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# Differential tissue regulation of peroxisome proliferator-activated receptor α (PPARα) and cannabinoid receptor 1 (CB1) gene transcription pathways by diethylene glycol dibenzoate (DEGB): preliminary observations in a seabream (*Sparus aurata*) *in vivo* model

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ABSTRACT

Today a variety of endocrine disrupting chemicals (EDCs) are recognized in the group of metabolic disruptors, a wide range of environmental contaminants that alter energy balance regulation by affecting the peroxisome proliferator-activated receptor (PPAR)/retinoid X receptor (RXR) pathway. Herein, we investigated the effect of diethylene glycol dibenzoate (DEGB), a dibenzoate-based plasticizer used as alternative to phthalates, on the expression of key genes involved in lipid metabolism and energy balance by using *Sparus aurata* juveniles as models. We also evaluated the correlation between cannabinoid receptor 1 (CB1) and PPARα transcriptional patterns in both liver and brain tissues. Exposure to the highest DEGB concentration differentially modulated PPARα/CB1 transcriptional pathways in liver/brain tissues of seabream. We hypothesize that, at peripheral level (*i.e.* liver), DEGB acts as PPARα agonist resulting in a potential stimulation of key lipolytic genes and a concomitant down-regulation of endocannabinoid metabolic enzyme genes.

## 1. Introduction

Today a variety of endocrine disrupting chemicals (EDCs) are recognized in the group of metabolic disruptors that includes obesogens, a wide range of environmental contaminants capable of altering energy balance regulation leading to obesity (Grun and Blumberg, 2006). These chemicals act through multiple mechanisms, ranging from direct increase in number/size of adipocytes to indirect alteration of both basal metabolic rate and hormonal control of appetite and satiety (Heindel et al., 2017). Recently, obesogen list has expanded to include contaminants that deregulate lipid metabolism (Chamorro-Garcia and Blumberg, 2014). Collectively, experimental evidence from cell lines to *in vivo* models supports the peroxisome proliferator-activated receptor (PPAR)/retinoid X receptor (RXR) pathway, as target for metabolic disruption (Feige et al., 2006). Indeed, it is well established that environmental contaminants such as phthalates work as agonist of PPARγ promoting adipogenesis together with the up-regulation of genes involved in lipid metabolism (Feige et al., 2006; Hao et al., 2012). In this regard, we have recently shown that different chemicals used as plasticizers in food-contact materials can interfere with adipogenesis by inducing PPARγ transcriptional activity in 3T3-L1 adipocytes (Pomatto et al., submitted). Similar mode of action has also been demonstrated for other contaminants, particularly flame retardants (Fang et al., 2015) and organochlorine compounds (Mangum et al., 2015). Beside their classical PPARγ binding properties, obesogens can also show the ability to interact with PPARα influencing the expression of lipid metabolism genes (Hayashi et al., 2011). In this context, our early work demonstrated high binding efficiency of diisodecyl phthalate (DiDP) towards both PPARα and RXRα in teleost fish (Cocci et al., 2015). Similarly, we also observed that exposure to the organophosphate flame retardant trim-cresyl phosphate (TMCP) altered fatty acid synthesis/uptake and cholesterol metabolism through the induction of PPARs and liver X receptor (LXRα) target genes (Palermo et al., 2016). Emerging evidences also report the ability of some contaminants with obesogenic properties to induce metabolic alteration by up-regulating the endocannabinoid system (Martella et al., 2016). This finding suggests the existence of a potential cross-talk between PPARs and endocannabinoid systems that could be involved in mediating obesogen-related effects on central integration of energy balance (Grun and Blumberg, 2009). According to the European Chemical Agency, phthalates have been

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recently replaced by dibenzoates-based plasticizers (*e.g.* diethylene glycol dibenzoate (DEGB)) which show potentially higher biodegradation rates (Arendt and Lang, 1998; Deligio, 2009). However, previous studies have reported incomplete microbial degradation of dibenzoate-based plasticizers suggesting the risk of accumulation of toxic products (Gartshore et al., 2003). Thus, the present study was designed first to predict the DEGB binding capacity for PPARα/LXRα/RXRα by using computation methods and then to evaluate the effect of DEGB exposure on central and peripheral lipid metabolism by determining plasma lipid titers and expression levels of key genes involved in physiological mechanisms such as lipogenesis (*i.e.* stearoyl-CoA desaturase 1A (SCD1A), fatty acid desaturase 2 (FADS2)), cholesterol metabolism (*i.e.* sterol regulatory element-binding protein 1c (SREBP-1c)), fatty acid oxidation (*i.e.* carnitine palmitoyltransferase 1A (CPT1A)), and energy balance (*i.e.* neuropeptide Y (NPY)). Also, efforts were made to investigate the correlation between cannabinoid receptor 1 (CB1) and PPARα transcriptional patterns. Gilthead seabream (*S. aurata*) juveniles were used as study models.

## 2. Materials and methods

### 2.1. Molecular docking

To evaluate the affinity of DEGB/sea bream nuclear receptor complexes and their related geometries we performed a molecular docking analysis, using the Autodock Vina software (version 1.1.2) (Trott and Olson, 2010) on an Intel Core i7/Mac OS X 10.12-based platform, between the three-dimensional structure of DEGB and the homology modelled structures of PPARα, LXRα and RXRα as previously reported (Palermo et al., 2016). The final evaluation of predicted equilibrium dissociation constants ($K_{d,pred}$) was achieved using the NNscore 2.0 python script (Durrant and McCammon, 2010).

### 2.2. Fish and treatment

Juvenile gilthead seabream (*S. aurata*) (mean weight: 15.7 ± 4.3 g) were purchased from an Italian fish farm, Orbetello Pesca Lagunare s.r.l., (Orbetello, GR, Italy) and kept in 100-L glass aquaria filled with 80 L of seawater (21–23 °C, dissolved oxygen 5.7–7.8 ppm, salinity 34–36 g $L^{-1}$, pH 7.0–8.0, natural photoperiod) at University of Camerino labs. Following the acclimation period fish were randomly divided in three groups (n = 10 fish/group): control group received carrier solvent alone (ethanol, (EtOH); 0.01% final concentration), and the others were exposed to two relevant concentrations of DEGB (1 μg$L^{-1}$ or 100 μg$L^{-1}$). Concentrations of DEGB were chosen on the basis of molecular docking analysis and taken into account the doses that can potentially affect nuclear receptor signaling pathways (USEPA, 1981, 2016). Animals were exposed for 10 days in glass aquaria. The chemicals were added to the water every 24 h after draining the tanks to ~10% volume and refilling them with fresh water (Jordan et al., 2012). After the treatment each animal were euthanized with 3-aminobenzoic acid ethyl ester methanesulfonate (Sigma) and blood was immediately collected into heparinized tubes. Plasma for lipid analysis was isolated from whole blood by centrifugation (1500*g*/20 min/4 °C). Once sacrificed liver and brain were removed from seabream for each treatment and control group. The tissues were immediately snap-frozen in liquid nitrogen and subsequently kept frozen at −80 °C until use for molecular biology analyses. Animal manipulation was performed according to the recommendations of the University Ethical Committee, to the European Union directive (2010/63/EU) and under the supervision of the authorized investigators.

### 2.3. RNA extraction and real time PCR

Total RNA was extracted from 50 mg of liver or brain samples using Trizol Reagent (Invitrogen, Milan, Italy) according to the manufacturer's instructions. DNase digestion (2U, 30 min, 37 °C; Ambion, Austin, TX) was performed to eliminate genomic DNA contamination. RNA concentration and purity were assessed spectrophotometrically at absorbance of 260/280 nm, and the integrity was confirmed by electrophoresis through 1% agarose gels stained with ethidium bromide. The complementary DNA (cDNA) was synthesized from 2 μg of total RNA in 20 μL of total volume reaction using random hexamers (50 ng μ$L^{-1}$) and 200 U of SuperScript™ III RT according to manufacturer's instruction (Invitrogen Life Technologies, Milan, Italy). SYBR green-based real-time PCR (q-PCR) was used to evaluate expression profiles of CB1, NPY, LXRα, CPT1A, FADS2, SREBP-1c, SCD1A, PPARα, target genes. Analysis of the 18 s rRNA gene expression confirmed that its expression was unaffected by exposure to phthalates (Cocci et al., 2015), and thus, it is considered to be an appropriate reference gene for the qPCR analysis. All of the primer sequences are reported in Table 1.The expression of individual gene targets was analyzed using the Mx3000P Real-time PCR system (Stratagene, La Jolla, CA, USA). Thermo-cycling for all reactions was for 10 min 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 57 °C. Fluorescence was monitored at the end of every cycle. Results were calculated using the relative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and means of mRNA levels are expressed with respect to control fish ± standard deviation (SD).

**Table 1**
List of primers used in this study.

| Gene | Primer sequence (5′ - 3′) | GenBank | Reference |
|---|---|---|---|
| *CB1* | GCTGGGCTGGAACTGTAAAC<br>TTCCACAGGATGTATATGTAGGC | EF051620 | (Piccinetti et al., 2010) |
| *NPY* | GGAGCTGGCCAAGTACTACTCA<br>GAGACCAGCGTGTCCAGAAT | FJ946988 | (Piccinetti et al., 2010) |
| *LXRα* | GCACTTCGCCTCCAGGACAAG<br>CAGTCTTCACACAGCCACATCAGG | FJ502320 | (Benedito-Palos et al., 2013) |
| *CPT1A* | GTGCCTTCGTTCGTTCCATGATC<br>TGATGCTTATCTGCTGCCTGTTTG | JQ308822 | (Perez-Sanchez et al., 2013) |
| *FADS2* | GCAGGCGGAGAGCGACGGTCTGTTCC<br>AGCAGGATGTGACCCAGGTGGAGGC | AY055749 | (Benedito-Palos et al., 2013) |
| *SREBP-1c* | AGGGCTGACCACAACGTCTCCTCTCC<br>GCTGTACGTGGGATGTGATGGTTTGG | JQ277709 | (Benedito-Palos et al., 2013) |
| *SCD1A* | CGGAGGCGGAGGCGTTGGAGAAGAA<br>AGGGAGACGGCGTACAGGGCACCTA | JQ277703 | (Benedito-Palos et al., 2013) |
| *PPARα* | GCAGCCTGTGAGTCTTGTGAGTGA<br>CTCCATCAGGTCTCCACACAGC | AY590299 | (Fernandez et al., 2013) |
| *18s* | GCATTTATCAGACCCAAAACC<br>AGTTGATAGGGCAGACATTCG | AY993930 | (Perez-Sanchez et al., 2011) |

### 2.4. Plasma lipid profile

Plasma cholesterol concentrations, LDL-C and HDL-C, were determined enzymatically by a cholesterol oxidase–peroxidase aminophenazone (CHOD-PAP) method, using commercial enzymatic kits (Giesse diagnostics, Rome, Italy), and TG concentration was determined according to the fully enzymatic glycerol phosphate oxidase (GPO)-PAP method, using a commercial enzymatic kit (Giesse diagnostics) as described by Palermo et al. (2013).

### 2.5. Statistical analysis

Data were first examined for their fit to a normal distribution and homogeneity of variance using Kolmogorov–Smirnov and Levene median tests. A one-way analysis of variance (ANOVA) was used to compare results between groups, followed by the Dunnett's post hoc test. Differences between means were considered significant when $P < 0.05$. All statistical analyses were performed using R (R, 2008).