Endocrine disruptors in the diet of male Sparus aurata: Modulation of the endocannabinoid system at the hepatic and central level by Di-isononyl phthalate and Bisphenol A

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ABSTRACT

The increasing manufacture of plastics and their mismanagement has turned plastic into a ubiquitous waste in the marine environment. Among all the substances conforming the plastic items, the effects of a dietary Bisphenol A (BPA) and Di-isononyl phthalate (DiNP) have been evaluated in adult male gilthead sea bream, focusing on their effects in the modulation of the Endocannabinoid System (ECS). In zebrafish, the ECS has been recently chosen as a new target for the activity of some Endocrine Disrupting Chemicals (EDC), since it represents a complex lipid signaling network essential for the well-being of the organisms. The results obtained in gilthead seabream showed that BPA and DiNP altered the structure and the biochemical composition of liver, increasing the presence of lipids and triglycerides and decreasing the glycogen and phospholipids. Moreover, the addition of BPA or DiNP in the gilthead sea bream diet altered the levels of endocannabinoids (EC) and EC-like mediators in the liver. These alterations were also associated to changes at the transcriptomic level of genes involved in lipid biosynthesis and ECS metabolism. At the central level, both BPA and DiNP reduced the expression of the endocannabinoid receptor type I (cnr1) and the neuropeptide Y (npy) as well as the levels of the endocannabinoid Anandamide (AEA), suggesting a downregulation of appetite. The results herein reported highlighted the negative effects of chronic dietary exposure to DiNP or BPA on ECS functions and lipid metabolism of male gilthead sea bream liver, showing a similar disruptive activity of these contaminants at metabolic level. Moreover, the novelty of the biomarkers used evidenced possible innovative endpoints for the development of novel OEDCS test guidelines.

1. Introduction

With a production of 1.5 million tonnes of plastics in 1964, world plastic manufacture has exponentially increased reaching 335 million tonnes in 2016 (Plastics Europe, 2015, 2018) In this context, it was estimated that between 4.8 and 12.7 million tonnes entered to the ocean in 2010, corresponding to 1.7 to 4.6% of total plastic waste generated in coastal countries (Jambeck et al., 2015), and by 2050 it is expected that our planet will hold about 33 billion tonnes of plastic (Rochman et al., 2013). As consequence, plastic debris have been found in the stomachs of over 220 different marine species (FAO, 2017).

For years, plastic has been considered biochemically inert (Lithner et al., 2011), however plastic additives or “plasticizers” may leach out to the marine environment (Rani et al., 2015) and thus, introducing potentially hazardous chemicals to biota, since plastics are both a mixture of chemicals and a platform to accumulate other nearby toxic components (Bakir et al., 2014; Cole et al., 2011; Engler, 2012; Fossi et al., 2012, 2014; Rochman et al., 2014; Yamashita et al., 2011).

Among the myriad of substances used for plastic production, our emphasis will be placed on two plastic additives, the Bisphenol A (BPA) and a phthalate, the Di-isononyl phthalate (DiNP), because of their reported endocrine disrupting properties. Phthalates are chemical compounds primarily used for the manufacture of polyvinyl chloride (PVC) and characterized by their incapacity to be chemically bound to
the plastic matrix (Fujii et al., 2003; Roh et al., 2013). In this regard, DiNP can be found in freshwater as well as marine water (Blair et al., 2009; Mackintosh et al., 2004, 2006) and sediments (Oehlmann et al., 2008). Nevertheless, phthalates are considered to be non-persistent chemicals, to possess a short half-life and a lower bioconcentration factor in fishes than other contaminants (Staples et al., 1997). Although no data are available regarding teleost, in humans and murine models, the metabolism and excretion of DiNP appear to be in the successive hours after the oral dosing (McKee et al., 2002; Saravanabhavan and Murray, 2012). However, concerns about their toxicity exist due to their potential chronic effects on kidney and liver, since the liver is one of the target for DiNP toxicity (Ma et al., 2014), and its anti-androgenic properties.

On the other hand, BPA is mainly used for the manufacture of polycarbonate plastics (66%) and epoxy resins (30%). Similarly to DiNP, BPA is considered a non-persistent chemical with a short half-life (Staples et al., 1998), however, its presence in a large amount of products turns BPA into a ubiquitous chemical found in all the environmental compartments (Flint et al., 2012; Staples et al., 1998). Its release in the environment can take place during the manufacture and consumption via wastewater discharge, landfill leachate or natural breakdown from plastics (Crain et al., 2007). In the other hand, BPA presents a low potential of bioaccumulation in aquatic organisms (Staples et al., 1998). Indeed, when BPA is orally administered in rainbow trout, it showed a fast metabolism due to its relatively short half-life (Bjerregaard et al., 2007). Even the bioaccumulation does not seem to pose a problem, studies on wildlife indicate that it may have negative effects at environmentally relevant concentrations (Crain et al., 2007; Flint et al., 2012; Lahnsteiner et al., 2005; Mandich et al., 2007; Sohoni et al., 2001; Santangeli et al., 2016). In humans, BPA is also rapidly metabolized and excreted in the subsequent hours after administration (Thayer et al., 2015; Völkel et al., 2002). Even so, in January 2015, the European Food Safety Authority established a new Tolerable Daily Intake for humans (TDI), lowering the intake from 50 to 4 μg of BPA per kg of body weight per day.

BPA and DiNP have been recently reported to induce alterations in the Endocannabinoid System (ECS), indeed, exposure to BPA and DiNP deregulate the endocannabinoid tissue levels, the enzymatic activity and the gene expression of the ECS elements, in the liver and brain (Forner-Piquer et al., 2017, 2018; Martella et al., 2016). In this context, in vertebrates, the ECS is a lipid signaling network involved in a plethora of physiological roles, such as the regulation of energy homeostasis and food intake (Cristino et al., 2014; Di Marzo et al., 2001; Watkins and Kim, 2015) at central (i.e. hypothalamic) and peripheral level (i.e. liver). Briefly, the ECS is composed by two G-protein coupled receptors: the cannabinoid receptor type I (CB1) and type II (CB2); their endogenous agonists, the endocannabinoids (EC): N-arachidonoyl-ethanolamine (Anandamide or AEA) and 2-Arachidonoyl-glycerol (2-AG), which are often accompanied by biochemically but not necessarily functionally related compounds (or endocannabinoid-like mediators), such as N-oleoylethanolamine (OEA) and N-palmitoylethanolamine (PEA), all regulated by a complex enzymatic machinery composed by biosynthetic and catabolic enzymes. A correct tone of the ECS is fundamental for the well-being and performance of the organism, since the deregulation of the ECS tone is the starting point of several, i.e. metabolic, disorders (Osei-Hyiaman et al., 2005, 2008; Piccinetti et al., 2010).

Additionally, BPA and DiNP have been reported to alter hepatic lipid metabolism in zebrafish Danio rerio and gilthead sea bream Sparus aurata. In this regard, BPA and DiNP induced alterations in the expression of genes coding for enzymes involved in the triglycerides and fatty acids metabolism, as well as, both chemicals induced hepatosteatosis and altered the hepatic biochemical composition (Forner-Piquer et al., 2017, 2018; Maradonna et al., 2015; Martella et al., 2016; Santangeli et al., 2018).

On the other hand, given the low aqueous solubility of DiNP (and the moderate water solubility of BPA) it is expected the diet as a relevant route of exposure to these chemicals for wildlife (Staples et al., 1998; Thomann, 1989), although, as Bjerregaard reported (Bjerregaard et al., 2007), the oral route has been practically neglected. Thus, based on previous studies performed in gilthead sea bream (Carnevali et al., 2017; Maradonna et al., 2014, 2015) and the recently published studies of the effects of BPA and DiNP on the ECS mentioned above, the main goal of the present study was to elucidate whether DiNP or BPA, assumed by diet through a chronic treatment, might modify the lipid metabolism in Sparus aurata by altering the ECS and then, validate this system as a new target for EDCs exposure.

2. Material and methods

2.1. Fish maintenance

Two-year-old gilthead sea bream males (mean weight 458.8 ± 56.8 g; Total length 301.7 ± 10.7 cm) were maintained at the AQUALABS facilities of the Institute of Marine Biology, Biotechnology and Aquaculture of the Hellenic Centre for Marine Research (HCMR), Iraklion, Crete, Greece. The fish were maintained in 2-m³ tanks supplied with shallow well-water under ambient photo-period and stable temperature ranging between 19 and 20 °C. Measurements of dissolved oxygen, pH and NH₃-N and NO₂-N were done weekly during the maintenance period. The fish were fed to apparent satiation twice per day with commercial feed (IRIDIA SA, Greece) during the three-month acclimation previous to the experimentation.

2.2. Experimental design

The experimental feed containing the EDCs was prepared according to Bjerregaard et al. (2007) and Maradonna et al. (2014) using the same commercial feed that fish were eating during the acclimation period. Briefly, commercial feed (IRIDIA SA, Greece) was crushed and mixed with distilled water. For the treated groups, both pollutants were dissolved in ethanol and added to the food to obtain the final concentration described below. In the control group only the vehicle (ethanol) was added. Then, gelatin powder (Sigma, Milan, Italy) was dissolved in distilled water, warmed and added to the crushed food (0.2 g gelatin/5 g food). The final mix was dispensed and after cooling at room temperature for 2 h under the hood, stored at −20 °C till use.

The amount of DiNP and BPA added to the feed were adjusted to give the experimental dose according to the feed ration of fish of that size and at the ambient water temperatures.

Male fish were divided in 5 treatment groups of 10 individuals in duplicates. Then, fish were stocked in separate 2-m³ tanks and treated for 21 days. Fish were fed 0.7% of their body weight (bw), divided in five feedings per day, in order to ensure all the feed administered was consumed. The treatments were as follows: Control individuals (CTRL) were fed with the commercial feed mixed with the vehicle (1,4 ml of EtOH kg⁻¹ feed); BPA LOW were fed with commercial feed enriched with BPA so that fish consuming their daily ration of feed would receive 4 μg BPA kg⁻¹ bw day⁻¹; BPA HIGH were fed with commercial feed enriched with BPA so that fish consuming their daily ration of feed would receive 4000 μg BPA kg⁻¹ bw day⁻¹; DiNP LOW were fed with commercial feed enriched DiNP, so that fish consuming their daily ration of feed would receive 15 μg DiNP kg⁻¹ bw day⁻¹; DiNP HIGH were fed with commercial feed enriched DiNP so that fish consuming their daily ration of feed would receive 1500 μg DiNP kg⁻¹ bw⁻¹.

The choice of BPA and DiNP doses was based on the Tolerable Daily Intake (TDI) for humans ruled by the European Food Safety Authority, which is 4 μg/kg day⁻¹ and 150 μg/kg day⁻¹ respectively. The experimentation was done in the same conditions (tanks, temperature and photoperiod) than the previous acclimation of the animals. Water pH (7,52 ± 0.02) and dissolved oxygen levels (89.2 ± 1.8%) were monitored daily during the experimentation. The water NH₃-N and NO₂-N...
levels were measured weekly and were always kept under the safety levels during all the experimentation.

After 21 days, 5 fish per tank (n = 10 per experimental condition) were randomly taken and anesthetized in a bath of 30 mg 1⁻¹ clove oil until they ceased ventilating (Mylonas et al., 2005). Then the fish were weighed and decapitated swiftly and the brain and liver extracted. The livers were extracted and weighed in order to calculate the Hepatosomatic Index (HSI) according to the following equation: [(Liver weight (g) / Fish weight (g)) × 100]. Pieces of livers and brains were collect and stored at −80 °C for further analyses. Pieces of livers were also fixed in a solution of Formaldehyde:glutaraldehyde (4:1) for histological analysis. For gene expression analysis, aliquots of liver and brains were stored within Rnalater (Ambion Inc., Texas, US) at 4 °C until processing.

All procedures involving animals were conducted in accordance the “Guidelines for the treatment of animals in behavioral research and teaching” (2001). The experimental protocol was approved by the Greek National Veterinary Agency with the Protocol Number #255361 to the experimental facility EL91-BIOexp-04.

2.3. Histology and area covered with lipid vacuoles in the liver (ACVL)%

Before embedding in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany), pieces of liver (n = 10) were dehydrated in gradually increased ethanol solutions (70–96%). Sections of 4 μm were obtained with a microtome (Leica RM2245, Germany) and stained with Methylene Blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic fuchsin (Polysciences, USA) according to Bennett et al. (1976). The histological sections were examined under a light microscope (Nikon Eclipse 50i) and microphotographed with a digital camera (Jenoptik progress C12 plus).

The estimation of hepatic lipid area was done according to the methodology of Papadakis (Papadakis et al., 2013). For each liver, 6 microphotographs were obtained at ×40 magnification from different areas of the liver.

2.4. Fourier transform - infrared spectroscopy (FT-IR)

FT-IR measurements were carried out following the procedure reported by Gioacchini et al. (2014). The spectra extracted from the chromatic maps were integrated under 2990–2836 cm⁻¹ (lipids), 1765–1723 cm⁻¹ (COO groups, triglycerides) and 3027–2995 cm⁻¹ (=CH groups), where the sum of the integrated areas 2990–2836 cm⁻¹ and 1765–950 cm⁻¹ was indicative of the total cellular biomass of the selected area considered (cell). All the values indicative of hepatic fat characteristics were normalized to the cell value. These bands were chosen in order to quantitatively locate the presence and the characteristics of fat and were integrated giving a visible display with a false color scale. The intensity of the signal associated with a specific molecular/chemical group provides information of the presence and on the localization of the molecular/chemical group.

2.5. Measurement of endocannabinoids (AEA, 2-AG) and endocannabinoids-like mediators (OEA, PEA) in liver and brain

First, tissues were dounce-homogenized and extracted with chloroform/methanol/Tris-HCl 50 mM pH 7.4 (2:1:1, v/v) containing internal deuterated standards for AEA, 2-AG, PEA and OEA quantification by isotope dilution (5 pmol of d⁴-AEA, 50 pmol of d⁴-2AG, d⁴-PEA and d⁴-OEA Cayman Chemicals, MI, USA). The lipid-containing organic phase was dried down, weighed and pre-purified by open bed chromatography on silica gel. Fractions were obtained by eluting the column with 99:1, 90:10 and 50:50 (v/v) chloroform/methanol. The 90:10 fraction was used for AEA, 2-AG, PEA and OEA quantification by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS), as previously described and using selected ion monitoring at M + 1 values for the four compounds and their deuterated homologues, as described in Piscitelli et al. (2011).

2.6. Fatty acid amide hydrolase (FAAH) enzymatic activity in liver

Very briefly, livers were homogenized at 4 °C in 50mMTris-HCl buffer, pH 7.4, centrifuged at 1000 × g for 10 min and the supernatant was collected and centrifuged at 12000 × g for 30 min. Protein concentration was measured by Bradford assay. Membrane fractions (400 μg/sample) were incubated with [¹⁴C] AEA (10,000 cpm, 1.8 μM) in 50 mM Tris-HCl, pH 9, for 30 min at 37 °C. [¹⁴C] Ethanolamine produced from [¹⁴C] AEA hydrolysis was then extracted from the incubation mixture with 2 volumes of CHCl₃/CH₃OH (1:1 by volume) and the subsequent aqueous phase measured by scintillation counting in order to calculate FAAH activity.

2.7. RNA extraction and cDNA synthesis

Total RNA was isolated from liver and brain with RNAzol solution (Sigma Aldrich, Milan, Italy) according to the manufacturer’s instructions as previously reported (Santangeli et al., 2016). The quantity of purified RNA was determined using a nanodrop spectrophotometer (NanoPhotometer™ P-Class, IMPLEN, Germany) and the quality with a 1% agarose gel electrophoresis.

cDNA synthesis was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) using random decamers and 500-ng total RNA in a final volume of 100 μl. Reverse transcriptase (RT) reactions were incubated 10 min at 25 °C and 2 h at 37 °C. Negative control reactions were run without RT.

2.8. Real-time quantitative PCR

Real-time quantitative PCR was carried out with an Eppendorf Mastercycler Ep Realplex real-time PCR system (Eppendorf, Wesseling-Berzdorf, Germany), using a 96-well PCR array layout designed for the simultaneously profiling of a panel of 25 regulated genes for liver and 20 for brain, selected as markers of: i) endocannabinoid system: cb1, cb2, ppara, pparβ, pparγ, faah, nape-pld, abdh4, cyc-pla2, cox2, dalg, adbh6a, adbh12a, trpv1 ii) lipoytic/lipogenic markers: lepr, lep, fasn, agpat4, dagat1, dagat2, gpat, sphk1, bl, brr, accx3 iii) appetite: lepr, nuch2, nuch1, aqp, pamp, cart, npy. Primers were designed to obtain ampiclons of 50–150 bp in length (Table 1). The array included 24 new sequences for gilthead sea bream, already represented in the IATS-Nutrigroup gilthead sea bream transcriptomic database [www.nutrigroup-iats.org/seabreamdb; (Calduch-Giner et al., 2013)] and uploaded to GenBank with the accession numbers from MG570167 to MG570186 (Table 2). In them, 21 are full codifying sequences. Each PCR-well contained 12.5 μl SYBR Green Master Mix (Bio-Rad), 5 μl of specific primers at a final concentration of 0.9 μM, and 7.5 μl diluted RT reaction. PCR amplification included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/ extension for 60 s at 60 °C. The specificity of reactions was verified by analysis of melting curves. Housekeeping gene (β-actin) and controls for general PCR performance were included on each array, and all the pipetting operations were performed with the EpMotion 5070 Liquid Handling Robot (Eppendorf). The efficiencies of all PCR runs were always higher than 90% and specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5 °C/10 over a temperature range of 55–95 °C). Data acquired during the PCR extension phase were normalized using the delta-delta Ct method (Livak and Schmittgen, 2001).

2.9. Statistical analysis

Means between the five treatments (in duplicates) of the examined parameters were analyzed statistically using one-way analysis of
variance (ANOVA) followed by Tukey’s multiple comparison tests. Two-ways ANOVA was only used to compare the body weight of the animal before and after the treatments. Statistical significance was set at \( p < 0.05 \). Results are shown as means ± standard error of the mean (SEM) excepting the HSI and hepatic lipid area which are expressed in percentage and reported as mean ± SEM. Data fulfilled the condition for applying a parametric test, given the log-normalization to homogenize the variance when needed and performing ANOVA on ranks were the log-normalization failed. Expression heatmaps of analyzed genes were constructed by means on Genesis software (v1.7.7). All statistical procedures were run using GraphPad Prism 6 and SigmaStat 3.5.

### Table 1

| Gene name | Symbol | Tissue | Primer sequence (5′ - 3′) |
|-----------|--------|--------|---------------------------|
| Cannabinoid receptor 1 | cb1 | Liver, brain | GCTCAA CATCAC GCT CAACCC ACTAC |
| Cannabinoid receptor 2 | cb2 | Liver, brain | TGGTCTCCGAGAGGAGGAGAGA |
| Peroxisome proliferator activated receptor α | ppara | Liver, brain | TCTCTCACACCACCCTACCA |
| Peroxisome proliferator activated receptor β | pparβ | Liver, brain | AGGCAGAGAGATGGAGGAGAGGAG |
| Peroxisome proliferator activated receptor γ | pparγ | Liver, brain | GCCGAGGAGGAGGAGGAGGAGGAG |
| Transient receptor potential cation channel subfamily V member 1 | trpv1 | Liver | GGGTACCTGCTCTACCAGTCTAC |
| Fatty-acid amide hydrolase 1 | faah | Liver, brain | CCTGCCTGCTGCTGATGAG |
| N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D | nape-pld | Liver, brain | CGGCATCGCTCTCCACCA |
| Abhydrolase domain containing 4 | abhd4 | Liver, brain | GTGACCCTACCAACCAGATGGAGAG |
| Cytosolic phospholipase A2 | cyt-pla2 | Liver, brain | GCCGATGAGAGGAGGAGGAGGAGGAG |
| Cyclooxygenase-2 | cox2 | Liver, brain | GGGAGGAGTATAGTTGATGTAGTG |
| S1P-specific diacylglycerol lipase alpha | dagα | Liver, brain | GCCGCTCTGTTGTTGTTGTTGTT |
| Monoacylglycerol lipase abhd6a | abdh6a | Liver, brain | CATCATCCTGCTGCTGAG |
| Monoacylglycerol lipase abhd12a | abdh12a | Liver, brain | CGTGGTGGTGGTGGGAG |
| Fatty acid synthase | fas | Liver | ACAAGCCGATCCAGGCTCTGATACTCA |
| 1-acyl-sn-glycerol-3-phosphate acyltransferase delta | agpat4 | Liver | CTCTGCTATGCTATTGTT |
| Diacylglycerol O-acyltransferase 1 | dgat1 | Liver | GGGATGGAGGAGGAGGAGGAGGAG |
| Diacylglycerol O-acyltransferase 2 | dgat2 | Liver | GTAACCCCTGCTGATCATC |
| Hepatic lipase | hl | Liver | AGCAAAGGAGATGGAGAGAG |
| Liver X receptor α | lxrα | Liver, brain | GCACTGCTGCTGCTGAGGAG |
| Leptin receptor | lepr | Liver | GGGGAGTCTGCTGCTGCTGCT |
| Leptin | lep | Liver | GAGGAGGAGGAGGAGGAGGAGGAG |
| Glycerol-3-phosphate acyltransferase 1 | gpat1 | Liver | CTTCATCATCATGATGAGGAG |
| Peroxisomal acyl-coenzyme A oxidase 3 | acox3 | Liver | GGGGAGGAGGAGGAGGAGGAGGAG |
| Sterol regulatory element – binding proteins 1 | srebp1 | Liver | GCTGCTGCTGCTGCTGCTGCTGCTGCT |
| Nesfatin-1 | nuch2 | Brain | CGCTGCTGCTGCTGCTGCTGCTGCTGCT |
| Nesfatin-1-like peptide | nuch1 | Brain | CGCTGCTGCTGCTGCTGCTGCTGCTGCT |
| Neuropeptide Y | npy | Brain | AGGACCGAGTAGGAGGAGGAGGAG |
| Agouti-related protein | agr | Brain | ATGTGCTGCTGCTGCTGCTGCTGCTGCT |
| Pro-opiomelanocortin | pomc | Brain | TCGCTGCTGCTGCTGCTGCTGCTGCTGCT |
| Cocaine- and amphetamine-regulated transcript | cart | Brain | CGCTGCTGCTGCTGCTGCTGCTGCTGCT |
| β-actin | actb | Liver, brain | GCCGAGGAGGAGGAGGAGGAGGAG |

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3. Results

3.1. BPA and DiNP administered with the diet have no effects on body weight and hepatosomatic index (HSI)

The weight of the specimens was measured before and after the treatment, showing that after 3 weeks of EDC exposure the body weight (Fig. 1S-A) and the HSI (Fig. 1S-B) were similar to the control group.

3.2. Alteration of hepatic structure and biochemical composition

Histological analysis of the liver, one of the main target tissues of BPA and DiNP, was performed to determine whether the selected pollutants could induce morphological changes. The histological analysis (Fig. 1A) showed that both EDCs altered the structure of the liver by causing the loss of the typical cord conformation and the hexagonal shape of the hepatocytes. Regarding the treated groups, the hepatocytes by causing the loss of the typical cord conformation and the hexagonal lysis (Fig. 1A) showed that both EDCs altered the structure of the liver pollutants could induce morphological changes. The histological analysis was performed to determine whether the selected pollutants induced changes in the liver. Considering the biochemical composition of the liver assessed by considering the biochemical composition of the liver assessed by FT-IR (Fig. 2), both DiNP and BPA increased the presence of lipids and fatty acids, although the increase induced by BPA HIGH was higher than in the other groups. Surprisingly, the presence of glycogen was dramatically reduced by all the treatments. In accordance with the FT-IR imaging data, the quantitative analysis of lipids and triglycerides (Fig. 3) showed an increase in all treatments evidencing the highest levels in the BPA HIGH group. However, the presence of phospholipids was reduced in all the treated groups, being lowest in the BPA LOW group. The fatty acid (f.a.) chain length was significantly increased in all groups exposed to the EDCs (Fig. 3).

3.3. Changes in EC and EC-like mediator levels after EDC intake

The concentrations of the endocannabinoids were measured at central level (brain), where AEA, 2-AG and PEA were drastically reduced in all treated groups except for OEA, whose levels remained unchanged with the exception for the DiNP LOW group (Fig. 4A). At the hepatic level, AEA was generally decreased by all treatments and the same pattern was observed for PEA and OEA, while 2-AG levels were significantly decreased only in the BPA HIGH group (Fig. 4B). Surprisingly, an unaltered hepatic FAAH enzymatic activity was observed after the treatments (Fig. 5).

3.4. DiNP and BPA-induced changes at transcriptional level

Genes coding for ECS components, food intake and lipid metabolism biomarkers were investigated in the brain and liver of gilthead sea bream by qPCR array. Regarding the brain, relative expression levels were stated in Table 3 and summarized as a heatmap in Fig. 6A. A significant down-regulation of the hepatic expression of pparb in comparison with control, and that of the orexigenic agrp, was also significantly decreased in all four experimental conditions. By contrast, both BPA treatments largely increased the expression of the anorexigenic cart. Another anorexigenic biomarker, pomc, was also significantly upregulated by the BPA HIGH treatment.

Concerning the liver, the relative expression levels shown in Table 4 (heatmap in Fig. 6B), evidenced that hepatic expression of crr2 was higher than that of crr1 and that both DiNP treatments and BPA HIGH resulted in a significant downregulation of the hepatic expression of crr1. Regarding peroxisome proliferator-activated receptors, ppara was by far the most expressed among the three sub-types. For the AEA metabolism, the biosynthetic enzymes nape-plt and adh4 were more expressed than the catabolic faah. About the synthetic enzymes involved in 2-AG levels, the levels of the mRNA coding for the catalytic

Table 2
Characteristics of new assembled sequences according to BLAST searches. *Gene identity determined through BLAST searches: cb1: cannabinoid receptor 1; cb2: cannabinoid receptor 2; ppara: peroxisome proliferator activated α; pparβ: peroxisome proliferator activated β; pparγ: peroxisome proliferator activated receptor γ; faah: fatty-acid amide hydrolase; nape-pld: N-acylphosphatidylethanolamine phospholipase D; adh4: aldehyde reductase domain containing 4; cyt-pla2: cytosolic phospholipase A2; cyclooxygenase 2; dagla: Sn1-specific diacylglycerol lipase alpha; trpv1: transient receptor potential cation channel subfamily V member 1; agpat4: 1-acyl-sn-glycerol-3-phosphate acyltransferase delta; dag1: diacylglycerol O-acyltransferase 1; dag2: diacylglycerol O-acyltransferase 2; lep: leptin receptor; lep: leptin; gata: glyceraldehyde – 3 – phosphate acyltransferase: acox3: peroxisomal acyl-coenzyme A oxidase 3; nucb1: N-acyl-phosphatidylethanolamine phospholipase D; nucb2: N-acyl-phosphatidylethanolamine phospholipase D; nucb2: N-acyl-phosphatidylethanolamine phospholipase D; fi: fatty-acid amide hydrolase; fi: fatty-acid amide hydrolase; fi: fatty-acid amide hydrolase.

| Contigs | Size (nt) | Annotation | Best match | E | CDS | GenBank accession |
|---------|----------|------------|------------|----|-----|------------------|
| C2_14935 | 1491 | cb1 | XP_010750483 | 0.0 | 1-1407 | MG570367 |
| C2_34590 | 1233 | cb2 | XP_019130217 | 0.0 | < 1-1059 | MG570168 |
| C4_03715 | 2749 | faah | XP_019130584 | 0.0 | 253-2013 | MG570169 |
| C2_2102 | 2659 | nape-pld | XP_018549114 | 0.0 | < 1-1224 | MG570170 |
| C2_21189 | 1255 | adh4 | XP_010745261 | 0.0 | 118-1221 | MG570171 |
| C3_2781 | 2479 | cox-2 | AHC09389 | 0.0 | 51-1874 | MG570172 |
| C2_55849 | 3326 | dagla | XP_008279582 | 0.0 | 1-3222 | MG570173 |
| C2_21917 | 3798 | trpv1 | XP_008305127 | 0.0 | 159-2516 | MG570174 |
| C4_06531 | 1753 | agpat4 | XP_019131188 | 0.0 | 229-1356 | MG570175 |
| C2_46062 | 1624 | dag1 | XP_019115372 | 0.0 | 19-1590 | MG570176 |
| C2_8832 | 2168 | dag2 | XP_008285514 | 0.0 | 138-1220 | MG570177 |
| C2_51183 | 4763 | lep | AIH86619 | 8e-147 | 239-3652 | MG570178 |
| C2_31952 | 954 | lep | AIH86602 | 2e-66 | 355-897 | MG570179 |
| C2_30122 | 1394 | gata3 | XP_018558829 | 0.0 | 218-2698 | MG570180 |
| C2_15240 | 3121 | acox3 | XP_02267241 | 0.0 | 121-2196 | MG570181 |
| C2_14540 | 1804 | nucb2 | XP_00828466 | 0.0 | 74-1555 | MG570182 |
| C2_8104 | 1545 | nucb1 | XP_019113030 | 0.0 | 83-1438 | MG570183 |
| C2_56854 | 480 | npy | XP_018523120 | 0.0 | 1-291 | MG570184 |
| C2_31112 | 1051 | agrp | CC78544 | 6e-66 | 75-422 | MG570185 |
| C3_27777 | 611 | cart | XP_017047599 | 4e-68 | 105-425 | MG570186 |
enzymes (abdh6, abdh12) were higher respect the biosynthetic ones (dagla). Among the two isoforms of DGAT, dgat2 was more expressed than dgat1. BPA and DiNP treatments had a large impact on the analyzed hepatic expression, with significant differences in 14 of the 25 selected genes. Dietary treatments with DiNP significantly decreased the hepatic expression of fasn and gpat (only DiNP LOW) and abdh6 (DiNP HIGH). All four dietary treatments significantly decreased srebp1 expression. On the contrary, BPA treatment increased the expression of pparb, pparg (BPA HIGH), cyt-pla2 (BPA LOW), dgat2 and lepa (BPA LOW). This latter gene was also significantly upregulated with the DiNP HIGH treatment.

4. Discussion

Plastic debris is a cocktail of different contaminants, included phthalates and BPA, which are not chemically bound to the polymer matrix and can be released to the nearby environment and consequently, being bioavailable for marine life and being potentially introduced along in the food chain. In this context, the release of BPA from plastics can be affected by the temperature, pH or contact time (Danish Environmental Protection Agency, 2015). Additionally, leachates of plastics additives can depend on the characteristics of the polymer matrix and the additives, including size, solubility or volatility (Lithner et al., 2011).

Thus, in this regard, the results herein obtained show that both chemicals induce alterations in the central and hepatic ECS, as well as produce morphological and biochemical changes in the liver.

In the brain, the ECS plays mostly an orexigenic role by favoring food intake via CB1 and AEA/2-AG signaling (Williams and Kirkham, 1999; Colombo et al., 1998; Di Marzo and Matias, 2005; Kirkham et al., 2002). In the present study, both BPA and DiNP induce a decrease of AEA and 2-AG levels associate with a downregulation of cnr1 and npy (orexigenic signal) mRNA in all treated groups. Based on the expression of genes linked with the appetite control, the alterations seem more notable in the BPA than in the DiNP groups due to the significant up-regulation of pomc and cart, both coding for anorexigenic signals (Lau...
To gain further insight in the effects of the two EDCs, we also measured the levels of PEA and OEA, which is known as an endogenous anorexic molecule acting at the peripheral level (Hansen, 2014). PEA and OEA exert their activity, among others, through PPARα, with PEA being significantly less effective than OEA in food intake suppression (Avraham et al., 2013). Therefore, the increase of OEA in the DiNP LOW group could be considered a further marker of satiety being induced by this additive.

These data may explain why BPA can negatively regulate the appetite as previously evidenced in S. aurata with a dose of 5000 μg/kg/day (Maradonna et al., 2015). Similarly, in male mice exposed to BPA at 5 or 5000 μg/kg/day during fetal development, food intake remained unaltered or decreased, respectively (Angle et al., 2013) whereas in rats (both male and female), perinatal exposure to 50 μg/kg/day did not alter the food intake (Wei et al., 2011). Regarding DiNP, its administration at higher dosages in male rats for two weeks and for two years, decreased or did not alter the food intake (Kwack et al., 2010; Lington et al., 1997). Interestingly, a recent in vitro study using pico- and nano-molar doses of DiNP reported a decrease of npy mRNA expression in human neuronal cells (Rendel et al., 2017), confirming its negative effect on appetite control. Despite a reduction of appetite might be hypothesized also in gilthead sea bream, all the treatments induced a higher accumulation of lipids (i.e. fatty acids, triglycerides, ACVL) in the liver. These findings suggested an over-activity of the ECS at the hepatic level. In fact, in different animal models, it has been demonstrated that the induction of CB1 increases de novo fatty acid biosynthesis through the transcription factor SREBP1c and its target enzyme, FAS (Osei-Hyiaman et al., 2005) jointly with an elevation of AEA. On the contrary, CB1 antagonists improve hepatic lipid metabolism and stimulate β-oxidation (Jourdan et al., 2012). Controversially, our data indicated a reduction of the ECS activity, with reduced cnr1 mRNA and AEA levels and, subsequently, srebpl expression, suggesting a negative feedback among the ECS signaling and the liver. In addition, the genes coding for the enzymes of de novo phospholipid synthesis pathway, such as agpat4 and gpat were downregulated. However, dgat2, which is in charge of the last step of triglycerides (TAG) biosynthesis was upregulated in parallel with the elevated presence of TAG in the tissue, although phospholipids were decreased. Phospholipids such as phosphatidic acid (PA) and its derivatives, as the phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phosphatidylcholines (PC), are the precursors of the endocannabinoids (AEA and 2-AG) and EC-like mediators (PEA and OEA) (Astarita et al., 2008; Cadas et al., 1996; Fonseca et al., 2013; Hermansson et al., 2011). Furthermore, PA is an intermediate step for the biosynthesis of TAG. Considering these data, we speculate that BPA and DiNP downregulate the lipid biosynthesis, especially until the production of PA, thereby inducing, among others, a

Fig. 2. False color maps integrated under the lipids stretching regions (2990–2836 cm⁻¹) for lipids, fatty acid and glycogen (Gly) in livers from fish treated with BPA and DiNP. False color scale on the right.

Fig. 3. Graphics showing the results of FT-IR imaging analysis. Data are expressed as means ± SEM in a.u. (dimensional units); letters above each column indicate statistical difference among groups (p < 0.05, one-way ANOVA).
downregulation of endocannabinoids levels. However, TAGs are still biosynthesized, possibly from fatty acids coming from the mobilization of glycogen as a precursor, whose levels resulted significantly down-regulated, as observed by the FT-IR imaging. Fatty acids, whose levels were accordingly increased, would be incorporated in the last step of TAG biosynthesis catalyzed by the enzyme codified by dgat2 gene, which was upregulated in fish exposed to both contaminants, as summarized in Fig. 7. Interestingly, of the two genes coding for DGAT, dgat1 and 2, the latter exhibited higher expression than the former. Indeed, DGAT2 is likely more important for TAG biosynthesis than DGAT1 due to its higher affinity for substrates (Yen et al., 2008). In addition, fine regulation of TAG biosynthesis is achieved by SREBP1c, PPARγ and LXR’s (Coleman and Lee, 2004). SREBP-1c activates genes involved in fatty acid and TAG biosynthesis, such as FAS or GPAT. However, despite the high levels of TAG observed in the livers, srebp1 was downregulated, possibly due to a negative feedback mechanism, as mentioned above. Regarding PPAR’s, generally, all the treatments in the present study increased their expression, being surprising, due to the distinct roles of ppara and pparγ. In fact, ppara stimulates fatty acid β-oxidation while pparγ favors lipid biosynthesis and storage, leading to hepatic steatosis. Such upregulation may be explained since xeno-contaminants can interfere with nuclear transcriptional regulators, such as PPAR’s, indeed DiNP and BPA are per se considered to possess PPAR activity in humans and rodents (Grün and Blumberg, 2009; Kaufmann et al., 2002; Valles et al., 2003). As previously reported (Grün and Blumberg, 2009), PPAR’s contain some of the largest and most promiscuous ligand binding pockets, allowing a large variety of agonist, including EDCs.

Regarding other markers of hepatosteatosis, the expression of lepa seems not to follow the same pattern as the receptor (lepr), since the ligand was unaltered. Huang et al. (2006) demonstrated that leptin decreases hepatic TAG levels and TAG secretion, and hence, increases fatty acid oxidation, but our selected markers for fatty acid mobilization including acox-3 or hl were unaffected. Only OEA levels, which mitigates steatosis in liver (Li et al., 2015), was markedly increased following the dietary intake of the low dose of DiNP. More than with TAG metabolism, human leptin levels have been positively associated with BPA concentration in serum (Rönn et al., 2014) or in perinatal exposure rats to 50 μg BPA/bw/day (Wei et al., 2011), in agreement with the upregulation of lepa in the BPA LOW group observed here.

5. Conclusions

In summary, herein we demonstrated that in gilthead sea bream, as previously found in zebrafish, the intake of two common plastic compounds reported to have distinct disruptive activity, the BPA with estrogenic like activity and the DiNP with anti-androgenic like activity, alters the endocannabinoid signaling, the liver composition and the lipid biosynthesis, exerting a similar pro-hepatosteatotic role, probably not through the increase of food intake, but rather through altering the mobilization of the internal resources as described in Fig. 7. Being the
Table 3
Relative expression of genes expressed in the brain of gilthead sea bream. Data are expressed as means ± SEM. All data are normalized to the expression level of \textit{lepr} of control fish with an arbitrarily assigned value of 1. Asterisks \( (*) \) indicate significant differences between the treatment and control group, \( * (p < 0.05) \); \( ** (p < 0.01); *** (p < 0.001); **** (p < 0.0001) \). Evaluated by one-way ANOVA with Tukey’s post-test \( (p < 0.05) \).

| Gene   | Control       | BPA low | BPA high | DiNP low | DiNP high |
|--------|---------------|---------|----------|----------|-----------|
| cnr1   | 7.44 ± 1.06   | 4.17 ± 0.50\( * \) | 5.45 ± 0.90 | 2.16 ± 0.25\( ** \) | 5.45 ± 0.98 |
| cnr2   | 0.017 ± 0.003 | 0.021 ± 0.001 | 0.022 ± 0.002 | 0.025 ± 0.003 | 0.017 ± 0.002 |
| ppar \( \alpha \) | 5.23 ± 0.93   | 5.34 ± 0.63 | 3.49 ± 0.52 | 7.09 ± 1.05 | 5.56 ± 0.49 |
| ppar \( \beta \) | 1.22 ± 0.10   | 1.44 ± 0.10 | 1.26 ± 0.15 | 1.76 ± 0.14 | 1.45 ± 0.12 |
| ppar \( \gamma \) | 1.60 ± 0.11   | 1.45 ± 0.08 | 1.40 ± 0.03 | 1.91 ± 0.09 | 1.42 ± 0.09 |
| faah   | 0.075 ± 0.01  | 0.121 ± 0.01 | 0.091 ± 0.02 | 0.111 ± 0.01 | 0.105 ± 0.01 |
| nape-pld | 4.31 ± 0.23   | 4.68 ± 0.22 | 4.68 ± 0.66 | 5.60 ± 0.28 | 4.77 ± 0.28 |
| abhd4  | 3.31 ± 0.07   | 1.67 ± 0.19 | 1.17 ± 0.11 | 1.77 ± 0.14 | 1.40 ± 0.07 |
| cry-pla2 | 0.139 ± 0.02  | 0.141 ± 0.01 | 0.139 ± 0.02 | 0.116 ± 0.01 | 0.105 ± 0.01 |
| cox2   | 0.042 ± 0.01  | 0.040 ± 0.01 | 0.061 ± 0.01 | 0.044 ± 0.01 | 0.029 ± 0.01 |
| dagla  | 7.72 ± 0.97   | 8.35 ± 0.59 | 8.83 ± 0.55 | 8.32 ± 1.13 | 7.87 ± 0.58 |
| abhd 6a | 2.36 ± 0.15   | 2.31 ± 0.09 | 2.68 ± 0.35 | 2.68 ± 0.34 | 2.25 ± 0.14 |
| abhd 12a | 0.81 ± 0.10   | 1.16 ± 0.15 | 1.02 ± 0.12 | 1.01 ± 0.07 | 0.88 ± 0.10 |
| nucb2  | 3.73 ± 0.40   | 4.02 ± 0.21 | 3.71 ± 0.19 | 4.51 ± 0.44 | 4.28 ± 0.24 |
| nucb1  | 6.97 ± 0.46   | 7.37 ± 0.34 | 6.61 ± 0.45 | 7.95 ± 0.28 | 7.00 ± 0.21 |
| npy    | 22.41 ± 3.68  | 11.40 ± 1.15\( ** \) | 10.32 ± 2.38\( ** \) | 7.99 ± 1.56\( ** \) | 10.01 ± 1.36\( ** \) |
| lepr   | 1.06 ± 0.09   | 1.38 ± 0.11 | 1.37 ± 0.12 | 1.12 ± 0.05 | 1.09 ± 0.07 |
| agrp   | 2.37 ± 0.42   | 2.37 ± 0.28 | 3.37 ± 0.41 | 1.57 ± 0.22 | 1.86 ± 0.28 |
| pome   | 0.060 ± 0.02  | 0.068 ± 0.02 | 0.58 ± 0.07\( **** \) | 0.045 ± 0.01 | 0.037 ± 0.01 |
| cart   | 0.815 ± 0.12  | 4.75 ± 0.65\( **** \) | 3.11 ± 0.56\( ** \) | 0.317 ± 0.12 | 0.027 ± 0.01 |

Fig. 6. Heatmaps of the effects of dietary treatments on the gene expression of selected genes in the brain (A) and liver (B). Scales of colors represent fold-change values in comparison to control (green, down-regulation; red, up-regulation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Table 4
Relative expression of genes expressed in the liver of gilthead sea bream. Data are expressed as means ± SEM. All data are normalized to the expression level of Table 4.

| Gene       | Control     | BPA low       | BPA high      | DiNP low    | DiNP high    |
|------------|-------------|---------------|---------------|------------|-------------|
| cer1       | 0.31 ± 0.04 | 0.31 ± 0.04   | 0.13 ± 0.03***| 0.16 ± 0.03***| 0.11 ± 0.00***|
| cer2       | 0.56 ± 0.07 | 0.42 ± 0.04   | 0.54 ± 0.06   | 0.31 ± 0.06* | 0.43 ± 0.08  |
| trpv1      | 1.03 ± 0.08 | 0.96 ± 0.17   | 1.40 ± 0.19   | 1.01 ± 0.14 | 1.02 ± 0.17  |
| ppar α     | 135.1 ± 10.71| 144.3 ± 18.29 | 157.6 ± 10.95| 134.9 ± 6.01| 173.7 ± 18.71|
| ppar β     | 32.59 ± 4.31| 63.61 ± 2.06***| 60.81 ± 6.08***| 36.28 ± 4.02| 35.73 ± 3.72  |
| ppar γ     | 28.70 ± 2.72 | 31.67 ± 2.21  | 53.91 ± 4.00***| 33.44 ± 3.06| 36.77 ± 4.75  |
| fasb       | 0.80 ± 0.10 | 0.93 ± 0.07   | 1.06 ± 0.10   | 0.74 ± 0.08 | 0.93 ± 0.08   |
| nupr-pkd   | 75.51 ± 6.35 | 51.29 ± 5.31*  | 58.83 ± 4.12  | 59.67 ± 7.27  | 63.34 ± 6.74  |
| aldh6        | 18.80 ± 2.23 | 21.05 ± 2.32  | 23.15 ± 2.27  | 18.41 ± 2.71  | 19.15 ± 2.25  |
| cyt-pla2    | 1.19 ± 0.09 | 1.71 ± 0.12*  | 1.58 ± 0.13   | 1.50 ± 0.13   | 1.52 ± 0.16   |
| cox2       | 0.78 ± 0.07 | 0.98 ± 0.05   | 0.57 ± 0.03   | 0.62 ± 0.08   | 1.00 ± 0.13   |
| dagl       | 1.08 ± 0.09 | 0.76 ± 0.12   | 0.88 ± 0.09   | 0.94 ± 0.11   | 1.08 ± 0.11   |
| aldh12a     | 56.68 ± 5.37 | 40.87 ± 4.57  | 40.37 ± 3.24  | 45.34 ± 5.39  | 37.16 ± 3.87* |
| aldh1     | 128.9 ± 5.95 | 163.4 ± 14.44 | 166.6 ± 12.88 | 141.1 ± 9.03  | 128.9 ± 10.81 |
| fasn       | 40.47 ± 3.27 | 44.36 ± 3.24  | 34.60 ± 6.24  | 18.75 ± 2.57**| 41.17 ± 5.69  |
| agpat4     | 0.60 ± 0.05 | 0.43 ± 0.05   | 0.40 ± 0.06*  | 0.43 ± 0.04*  | 0.34 ± 0.03*  |
| dga2       | 4.69 ± 0.64 | 4.33 ± 0.55   | 4.53 ± 0.46   | 6.13 ± 0.63   | 3.56 ± 0.39   |
| gpat       | 140.4 ± 6.90 | 195.5 ± 11.67*| 195.4 ± 12.98*| 165.6 ± 11.51| 168.3 ± 12.18|
| hl         | 24.89 ± 2.13 | 18.39 ± 1.81  | 23.60 ± 3.06  | 16.23 ± 1.43*| 18.88 ± 1.95  |
| lrr         | 528.7 ± 36.49| 552.0 ± 35.94 | 638.8 ± 40.14 | 409.7 ± 38.53| 499.0 ± 32.43|
| ispr       | 77.96 ± 5.25 | 91.50 ± 5.20  | 101.90 ± 6.80*| 68.53 ± 5.71| 85.48 ± 6.82  |
| lpa         | 31.53 ± 2.98 | 31.73 ± 2.61  | 34.28 ± 3.07  | 28.08 ± 2.46  | 30.23 ± 2.48  |
| acox3      | 4.74 ± 0.87 | 15.87 ± 2.58***| 8.36 ± 1.95*   | 6.13 ± 1.54  | 12.64 ± 2.24* |
| sreb1      | 89.00 ± 9.64 | 63.77 ± 6.29*  | 62.30 ± 6.01*  | 49.87 ± 5.09***| 59.30 ± 5.06*|

Fig. 7. Summarized findings for BPA and DiNP. Alteration of the biosynthesis of endocannabinoids, triacylglycerol and its metabolic pathways. CL, cardiolipin; DAG, diacylglycerol; FA, fatty acid; G-3-P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphati-dylserine; TAG, triacylglycerol.

effects here evidenced similar to those observed in zebrafish, we suggest the ECS as a novel target for the study of chemicals with estrogenic and anti-androgenic effects, and as new innovative endpoints for the development of upcoming OECDs test guidelines.

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Conflicts of interest

None.

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References

Aambre, B.M., Do, R.P., Ponzi, D., Stahlhut, R.W., Drury, B.E., Nagel, S.C., Welshons, W.V., Besch-Williford, C.L., Palanza, P., Parmigiani, S., vom Saal, F.S., Taylor, J.A., 2013. Metabolic disruption in male mice due to fetal exposure to low but not high doses of bisphenol A (BPA): evidence for effects on body weight, food intake, adipocytes, adipocytokines, leptin, adiponectin, insulin and glucose regulation. Reprod. Toxicol. 42, 256–268. http://dx.doi.org/10.1016/j.reprotox.2013.07.017.

Astarita, G., Ahmed, F., Piomelli, D., 2008. Identiﬁcation of biosynthetic precursors for the endocannabinoid anandamide in the rat brain. J. Lipid Res. 49, 48–57. http://dx.doi.org/10.1194/jlr.M700354-JLR200.

Avraham, Y., Katzhendler, J., Vorobeiv, L., Merchavia, S., Listman, C., Kunkes, E.,
