP3 expression is enhanced when the energy supply exceeded the energy demand, which leads to the concept that UCP3 is mostly involved in fat metabolism rather than in the regulation of energy expenditure as recently reviewed by Nabben and Hoeks (2008).

Previous investigations in mammals (Samec et al. 2002) and birds (Abe et al. 2006), irrespective of the postulated mechanism of action, have shown a link between the uncoupling protein expression and fatty acid metabolism as an attempt to protect the cell against the detrimental effects of fatty acid accumulation. In the present study, we also addressed over a range of physiological models the specific expression of UCP1 and UCP3 in liver and white skeletal muscle, respectively. In this respect it must be noted that in gilthead sea bream the up-regulation of UCP1 and UCP3 with age was linked to increases in whole-body adiposity and reallocation of body fat depots. This was especially relevant for UCP3 (fourfold increase), which might mediate major changes in local free fatty-acid availability. This is not surprising given that rate-limiting enzymes on tissue fatty-acid uptake (e.g. lipoprotein lipase, LPL) are also highly expressed in the white skeletal muscle of 3-year-old gilthead sea bream (Saera-Vila et al. 2007). Hence, the rise

in UCP3 transcripts can be viewed as a counter-regulatory response against the increased risk of lipid-oxidative stress. Similarly, the specific induction of LPL in the skeletal muscle of mouse leads to a rise in UCP3 mRNA (Kratky et al. 2001). Experimental evidence also indicates that UCP3 overexpression in transgenic mice blunts the age-induced increase in ROS production (Nabben et al. 2008). Overexpression of UCP2 and UCP3 also improves mice insulin sensitivity (Clapham et al. 2000; Horvath et al. 2003; Choi et al. 2007) and steatotic livers have chronically elevated levels of ROS and a higher counter-regulatory expression of UCP2 (Evans et al. 2008). Thus, while some doubts persist about the expression of UCP2 in hepatocytes of lean mice, UCP2 mRNA is approximately sixfold higher in genetically obese animals (Pecqueur et al. 2001).

A close association between UCP induction and dietary fatty acid overloads was also found in the current study after the total replacement of fish oil with vegetable oils. This nutritional model of deficiencies in omega-3 polyunsaturated fatty acids increases the flux of fatty acids from adipose tissue towards liver and skeletal muscle. Therefore, the enhanced expression of UCP3 in skeletal muscle and to a lower extent in the liver tissue, which also showed clear signs of lipoid liver degeneration as evidenced elsewhere (Benedito-Palos et al. 2008), is not surprising. The opposite situation with the increase of energy demand was also analysed, and we found that the enhancement of metabolic rates after chronic confinement exposure significantly reduced the expression level of UCP1 in the liver tissue. At the same time, however, other life-essential mitochondrial proteins (e.g. glucose-regulated protein 75; mitochondrial stress-protein of the HSP70 family), with a wide range of antioxidant functions, were up-regulated (Bermejo-Nogales et al. 2007). Therefore, in this experimental model, the primary strategy of mitochondria for reducing oxidative stress would be the improvement of ROS scavenging and oxidative repair rather than the reduction of ROS production by respiration uncoupling. Similarly, aerobic exercise training decreases the muscle expression of UCP3 in rats (Peterson et al. 2008) but opposite effects after exercise exhaustion have been reported (Jiang et al. 2009), which suggests that the antioxidant strategy depends on the intensity, duration and nature of the oxidative-stressor.

UCP-mediated effects on whole-body thermogenesis have been considered in Antarctic fish (Mark et al. 2006). The involvement of fish UCP1 on local brain thermogenesis has also been suggested in carp (Jastroch et al. 2007), but the present study in gilthead sea bream does not support a significant thermogenic role for hepatic UCP1 in ectothermic fish. This was based on the observation that the expression of hepatic UCP1 was highest in summer and autumn and markedly lower (2–3-fold) during cold exposure in winter. In this multivariate experiment, the down-regulated expression may be indicative that hepatic UCP1 is primarily under a negative in-season regulation that follows the temperature-mediated changes in metabolic rates and feeding levels. In the same line, hepatic expression of UCP1 is reduced by cold exposure in carp (Jastroch et al. 2005), but in both cases the experimental design does not allow to determine the specific effects due to changes in feed intake. Regarding white muscle expression in season, a similar conclusion can be drawn for UCP3 with a highest expression level in autumn rather than in winter. Moreover, the lowest expression was found in summer in coincidence with the increased demand of energy substrates for growth purposes, This expression pattern may be indicative that UCP3 in skeletal muscle is primarily under a positive regulation with the increased intracellular concentration of fatty acids to protect the cell against lipotoxicity. In gilthead sea bream and other fish species, we (Mingarro et al. 2002; Pérez-Sánchez et al. 2002) and other authors (Björnsson et al. 2002; Lynn et al. 2009) have demonstrated that growth hormone (GH), prolactin (PRL) and somatolactin provide an integrated signal for nutrient utilization and partitioning all year round, and it would be of interest to analyse the links between UCPs and fish GH/PRL family as key points of the endocrine cascade with overlapping functions in growth, fat deposition and reproduction.

To sum up, gilthead sea bream UCPs were unequivocally recognized as UCP1 and UCP3 counterparts. Each transcript had a tissue-specific expression pattern although more probably they show overlapping functions. Thus, major changes in both UCP1 and UCP3 were associated to switches in oxidative capacities in order to match both energy demand and antioxidant defence. This dual role is probably closely related to the ancestral protein UCP function, and allows the use of UCPs as lipotoxicity markers in ectothermic fish; although, the exact physiological function is so far unknown and requires more precise causal studies.

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