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Metabolic and transcriptional responses of gilthead sea bream (*Sparus aurata* L.) to environmental stress: New insights in fish mitochondrial phenotyping

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ABSTRACT

The aim of the current study was to phenotype fish metabolism and the transcriptionally-mediated response of hepatic mitochondria of gilthead sea bream to intermittent and repetitive environmental stressors: (i) changes in water temperature (T-ST), (ii) changes in water level and chasing (C-ST) and (iii) multiple sensory perception stressors (M-ST). Gene expression profiling was done using a quantitative PCR array of 60 mitochondria-related genes, selected as markers of transcriptional regulation, oxidative metabolism, respiration uncoupling, antioxidant defense, protein import/folding/assembly, and mitochondrial dynamics and apoptosis. The mitochondrial phenotype mirrored changes in fish performance, haematology and lactate production. T-ST especially up-regulated transcriptional factors (PGC1α, NRF1, NRF2), rate limiting enzymes of fatty acid β-oxidation (CPT1A) and tricarboxylic acid cycle (CS), membrane translocases (Tim/TOM complex) and molecular chaperones (mtHsp10, mtHsp60, mtHsp70) to improve the oxidative capacity in a milieu of a reduced feed intake and impaired haematology. The lack of mitochondrial response, increased production of lactate and negligible effects on growth performance in C-ST fish were mostly considered as a switch from aerobic to anaerobic metabolism. A strong down-regulation of PGC1α, NRF1, NRF2, CPT1A, CS and markers of mitochondrial dynamics and apoptosis (BAX, BCLX, MFN2, MIRO2) occurred in M-ST fish in association with the greatest circulating cortisol concentration and a reduced lactate production and feed efficiency, which represents a metabolic

Abbreviations: ACAA2, 3-ketoacyl-CoA thiolase, mitochondrial; AIFM1, apoptosis-related protein 1; AIFM3, apoptosis-related protein 3; ANOVA, analysis of variance; BAX, apoptosis regulator BAX; BCL2, apoptosis regulator Bcl-2; BCLX, Bcl-2-like protein 1; CAT, catalase; Cox4a, cytochrome C oxidase subunit IV isoform 1; CPT1A, carnitine palmitoyltransferase 1A; CS, citrate synthase; DER-1, derlin-1; DnaJA3Aa, 40 kDa heat shock protein DnaJ (Hsp40) homolog, subfamily A, member 3A; DnaJC20, iron–sulfur cluster co-chaperone protein HscB; ECH, Enoyl-CoA hydratase, mitochondrial; ER, endoplasmic reticulum; ERdj3, ER-associated Hsp40 co-chaperone; FIS1, mitochondrial fission 1 protein; GPX4, glutathione peroxidase 4; GR, glutathione reductase; Grp-170, 170 kDa glucose-regulated protein; GST3, glutathione S-transferase 3; HADH, hydroxyacyl-CoA dehydrogenase; IDH3A, isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial; IDH3B, isocitrate dehydrogenase [NAD] subunit beta, mitochondrial; IDH3G, isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial; MFN1, mitofusin 1; MFN2, mitofusin 2; MIFFB, mitochondrial fission factor homolog B; MIRO1A, mitochondrial Rho GTPase 1; MIRO2, mitochondrial Rho GTPase 2; mtHsp10, 10 kDa heat shock protein, mitochondrial; mtHsp60, 60 kDa heat shock protein, mitochondrial; mtHsp70, 70 kDa heat shock protein, mitochondrial; mtTFA, transcription factor A, mitochondrial; NRF1, nuclear respiratory factor 1; NRF2, nuclear respiratory factor 2; OXPHOS, oxidative phosphorylation; PERP, p53 apoptosis effector related to PMP-22; PGC1α, proliferator-activated receptor gamma coactivator 1 alpha; PGC1β, proliferator-activated receptor gamma coactivator 1 beta; PRDX3, peroxiredoxin 3; PRDX5, peroxiredoxin 5; ROS, reactive oxygen species; SOD2, superoxide dismutase [Mn]; Tim10, translocase of inner mitochondrial membrane 10 homolog; Tim13, mitochondrial import inner membrane translocase subunit 13; Tim14, mitochondrial import inner membrane translocase subunit 14; Tim16, mitochondrial import inner membrane translocase subunit 16; Tim17A, mitochondrial import inner membrane translocase subunit Tim17-A; Tim22, mitochondrial import inner membrane translocase subunit Tim22; Tim23, mitochondrial import inner membrane translocase subunit 23; Tim44, mitochondrial import inner membrane translocase subunit Tim44; Tim8A, mitochondrial import inner membrane translocase subunit Tim8 A; Tim9, mitochondrial import inner membrane translocase subunit Tim9; Tom22, mitochondrial import receptor subunit Tom22; Tom34, mitochondrial import receptor subunit Tom34; Tom5, mitochondrial import receptor subunit Tom5 homolog; Tom7, mitochondrial import receptor subunit Tom7 homolog; Tom70, mitochondrial import receptor subunit Tom70; UCP1, uncoupling protein 1; UCP2, uncoupling protein 2; UCP3, uncoupling protein 3; UPR, unfolded protein response.

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condition with the highest allostatic load score. These findings evidence a high mitochondrial plasticity against stress stimuli, providing new insights to define the threshold level of stress condition in fish.

1. Introduction

Mitochondria are cellular organelles that play a variety of important roles in eukaryotic cell physiology, ranging from production of ATP and redox homeostasis to biosynthesis of macromolecules and intracellular calcium regulation, which are related to different pathways that influence cellular homeostasis and fate, including cell death cascades (Galluzzi et al., 2012). Dysfunction of this cell organelle is, thereby, associated with the natural chronic process of aging, as well as with neurodegenerative disorders, metabolic diseases and toxic insults (Scharfe et al., 2009). The number of mitochondria and their level of activity also vary depending on tissue and cell type, reflecting the energy requirements of the cell. Both can be modulated by internal and external factors through the tight transcriptional and translational regulation of nuclear and mitochondrial proteins (Bolender et al., 2008; Garesse and Vallejo, 2001; Scheffler, 2001). This includes induction of protein transcriptional co-activators, import of precursor proteins into mitochondria, as well as incorporation of both mitochondrial and nuclear gene products into the expanding organelle reticulum. Each of these steps adapts to altered physiological conditions in order to regulate cellular homeostasis, and recent reviews in humans and other animal models have summarized the current knowledge on most of these processes. Thus, mitochondria biogenesis can be activated by physiological and pathological stimuli, such as exercise, caloric restriction, thermogenesis, postnatal breathing, secretion of thyroid hormone and erythropoietin, oxidative stress and inflammation (Chen et al., 2009; Ljubicic et al., 2010; Piantadosi and Suliman, 2012a,b).

Literature on the regulation of mitochondrial activity and biogenesis is poorer in fish than in humans and higher vertebrates, although it appears that fish mitochondria are especially versatile (O'Brien, 2011). Hence, fish mitochondrial activity is highly modulated by thermal (Beck and Fuller, 2012; Egginton and Johnston, 1984; Guderley, 1997; Mueller et al., 2011; Orczewska et al., 2010), osmotic (Tse et al., 2012), chemical (Peter et al., 2013) or nutritional stressors (Enyu and Shu-Chien, 2011). In particular, mitochondrial function in gilthead sea bream (*Sparus aurata*) is highly regulated by dietary oils (Pérez-Sánchez et al., 2013), but it remains largely unclear how different stressors induce mitochondrial damage, energy failure and cell death, and more importantly, how these processes initiate retrograde signals for transcriptional regulation of mitochondrial biogenesis and cell-tissue repair. Furthermore, there is not a consensus endocrine profile for chronically stressed animals or how to asses it without invoking further stress (Dickens and Romero, 2013; Pankhurst, 2011). This notion is extensive to gilthead sea bream exposed to chronic and acute stress (Arends et al., 1999; Calduch-Giner et al., 2010; Fanouraki et al., 2011; Rotllant et al., 2000), but even in a higher extent when the less studied intermittent and repetitive stressors are considered (Ibarz et al., 2007; Tort et al., 2001). These type of stressors typically include daily farming activities, such as people walking alongside tanks and removal of dead fish, as well as activities that involve changes in noise and/or light level, potentially giving rise to a wide variety of stimuli that most fish adapt to slowly and are difficult to quantify (Bratland et al., 2010; Nilsson et al., 2012).

The current methodological constrains can be partially overcome with the advent of improved genomic resources for the most important cultured fish species. This is the case of gilthead sea bream (Calduch-Giner et al., 2013), for which an updated reference transcriptome database with a high representation of mitochondrial-related transcripts is now available at www.nutrigroup-iats.org/seabreamdb. This has allowed the development and validation of a mitochondrial quantitative PCR array that profiles the expression of 60 genes, selected as markers of nuclear transcriptional regulation (5 genes), oxidative metabolism/respiration uncoupling (13 genes), antioxidant defense (7 genes), protein import/folding/assembly (23 genes), and mitochondrial dynamics and apoptosis (12 genes). These markers were selected on the basis of the transcriptionally-mediated responses of gilthead sea bream to crowding stress (Bermejo-Nogales et al., 2008; Calduch-Giner et al., 2010; Saera-Vila et al., 2009), and literature references in other animal models, including rodents and humans (Liesa et al., 2009; Ljubicic et al., 2010; Manoli et al., 2007; Wenz, 2013). This molecular phenotyping was then completed with measurements of haematological parameters, plasma hormones and metabolites, including cortisol, glucose and lactate as a marker of anaerobic metabolism. The final aim was to determine whether mitochondrial response could be used as a highly integrative and informative tool capable of phenotyping stress in fish, providing at the same time new tools and insights to define the threshold level of stress condition in cultured fish.

2. Materials and methods

2.1. Experimental setup

Juvenile gilthead sea bream of Atlantic origin (Ferme Marine de Douhet, France) were acclimatized to the indoor experimental facilities of the Institute of Marine Research (IMR), Matre Research Station (Norway) for 2 months. Fish (265–274 g average body weight) were then distributed into twelve 500 L tanks (27 fish per tank) at a stocking density of 14–15 kg/m^3. Each tank was closed with a lid fitted with two fluorescent light tubes (18 W each) and one automatic feeder (RVO-TEC T Drum 2000, Arvotec, Huutokoski, Finland). A 12D:12L photoperiod was maintained with lights on from 8:00 h to 20:00 h. All tanks were supplied with heated seawater (salinity 35‰) that was maintained at 20 °C with a flow rate of 24–32 L/min. Fish were fed 4.5 mm dry pellets (EFICO YM 554, BioMar, Dueñas, Palencia, Spain) twice a day (11:00 h and 16:00 h) near to satiation 7 days/week. Feed intake was collectively and daily monitored for each tank (experimental unit) through all the stress trial. Three weeks prior to the start of the stress trial, feed intake was also checked in order to ensure that there were no major tank effects in the trial.

Four groups, corresponding to control (CTRL) fish and three groups of stressed (ST) fish, were established in triplicate for an experimental period of 21 days. Fish assigned to the thermal stressed group (T-ST) were under water temperature cycles of 2 days at 12 °C to 3 days at 20 °C. Regulation of water temperature was done manually in the morning (start time 9:00 h), lasting approximately 4 h. In the chasing stress group (C-ST), water level in the tank was lowered twice a day (9:15 h and 14:15 h) to 10 cm and was kept at this level for 45 min. Thirty minutes after lowering water level, fish were intensively chased with a pole for

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