Linking chemical exposure to lipid homeostasis: a municipal waste water treatment plant influent is obesogenic for zebrafish larvae

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ABSTRACT

· *Abbreviations:* ACC, acetyl-CoA carboxylase; ACOX, acyl-CoA oxidase; C/EBPα, CCAAT/enhancer-binding protein α ; DBD, DNA binding domain; DEHP, (2-ethylhexyl) phthalate; DGAT2, diacylglycerol acyltransferase; DMSO, dimethylsulfoxide; Dre, *Danio rerio*; EDC, endocrine disrupting chemical; FAS, fatty acid synthase; HFD, high fat diet; HR, hinge region; Hs, *Homo sapiens;* LBD, ligand binding domain; LSD, least significant difference; LXR, liver X receptor; MoA, mode of action; MSC, mesenchymal stem cells; NR, nuclear receptor; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; SD, standard diet; SEM, standard error of the mean; SREBP, sterol regulatory element-binding protein; TBT, tributyltin; WAT, white adipose tissue; WWTP, wastewater treatment plant.

Obesity, a risk factor for the development of type-2 diabetes, hypertension, cardiovascular disease, hepatic steatosis and some cancers, has been ranked in the top 10 health risk in the world by the World Health Organization. Despite the growing body of literature evidencing an association between the obesity epidemic and specific chemical exposure across a wide range of animal taxa, very few studies assessed the effects of chemical mixtures and environmental samples on lipid homeostasis. Additionally, the mode of action of several chemicals reported to alter lipid homeostasis is still poorly understood. Aiming to fill some of these gaps, we combined an *in vivo* assay with the model species zebrafish (*Danio rerio*) to screen lipid accumulation and evaluate expression changes of key genes involved in lipid homeostasis, alongside with an *in vitro* transactivation assay using human and zebrafish nuclear receptors, retinoid X receptor α and peroxisome proliferator-activated receptor γ. Zebrafish larvae were exposed from 4 day post-fertilization until the end of the experiment (day 18), to six different treatments: experimental control, solvent control, tributyltin at 100 ng/L Sn and 200 ng/L Sn (positive control), and wastewater treatment plant influent at 1.25% and 2.5%. Exposure to tributyltin and to 2.5% influent led to a significant accumulation of lipids, with white adipose tissue deposits concentrating in the perivisceral area. The highest *in vitro* tested influent concentration (10%) was able to significantly transactivate the human heterodimer PPARγ/RXRα, thus suggesting the presence in the influent of HsPPARγ/RXRα agonists.

Our results demonstrate, for the first time, the ability of complex environmental samples from a municipal waste water treatment plant influent to induce lipid accumulation in zebrafish larvae.

Keywords: obesity, obesogen, endocrine disruptor, environmental mixture, nuclear receptor.

1. INTRODUCTION

Obesity, the result of a prolonged disturbance of energy homeostasis favoring triglyceride storage and adipocyte hypertrophy, has been reported to be reaching epidemic proportions, affecting developed and developing countries and increasing prevalence among children and animals (Chamorro-García and Blumberg, 2014; Grün and Blumberg, 2009; Landgraf *et al.*, 2017; Newbold *et al.*, 2007; Minchin & Rawls, 2011; Santos *et al.*, 2012). In addition of being a risk factor for type-2 diabetes, hypertension, cardiovascular disease, hepatic steatosis, premature mortality and some cancers, among other medical conditions (Grün and Blumberg, 2009; Landgraf *et al.*, 2017; Minchin and Rawls, 2011; Newbold *et al.*, 2007), obesity has a detrimental impact on economic development costing billions of dollars annually in direct or indirect care (Spencer & Tilbrook, 2011). Consequently, it is ranked in the top 10 health risks in the world by the World Health Organization, with the number of overweight people exceeding the number of undernourished ones worldwide (Newbold *et al.*, 2007).

An increasing body of evidence suggest that this condition does not develop solely from a chronic positive energy balance (high caloric intake *versus* lack of exercise), but is rather a consequence of complex interactions between genetic and environmental factors (Bašić *et al.*, 2012; Capitão *et al.*, 2017; Chamorro-García and Blumberg, 2014; Dimastrogiovanni *et al.*, 2015; Grün and Blumberg, 2009; Lyssimachou *et al.*, 2015; Minchin and Rawls, 2011; Newbold *et al.*, 2007; Santos *et al.*, 2012; Spencer and Tilbrook, 2011). The disruption of energy homeostasis by endocrine disrupting chemicals (EDCs) is an emerging new field and could explain the rapid increase in obesity rates observed in animals living in close proximity to human populations in industrialized societies (Chamorro-García and Blumberg, 2014; Grün and Blumberg, 2009; Klimentidis *et al.*, 2011; Lyssimachou *et al.*, 2015; Newbold *et al.*, 2007), which coincided with the massive release of industrial chemicals over the past decades (Newbold *et al.*, 2007; Santos *et al.*, 2012).

Lipid homeostasis is controlled by complex networks and feedback mechanisms involving several body organs, transcription factors (e.g. nuclear receptors (NR)), enzymes, hormones and nutrients, with a complex cross-talk between different signaling pathways (Capitão *et al.*, 2017, 2018; Moseti *et al.*, 2016). The central nervous system is the coordinator of all these processes, acting as a lipid and hormone sensor and integrating and transforming afferent information from other organs into signals that will ensure necessary adjustments, for example, by changing feeding behavior or energy expenditure (Lyssimachou *et al.*, 2015; Santos *et al.*, 2012). The adipose tissue, besides being one of the main tissues for the occurrence of lipogenesis and where the excess of fatty acids are stored in the form of triglycerides (Kersten, 2001; Lyssimachou *et al.*, 2015; Moseti *et al.*, 2016; Santos *et al.*, 2012), functions also as an endocrine and immune organ (Bašić *et al.*, 2012; Birsoy *et al.*, 2013; Santos *et al.*, 2012).

Members of the peroxisome proliferator-activated receptors (PPARs), transcription factors from the NR superfamily play essential roles in adipocyte differentiation and energy homeostasis, forming obligate heterodimers with the retinoid X receptor (RXR) which changes the transcription of target genes upon the binding of a ligand to the ligand binding domain (LBD) (Janesick and Blumberg, 2011; Santos *et al.*, 2018, 2012; Yoon, 2009). Each subtype possesses different ligand specificity, tissue distributions and biological functions (Santos *et al.*, 2012; Yoon, 2009).

Widely known as the “Master regulator of adipogenesis”, due to its absolute requirement for the process, PPARγ is mostly expressed in adipocytes and directly induces genes that regulate the adipogenic cascade, glucose uptake, lipid uptake, synthesis and storage (Birsoy *et al.*, 2013; Janesick and Blumberg, 2011; Lyssimachou *et al.*, 2015; Moseti *et al.*, 2016; Santos *et al.*, 2012). As its ligand-binding pocket is large enough to accept a variety of chemical structures, this NR is particularly sensitive to environmental chemicals (Capitão *et al.*, 2018; Janesick and Blumberg, 2011; Lyssimachou *et al.*, 2015) and since the

RXR:PPARγ heterodimer is permissive, either receptor pocket can be targeted by ligands and mediate its signaling (Grün & Blumberg, 2009). Moreover, PPARs occur in vertebrate and some non-vertebrate groups (Cephalochordates, Urochordates, Molluscs and Echinoderms) and RXRs in most metazoans, indicating a wide taxonomic scope of the inappropriate regulation of these pathways (Lyssimachou *et al.*, 2015; Santos *et al.*, 2012). Moreover, RXR is the heterodimeric partner of several other nuclear receptors that are involved in lipid homeostasis to some extent [e.g. Liver X Receptor (LXR) and Farnesoid X Receptor] and also form permissive heterodimers (Capitão *et al.*, 2017).

Obesogens are a class of EDCs capable of interfering with the lipid metabolic pathways either directly, through interaction with NR leading to changes in patterns of gene expression stimulating adipogenesis and fat accumulation, or indirectly by mimicking or blocking the action of natural hormones and regulating appetite and satiety (Grün and Blumberg, 2007; Janesick and Blumberg, 2011; Lyssimachou *et al.*, 2015; Newbold *et al.*, 2007). Yet, several other chemicals may affect lipid homeostasis through mechanisms that do not lead to lipid accumulation (Coimbra et al. 2015; Capitão et al., 2017). Natural or xenobiotics, these chemicals are widely used in everyday products (e.g. food packages, medical devices, toys, cosmetics) (Janesick & Blumberg, 2011), from which they might leach promoting their ubiquitous presence in the environment and threatening not only human populations worldwide, but also the ecosystems (Capitão *et al.*, 2017).

The exact MoA of some of these chemicals has not yet been determined, as they may act on the several distinct levels aforementioned. Exposure to lipid homeostasis regulators *in utero* and during early development can alter developmental programming leading to obesity later in life (Bašić *et al.*, 2012; Grün and Blumberg, 2007; Newbold *et al.*, 2007; Santos *et al.*, 2012), as evidenced by mesenchymal stem cells (MSC) with fates biased towards the adipogenic lineage following exposure (Chamorro-Garcia & Blumberg, 2014). Other possible MoA include epigenetic modifications which could modify the expression of genes

(Bašić *et al.*, 2012; Chamorro-Garcia & Blumberg, 2014; Newbold *et al.*, 2007) and lead to an obesogenic effect at a transgenerational level (Chamorro-Garcia *et al.*, 2017; Chamorro-García and Blumberg, 2014). Post-translational modifications, reported to occur in several enzymes and transcription factors involved in lipid metabolism (Carnitine palmitoyltransferase I (Guan *et al.* 2016), ACC (Capitão *et al.*, 2017; Wang *et al.*, 2015), PPARγ (van Beekum *et al.*, 2009), SREBP (Wang and Sul, 2013)), could affect the interactions of NRs with the transcriptional machinery and the processing, activity, stability and degradations of proteins, as well as their affinity for certain substrates (Guan *et al.*, 2016; Janesick and Blumberg, 2011; Lempradl *et al.*, 2015; Wang and Sul, 2013). In addition to the conformational change in the receptors and induction of transcriptional activation through the binding of a ligand, nuclear receptors can also be activated or unrepressed through these post-translational modifications in the absence of ligands (Janesick & Blumberg, 2011).

Amongst the 1300 chemicals identified as potential EDCs, very few have been evaluated through lipid homeostasis disruption endpoints (Capitão *et al.*, 2017). So far, identified obesogens include members of the organotins, thiazolidinediones, phthalates, bisphenol-A and its polybrominated and polychlorinated derivatives, organochlorine pesticides, organophosphates, carbamates, solvents and heavy metals such as cadmium and lead, as well as estrogenic chemicals and phytoestrogens (Biemann *et al.*, 2014; Grün and Blumberg, 2007; Janer *et al.*, 2007; Janesick and Blumberg, 2011; Jordão *et al.*, 2015, 2016 Newbold *et al.*, 2007; Riu *et al.*, 2014, 2011; Santos *et al.*, 2012; Watt and Schlezinger, 2015), but in addition to the lack of information regarding their MoA, data concerning their interactions in mixtures, as they are found in the environment, is scarce (Biemann *et al.* 2014; Capitão *et al.*, 2017).

The overall aim of this study was to evaluate the effects of complex environmental samples from a waste water treatment plant (WWTP) on lipid accumulation in zebrafish larvae. As a

147 proxy to better understand the underlying MoA of this complex samples, primary
148 approaches were also conducted using transactivation assays with PPARγ/RXRα, alongside
149 with the determination of expression of key lipogenic and adipogenic genes in zebrafish
150 larvae.

151 ## 2. METHODOLOGY

152 ### 2.1. *In vivo* assay

153 #### 2.1.1. Species selection

154 The zebrafish (*Danio rerio*), a freshwater cyprinid in the Actinopterygii class, is an
155 emerging model for the study of lipid metabolism and metabolic diseases (Hölttä-Vuori *et*
156 *al.,* 2010; Landgraf *et al.,* 2017). Besides the widely known advantages of this species (e.g.
157 small size, known ideal growth conditions, large number of eggs, short life cycle), it shows
158 a high conservation of genes, NRs and molecular processes regulating energy homeostasis
159 between mammals and teleosts and the ability to develop metabolic diseases also reported
160 in humans (e.g. hepatic steatosis) (Birsoy *et al.,* 2013; Hill *et al.,* 2005; Hölttä-Vuori *et al.,*
161 2010; Landgraf *et al.,* 2017; Lyssimachou *et al.,* 2015). Studying altered gene expression in
162 response to toxic insults by real-time PCR (qPCR) or genomic screens is facilitated since
163 the zebrafish genome is completely sequenced (Hill *et al.,* 2005; Veldman and Lin, 2008).
164 Another advantage is its optical clarity which allows the screening of lipid accumulations
165 using staining techniques with fluorescent dyes in small larvae without dissecting them.

166 #### 2.1.2. Animal breeding

167 All experiments with living fish were conducted in the facilities of BOGA (Biotério dos
168 Organismos Aquáticos – CIIMAR) and have been approved by the CIIMAR ethical
169 committee and by CIIMAR Managing Animal Welfare Body (ORBEA) according to the
170 European Union Directive 2010/63/EU.

A breeding stock of adult wild-type zebrafish (*Danio rerio*; Singapore) was kept in a 250L aquarium with dechlorinated aerated water (28 ± 1°C), under a photoperiod of 14:10h (light:dark). The fish were fed with commercial diet Tetramin (Tetra, Germany) twice a day. Nine females and nine males were placed in cages within a 30L aquarium, the cages possessing a net bottom covered with glass marbles, preventing eggs from being consumed by adults.

In the following morning, fertilized eggs were collected, cleaned and randomly allocated to different experimental aquaria.

2.1.3. Test conditions

The embryos obtained by natural mating were raised in 3L aquaria with dechlorinated water (28 ± 1 °C) with a 14L:10D photoperiod. Each aquarium contained 80 eggs.

From hatching, 4th day post-fertilization (dpf) until the end of the experiment (18^{th} dpf), the larvae were exposed to six different treatments in duplicate: experimental control, solvent control (0.0002% dimethylsulfoxide, DMSO), tributyltin (TBT) at 100 ng/L Sn and 200 ng/L Sn (positive control), and wastewater treatment plant influent at 1.25% and 2.5%. TBT was selected as a positive control given that Lyssimachou et al., (2015) previously demonstrated massive fat accumulation in zebrafish following a life-cycle exposure to environmentally relevant concentrations. TBT test solutions were obtained by successive dilutions of a stock solution containing TBT chloride (96%, Sigma-Aldrich) in DMSO (99.9%, Sigma-Aldrich). All aquaria had the same DMSO concentration with exception of experimental control. The 24-h composite WWTP influent was obtained at the Porto municipal waste water treatment plant facility in 2017, between 3 and 9 of May, encompassing a full week from Wednesday to Tuesday. Samples were collected daily for seven consecutive days, frozen immediately after collection to prevent degradation of the compounds and used within 1 month for the assays, following a similar sampling design of

196 that described by González-Mariño *et al.,* (2017) and Gracia-Lor *et al.,* (2017). This WWTP
197 serves a population of approximately 150.000 people (mean flux of 33000 m^3/day)
198 (González-Mariño *et al.,* 2017; Gracia-Lor *et al.,* 2017). The samples were characterized
199 upon collection (mean pH= 7,03; T= 21ºC; Ammonium (mg/L) 47,8). A preliminary
200 characterization of the composite influent was performed by Liquid Chromatography
201 Quadrupole Time-of-flight Tandem Mass Spectrometry (LC-QTOF-MS/MS) with a
202 particular focus on putative PPAR/RXR agonists as detailed in the Supporting Information
203 Table S1 (**Appendix A**). Detected compounds were classified in confirmatory levels as
204 proposed by (Schymanski *et al.,* 2014).

205 The medium was renewed daily, ammonia levels checked weekly using Palintest tablets and
206 Palintest Photometer 7000se (0.3625 ± 0.033 mg/L N and 0.4375 ± 0.0369 mg/L N for the
207 1.25% and 2.5% WWTP influent, respectively, and 0.1425 ± 0.0347 mg/L N for all
208 remaining treatments), while the temperature was monitored daily. Values of mortality were
209 registered daily and the dead larvae/eggs were removed. Total mortality did not differ
210 among groups (data not shown) and was maintained below 20% up to the end of the assays,
211 which is within the normal range of the species in laboratory assays (Coimbra *et al.,* 2015).
212 A light aeration was introduced to the aquaria on the 11th dpf.

213 Throughout the whole experiment, care was taken in order to avoid the suffering and
214 distress of the animals.

215 2.1.4. Feeding protocol

216 From 5 to 14 dpf larvae were fed with a standard diet (SD), Gemma Micro (Skretting),
217 supplemented with cod liver oil (Fagron) to obtain a final lipid content of 25% dry matter.
218 *Artemia spp.* cysts (Brine Shrimp Eggs; Ocean Nutrition) put to hatch for 16 hours were
219 supplied every other day from 7 to 13 dpf (0.2 grams fed 3 aquaria). From the 15^{th} to the
220 16^{th} dpf, larvae were fed lyophilized chicken egg yolk as a high fat diet (HFD), followed by

one day of starvation prior to the analysis on the 18th dpf. The specifications of the feeding regime are summarized in **table 1**.

Table 1. Feeding regime throughout the assay.

	Period (dpf)				
	0 to 4	5 to 11	12 to 14	15 to 16	17 to 18
Feeding type	Not fed	Gemma Micro 100-200 µm	Gemma 100-200 and 200-400 µm	Lyophilized egg yolk; Gemma 200-400 µm	Fasting state
Quantities	None	12 mg x3	24 mg (100-200 µm x2; 200-400 µm x1)	egg yolk 40 mg x1; Gemma 24 mg x2	None

2.1.5. Nile Red staining

On the 18th dpf, 7 larvae of similar size (6.55 ± 0.56 mm) were selected per aquarium and individually incubated with Nile Red (Sigma-Aldrich) (stock of 5 mg/mL in acetone diluted 1:1000 in dechlorinated water) in 3mL-wells plates, for 60 minutes, at 28 °C in the dark. The larvae were rinsed twice with dechlorinated water and anesthetized during 30 seconds with Tricaine 1000mg/g (Pharmaq) (stock of 168 mg/mL diluted 1:1000 in dechlorinated water).

The larvae were analyzed in water within approximately 2 minutes, under the microscope Nikon Eclipse TS100 with fluorescence filter B-2A: EX: 450-490nm, BA: 520nm. All images were captured under the same settings with imaging software NIS-Elements D (version 4.13). Fluorescence signals were determined using ImageJ software

(National Institutes of Health), by converting the images into 8-bit grayscales, measuring the background fluorescence, and subtracting to the value of larvae fluorescence obtained by selecting the white adipose tissue (WAT) and individual adipocytes in the perivisceral area (**Figure 1**).

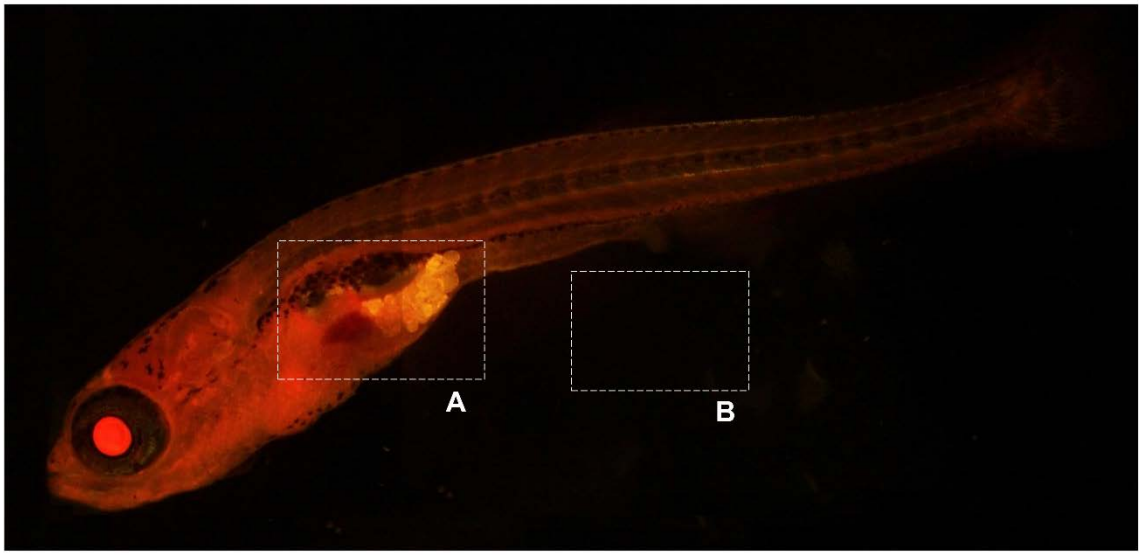

Figure 1. Zebrafish at the 18th dpf colored with Nile Red dye; A: Perivisceral area containing the analyzed WAT and individual adipocytes; B: Example of area for background fluorescence (note: image not converted to 8-bit grayscales).

The remaining zebrafish were euthanized in Ethyl 3-aminobenzoate methanesulfonate (98%; Sigma-Aldrich) at 200 mg/L to guarantee minimum pain and alleviation from discomfort, according to Annex IV of the EU Directive 2010/63/EU, and were stored in RNAlater (Sigma) for posterior molecular biology determinations.

2.2. Gene expression

2.2.1. RNA extraction and cDNA synthesis

The RNA extraction was performed for each body of larvae using illustra^{TM} RNA spin Mini RNA Isolation kit (GE Healthcare) and the RNA quality was confirmed with 1% agarose

gel. The concentration of total RNA was determined using Gen5™ software and equipment Synergy HT Multi-Mode Plate Reader (BioTek).

Total cDNA was synthesized from 200 ng of RNA using the iScriptTM cDNA Synthesis (Bio-Rad) kit and TGradient Thermocycler (Biometra). All assays were performed by following the kits' protocols.

2.2.2. Real-time PCR

The expression of reference gene β-actin and target genes PPARγ, RXRα, FASn, ACOX, SREBP1, CCAAT/enhancer-binding protein α (C/EBPα) and diacylglycerol acyltransferase (DGAT2), individually, was assessed for each body of larvae separately, using Mastercycler ep realplex system (Eppendorf). Primers (**Table S2**) were designed with Primer-Blast-NCBI tool and synthesized by STABVIDA (Portugal).

Each sample was amplified in duplicate, loaded into 96-well PCR plates containing 10 µL of reaction volume corresponding to 1 µL of cDNA (equals 10 ng), 5 µL of IQ TM SYBR® Green Supermix (Bio-Rad) and 200 nM of each primer.

The two-step qPCR program was performed according to the Taq polymerase's protocol, with a melting curve (55-95°C with 0.5°C increment) being generated at the end to confirm the specificity of each amplification. Products were also visualized through agarose gel electrophoresis, confirming the presence of single bands. The specificity of the amplification was verified by adding a "no template control" to each plate and the efficiency determined for each gene through standard curves with successive dilutions of cDNA pools from each sample (6 dilution steps with dilution factor 1:5). Efficiencies between 88-109% were considered acceptable. The reference gene was validated by its stability among the exposure groups and controls, verified through one-way ANOVA.

The relative quantification of target genes was performed according to Livak's method (Livak & Schmittgen, 2001). Relative changes in gene expression were calculated based on the amount of target normalized to β-actin and relative to an internal calibrator:

$$\text{Amount of target} = 2^{-\Delta\Delta Ct} = (2^{-(Ctsample-Ctcalibrator)_{target}}) / (2^{-(Ctsample-Ctcalibrator)_{reference}})$$

2.3. Transactivation assays

2.3.1. Plasmid constructs

Primers were designed using Primer 3 to amplify the Hinge Region (HR) plus LBD of human and zebrafish RXRα and PPARγ (**Table S3**), with insertion of additional oligonucleotides of recognition sequences for restriction enzymes XbaI and KpnI (Promega).

A rapid amplification of cDNA ends PCR was performed using zebrafish cDNA pool as a template for RXRα and zebrafish liver cDNA for PPARγ. The products were gel-purified and digested with restriction enzymes, for insertion of RXRα and PPARγ in pBIND vectors and insertion of RXRα in a pACT vector. Constructs' integrity and correct orientation of inserts were verified through Sanger sequencing.

2.3.2. Transactivation assay

The characterization of RXRα, PPARγ and heterodimer responses to different compounds was attained using the CheckMate™ Mammalian Two-Hybrid System (Promega). Viable COS-1 cells (kidney, *Chlorocebus sabaeus*), distributed at a density of $2x10^5$ cells/mL were grown in DMEM with phenol red (PANBiotech), supplemented with 1% Penicillin/Streptomycin (PANBiotech) and 10% fetal bovine serum (PANBiotech) for 24h at 37 °C and 5% CO_2. The plasmids were transfected into the cells (confluence around 80%), using lipofectamine (Invitrogen), Opti-MEM 1x (Gibco), 500 ng of pBind and pGL4, as well as 750 ng of pcDNA3 (or pACT for heterodimer assays) and incubated for 5 hours

(André *et al.*, 2017). Zebrafish and human NRs were exposed to the treatments for 24h: DMEM without phenol-red supplemented with 1% Penicillin/Streptomycin and 10% charcoal-treated Fetal Bovine Serum (PANBiotech) was contaminated with DMSO 0.1% (solvent control; 99.9%, Sigma-Aldrich), TBT 250 nM (positive control; TBT chloride 96%, Sigma-Aldrich) and 3 dilutions of WWTP influent (1.25%, 2.5%, 10%).

On the following day, cells were lysed with 1x Passive Lysis Buffer (Promega) and the luciferase activity was detected using the Dual-Luciferase® Reporter Assay System (Promega). The luminescence was measured using equipment Synergy HT Multi-Mode Plate Reader (BioTek), with Gen5™ software. Three replicates were performed for each transactivation assay.

2.4. Statistical analysis

The statistical analysis was performed on IBM SPSS Statistics 24 software, where the homogeneity of variance of all data was confirmed using Levene's tests, prior to analysis through one-way ANOVA.

The Nile-Red fluorescence data, real-time PCR results and transactivation results were further analyzed through Fisher's Least Significant Difference (LSD) post-hoc test.

When parametric assumptions were not attained, even after square-rooting or log-transformating the data, the results were analyzed using non-parametric Mann-Whitney *U* test.

In all cases, *p*-values lower or equal to 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Lipid accumulation

Despite the conservation of several signaling pathways regulating lipid homeostasis throughout metazoans, the majority of studies on the effects of obesogens has focused on

mammalian models (Capitão *et al.*, 2017). Hence for a better understanding of effects and possible MoA of chemicals impacting lipid homeostasis across vertebrates, this study combines *in vivo* assays alongside with molecular and biochemical techniques using the vertebrate model species *Danio rerio*. The study aimed to address two key questions: are complex environmental samples from WWTP able to induce an obesogenic response in zebrafish larvae? If so, and as a proxy to better understand the underlying molecular mode of action, does it involve the modulation of RXRα/PPARγ signaling pathways?

Figure 2 displays the results from the *in vivo* assay presenting the lipid accumulation, analyzed through Nile-red staining. Obtained values correspond to the Integrated Density (the product of area and mean gray value), representing the fluorescence captured for each larva on ImageJ Software (Figure 1). Higher values indicate greater accumulation of lipids.

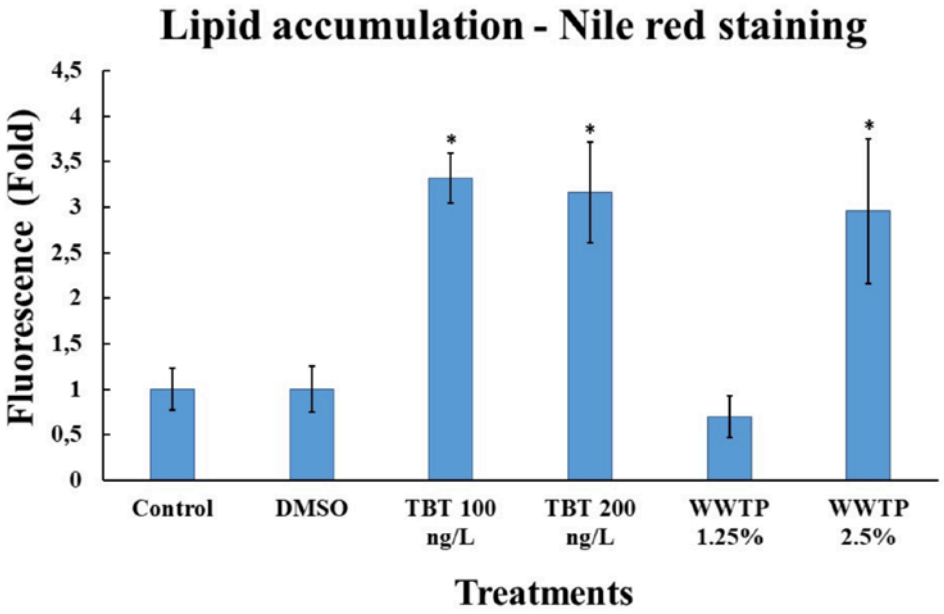

Figure 2. *In vivo* induction of lipid accumulation analyzed by Nile Red staining after exposure to TBT and WWTP influents. TBT and WWTP influent values were expressed

as average fold changes ± SEM (N=7) of the solvent control and experimental control groups, respectively. *$p < 0.05$ (one-way ANOVA, followed by Fisher LSD's test).

A statistically significant increase ($p < 0.05$) in fluorescence was detected in larvae chronically exposed to the environmentally-relevant concentrations of 100 and 200 ng/L Sn TBT and 2.5% WWTP influent, with WAT deposits concentrating in the perivisceral area. Whereas the subcutaneous fat accumulation is considered an adaptive process, exceeding the storage capacity of this adipose depot, diverts the excess of fatty acids towards the accumulation in the visceral cavity, muscle and liver, primary locations for correlations with metabolic disorders and increased mortality (Chamorro-Garcia and Blumberg, 2014; Grün and Blumberg, 2009; Lyssimachou *et al.*, 2015).

Preliminary assays performed by our group with regular diet (Gemma Micro, 59% protein, 19% oil) did not lead to an increase in fat accumulation in TBT exposed larvae (data not shown). Hence, the assays were performed with a SD supplied with additional lipids (25% of lipid content). Tingaud-Sequeira and colleagues (2011), reared zebrafish larvae on a standard diet and separated them into two groups which were fed for a day with different diets, SD or hard-boiled egg yolk as an HFD, followed by two days of starvation. During starvation, the initial adiposity was recorded and larvae were posteriorly exposed for 24h to 50 nM TBT and 1 nM rosiglitazone, with a final adiposity recording. The results indicated that HFD led to a more dramatic increase in lipid adiposity when the fishes were exposed, being more evident in the treatment with rosiglitazone in which, despite the fasting state, an increase in adiposity was obtained in the HFD group, oppositely to the smaller decrease recorded for the SD group when compared to the control. The present study corroborates previous findings of short-term exposures to higher TBT concentrations (Tingaud-Sequeira *et al.*, 2011) and to life-cycle zebrafish exposure to 10 and 50 ng/TBT (Lyssimachou *et al.*, 2015). Similarly, zebrafish larvae exposed for 15 days to tetrabromobisphenol A (TBBPA), tetrachlorobisphenol A, TBBPA-sulfate or TBT, and fed

with egg yolk, exhibited significant increases in lipid accumulation (Riu *et al.,* 2014). The authors also pointed out that when the larvae were fed with regular diets, instead of HFD, none of the treatments resulted in the staining of larvae, highlighting the impact of the synergy of both calories and chemical exposure in lipid accumulation.

As fat build-up results from the balance between lipogenesis and lipolysis (Kersten, 2001), this study assessed the impact of TBT and the WWTP influent on both lipogenesis and adipogenesis focusing in particular in PPARγ:RXRα associated pathways (i.e.,PPARγ:RXRα, SREBP1, C/EBPα, FASn, DGAT2) and also fatty acid oxidation (ACOX1). Bodies of zebrafish larvae from the *in vivo* assay were analyzed by qPCR to determine whether the lipid accumulation verified *in vivo* was accompanied by changes in levels of mRNA of key transcription factors and enzymes ruling lipid homeostasis.

Interestingly, the PCR results (**Figure 3**) only reveal statistically significant differences in the expression of the transcription factor SREBP1 at 100 ng/L Sn TBT and RXRα alongside DGAT2 at 200 ng/L Sn TBT, environmental concentrations that triggered significant lipid accumulations *in vivo*. Both the increase of SREBP1 levels at the lowest concentration and decrease of RXRα expression levels at the highest concentration, suggest a mechanism of negative feedback, whereas the overexpression of DGAT2, the enzyme that catalyzes the final reaction in triglyceride's synthesis (Lyssimachou *et al.,* 2015), suggests that lipid production has not ceased at the highest concentration of TBT. While the lack of altered significant gene expression for 1.25% influent matches the observed lack of lipid accumulation, the similar observation for the 2.5% concentration, which contrastingly induced significant accumulation, could also be due to a negative feedback mechanism. This could explain the 3-fold decrease in gene expression of C/EBPα in larvae exposed to both WWTP influent treatments.

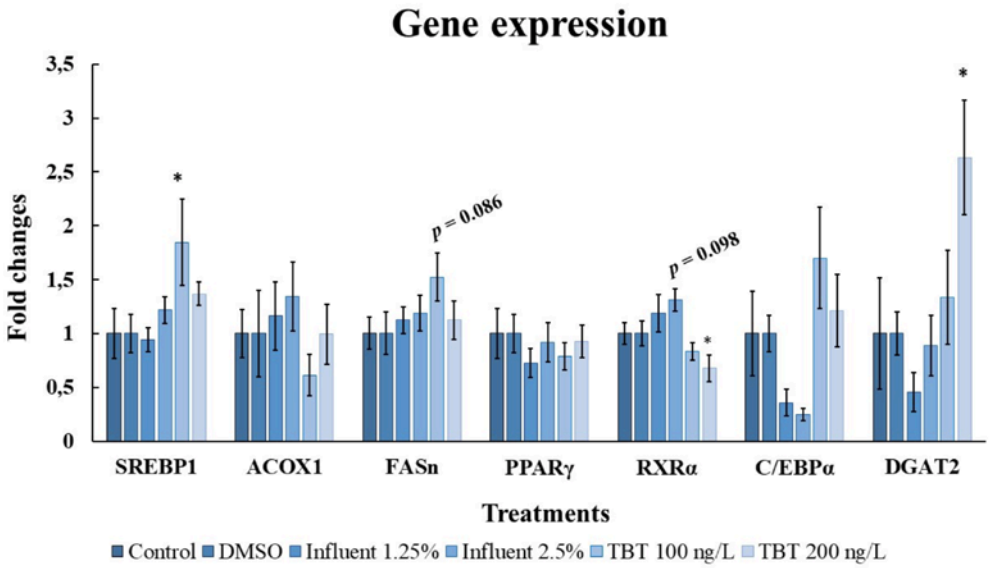

Figure 3. ***In vivo*** **induction of lipogenic and adipogenic genes in zebrafish, following exposure to TBT and WWTP influents**. Values normalized to β-actin and relative to an internal calibrator are expressed as the average fold changes ± SEM of the solvent control and experimental control groups for TBT and the WWTP influents, respectively. *$p < 0.05$ (one-way ANOVA, followed by Fisher LSD's test).

Similarly, Watt and Schlezinger (2015) reported lipid accumulations in bone marrow MSCs of male 9-week-old C57BL/6J mice following exposure to PPARγ-agonists rosiglitazone, TBT and triphenyltin, with no statistical significant increases in PPARγ mRNA, although downstream target genes were up-regulated (Watt & Schlezinger, 2015). Whereas PPARγ plays an important role in lipogenesis and adipogenesis in the adipose tissue, the levels of expression in other tissues are low, for example in the liver where the main mediator of the expression of lipogenic genes is SREBP-1 (Kersten, 2001). Thus, it is not surprising that several studies report organ-specific and sex-specific effects of exposure to TBT and other lipid homeostasis regulators in a variety of model animals (Bian *et al.*,

2017; Chamorro-García *et al.,* 2013; Guan *et al*., 2016; Lyssimachou *et al.,* 2015; Zhang *et al.,* 2012).

Nevertheless, the transcription factor SREBP1, which was overexpressed at 100 ng/L Sn TBT, is an early regulator of adipogenesis and stimulates PPARγ expression for the final adipocyte maturation where they will modulate the expression of adipogenic and lipogenic enzymes, such as ACCα and FASn (Birsoy *et al.,* 2013; Capitão *et al.,* 2017; Kersten, 2001; Lyssimachou *et al.,* 201; Moseti *et al.,* 2016; Santos *et al.,* 2012).

3.2. Transactivation assays

To further address if the WWTP influent and TBT could transactivate or repress the PPARγ/RXRα signaling pathways, we have performed transactivation assays using human (Hs) and zebrafish (Dre) PPARγ and RXRα receptors, as well as with the RXRα:PPARγ heterodimer. The results indicate that both influent and TBT are able to interact with the receptors *in vitro,* which might possibly be one of the mechanisms underlying the observed lipid accumulation and disruption of lipid homeostasis *in vivo*. The organotin TBT was the first obesogen to be associated with RXR and PPARγ signaling (Janesick and Blumberg, 2011; Santos *et al.,* 2012). Indeed, our positive control TBT transactivated HsRXRα, PPARγ and heterodimer at 250 nM (**Figure 4**). Interestingly, the results for the zebrafish receptors (**Figure 5**) show a different response when compared to HsPPARγ. While DreRXRα activity was significantly induced and PPARγ not affected by TBT, the heterodimer was massively repressed at the tested concentration.

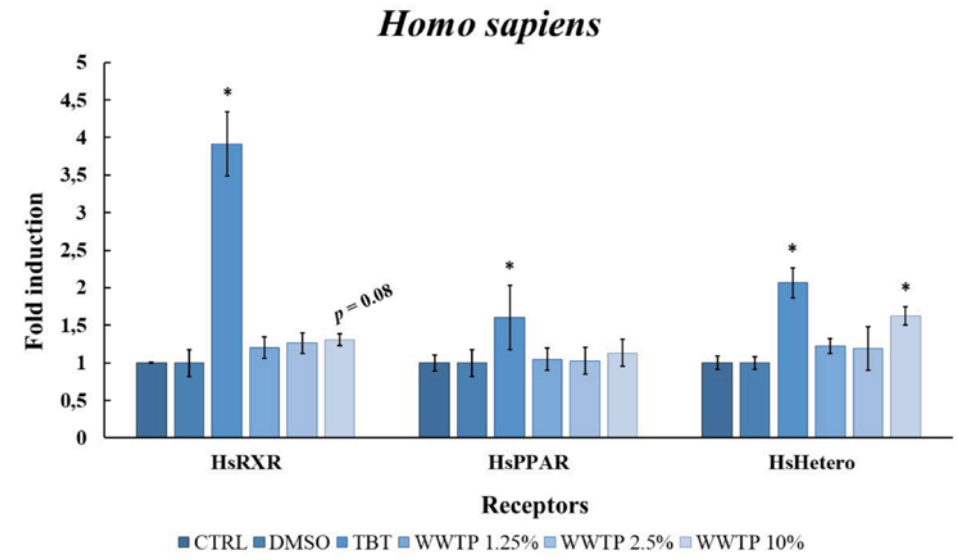

Figure 4. *In vitro* transactivation of *Homo sapiens* receptors by 250 nM TBT and WWTP influent. The activity of firefly luciferase (*Photinus pyralis*) was normalized to the activity of *Renilla* luciferase (*Renilla reniformis*) and then expressed as average fold changes ± SEM of the solvent control group. *$p < 0.05$ (one-way ANOVA, followed by Fisher's LSD test).

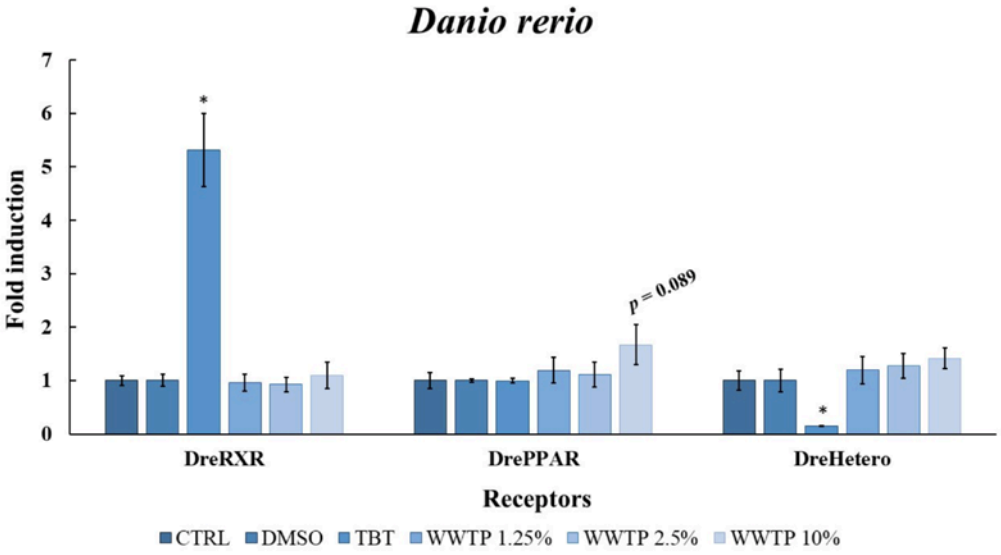

Figure 5. *In vitro* transactivation of *Danio rerio* receptors by 250 nM TBT and WWTP influent. The activity of firefly luciferase (*Photinus pyralis*) was normalized to the activity of *Renilla* luciferase (*Renilla reniformis*) and then expressed as average fold changes ± SEM of the solvent control group. *$p < 0.05$ (one-way ANOVA, followed by Fisher's LSD test).

Analyzing the amino acid sequences of human and zebrafish receptors, it was found a high percentage of similarities (99%) in both RXRα DNA-binding domain (DBD) and LBD, oppositely to the 94% similarities at the PPARγ DBD and 74% at the LBD (Ouadah-Boussouf & Babin, 2016; Zhao *et al.*, 2015), suggesting different ligand-binding affinities for PPARγ. Furthermore, the cysteine in position 285 of HsPPARγ LBD, which is mutated into a tyrosine in the zebrafish (Capitão *et al.*, 2017), has been shown as essential for the activation of this receptor by TBT (Harada *et al.*, 2015). This mutation could underlie the disparity between the transactivation results, as differences in nuclear receptors' structures may lead to distinct chemical binding and activation outcomes (Capitão *et al.*, 2017). The underlying mechanism of this finding has been recently discussed by Capitão *et al.*, (2018).

Indeed, Riu and collaborators have also noted that known HsPPARγ agonists thiazolidinediones (rosiglitazone, pioglitazone, ciglitazone and troglitazone), TBT and a prostaglandin could not be used as positive controls in assays involving DrePPARγ, as they failed to activate the receptor *in vitro* (Riu *et al.*, 2014).

The lack of DrePPARγ transactivation in comparison to RXRα suggest that the TBT's obesogenic effects reported here might be mediated by RXRα instead. In fact, this chemical was also reported to interact with other permissive heterodimers (LXR:RXRα heterodimer) (Capitão *et al.*, 2017; Le Maire *et al.*, 2009) and it has been suggested that the covalent binding to HsRXRα's Cys432 would allow a more efficient stabilization of the active receptor conformation, than its non-covalent ionic bond to HsPPARγ's Cys285 (Harada *et al.*, 2015; Le Maire *et al.*, 2009). Ouadah-Boussouf and Babin (2016) demonstrated that the

457 obesogenic effect of TBT on DreRXR:PPARγ was not inhibited by the HsPPARγ
458 antagonist T0070907, but was fully abolished by the HsRXR antagonist UVI3003 and by a
459 combination of HsRXR:RXR and HsRXR:PPARγ antagonists. The findings of Ouadah-
460 Boussouf and Babin (2016) support that RXR plays a central role in TBT-modulated
461 obesogenic outcomes and that *in vivo* obesogenic effects might occur through RXR-
462 dependent pathways which do not necessarily involve PPAR isoforms (Ouadah-Boussouf &
463 Babin, 2016).

464 TBT may modulate other NRs involved in lipid homeostasis such as LXR:RXR
465 heterodimer (Capitão *et al.*, 2017; Le Maire *et al.*, 2009). Interestingly, LXR is tightly
466 related to cholesterol homeostasis and regulates the expression of SREBP-1 (Capitão *et al.*,
467 2017), one of the upregulated target genes after exposure to TBT at 100 ng/L Sn.
468 Regardless, unveiling the crystallographic structure of TBT binding to the human RXR and
469 PPARγ has allowed a better understanding of the interaction with these receptors. Similar
470 studies should be performed with other chemicals and nuclear receptors to help elucidate
471 the different species-responses to a particular lipid homeostasis regulator.

472 Regarding the environmental samples, only the highest tested influent concentration (10%)
473 was able to significantly transactivate the heterodimer HsPPARγ/RXRα, thus indicating the
474 presence in the influent of HsPPARγ/RXRα agonists (**Figure 4**). It is important to have in
475 mind that the tested concentrations of 1.25% and 2.5% were used when zebrafish larvae
476 were chronically exposed from the 4^{th} to the 18^{th} dpf *in vivo*. Thus, the results are not easily
477 comparable, as transactivation assays comprise a short 24-hour window of exposure, which
478 could explain the lack of transactivation at these lower concentrations. In DrePPARγ a 1.5-
479 fold induction was observed for the 10% influent concentrations, although differences did
480 reach significance in comparison with control ($p = 0.089$).

481 A simultaneous exposure to a “cocktail” of chemicals can potentially lead to additive or
482 even synergistic effects (e.g. by acting on both RXR and PPARγ simultaneously) (Biemann

et al., 2014; Riu *et al.*, 2011) but also to antagonistic effects. Since several compounds have also been shown to repress the transactivation of nuclear receptors involved in lipid homeostasis, the possibility of antagonistic actions of different chemicals on the nuclear receptors cannot be disregarded (Biemann *et al.*, 2014). Indeed, the chemical characterization of the influent used in this study (**Appendix A,** Table S1) shows the presence of a variety of chemicals that have been reported to impact lipid homeostasis triggering lipid storage, i.e., (diclofenac (Puhl *et al.*, 2015), ibuprofen (Puhl *et al.*, 2015), dibutyl phthalate (Mjaeed *et al.*, 2017), methyl paraben (Hu *et al.*, 2016), acesulfame (Bian *et al.*, 2017), saccharin (Suez *et al*., 2015)) or lipid mobilization, i.e., (metformin (Park *et al*., 2009), piperine (Shah *et al*., 2011), irbesartan (Parhofer *et al*., 2007), valsartan (Cole *et al.*, 2010), chenodiol (Chen *et al.*, 2017), hippuric acid (Zhao *et al.*, 2016), fenofibric acid (Alagona, 2010)). Some of the compounds present in the influent have been reported to interact with the PPAR/RXR receptors, either through induction and/or agonism [PPARγ: acesulfame (Simon *et al.*, 2013), saccharin (Simon *et al.*, 2013), irbesartan (Afzal *et al.*, 2016), valsartan (Hasan *et al.*, 2014; Storka *et al.*, 2008), diclofenac (Ayoub *et al.*, 2009; Puhl *et al*., 2015), ibuprofen (Puhl *et al.*, 2015), dibutyl phthalate (Lapinskas *et al.*, 2005; Pereira-Fernandes *et al*., 2013), metformin (Elia *et al.*, 2011); methyl paraben (Pereira-Fernandes *et al.*, 2013); PPARα: metformin (Maida *et al.*, 2011); dibutyl phthalate (Lapinskas *et al.*, 2005), irbesartan (Rong *et al.*, 2010); fenofibric acid (Qiu *et al.*, 2017); Mouse PPARα: triclosan (Wu *et al.*, 2014);] or suppression and/or antagonism [PPARγ: piperine (Park *et al*., 2012); PPARα: chenodiol (Chen *et al.*, 2017); human PPARα: triclosan (Wu *et al.*, 2014)].

The preliminary mechanistic approach performed here to address the involvement of the PPARγ/ RXRα in the lipid accumulation observed *in vivo* in the 2.5% influent exposed larvae is not fully conclusive. Even though the chemical analysis of our environmental

samples confirms the presence of known PPARγ agonists and antagonists, it seems likely that other mechanisms might have also directed the *in vivo* effects observed.

So far, very few studies reported the impact of chemical mixtures and environmental samples on obesity and metabolic endpoints (Adeogun *et al.,* 2016; Biemann *et al.,* 2014; Lyche *et al.,* 2013, 2011; Maisano *et al.,* 2016), which is concerning, as only the exposure to mixtures depict real-case scenarios and the results are not always predictable by the effects of the individual compounds alone (Biemann *et al.,* 2014; Rodrigues et al., 2006; Santos et al., 2006, 2008; Sarria et al., 2011). In addition to organotins, other groups of compounds have been investigated for their variety of MoA on obesity outcomes and lipid homeostasis disruption: primary phthalates, such as (2-ethylhexyl) phthalate (DEHP), activate PPARα, favoring a lipolysis state, whereas their metabolites show preference for PPARγ (Grün & Blumberg, 2009); Halogenated Bisphenol A derivatives have been shown to disrupt the thyroid hormone receptor, estrogen receptors and the PPARγ signaling (Bašić *et al.,* 2012; Riu *et al.,* 2011). As lipogenesis and adipogenesis involve a complex network of transcription factors and enzymes, the disruption of the energy homeostasis can occur at many levels (Bašić *et al.*, 2012). Weighting these different factors together, through the study of complex samples of chemicals, is important to obtain a more realistic assessment of the impact of these mixtures on animal and human health. Interestingly, epidemiological and toxicological studies show a moderate to strong probability of human obesity being associated with exposure to DDE, Di-2-ethylhexylphthalate and BPA (Trasande *et al.,* 2015).

4. CONCLUSION

The present study demonstrates, for the first time, obesogenic responses in zebrafish larvae following exposure to complex environmental samples reported to contain an array of chemicals known to modulate lipid homeostasis. Yet, the mechanistic studies performed here are not fully conclusive. Hence, future studies should expand the screening to

additional nuclear receptors known to be engaged in vertebrate's lipid homeostasis and the study of pathway's other than the ones regulated by PPARγ/RXRα.

DECLARATION OF INTEREST

Conflicts of interest: none.

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Linking chemical exposure to lipid homeostasis: a municipal waste water treatment plant influent is obesogenic for zebrafish larvae

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ANNEX A

LC-QTOF-MS analysis of the influent wastewater

The wastewater sample was submitted to a solid-phase extraction (SPE) protocol prior to their analysis by Liquid Chromatography Quadrupole Time-of-flight Tandem Mass Spectrometry (LC-QTOF-MS/MS). Thus, 100 mL of the sample were passed through two 150 mg cartridges OASIS WCX and OASIS WAX (Waters, Milford, MA, USA) connected in series. The cartridges were previously conditioned following the manufacturer's specifications. After sample loading, the cartridges were dried under nitrogen stream for 20 minutes and eluted with 4 consecutive fractions consisting of 1) 10 mL of methanol, 2) 10 mL of methanol containing 5 % of ammonia, 3) 10 mL of methanol containing 2% of formic acid and 4) 10 mL of ethyl acetate. All these fractions were evaporated to dryness and reconstituted in 100 µL of methanol. The final extracts were injected (10 µL) into a LC-QTOF-MS 6520 system (Agilent Technologies, Santa Clara, USA). The chromatographic column was a Synergy Fusion-RP 100x2mm, 4µm (Phenomenex, Torrance, CA, USA) and the mobile phases consisted of ultrapure water (A) and methanol (B) both containing 0.1% formic acid. After the injection, the column was maintained at 95% A during 1 min, then, a linear gradient of 15 minutes was programmed from 95% A to 100% B (hold for 5 min) and the system was maintained at initial conditions again for other 10 min before the following injection. The QTOF-MS system worked in the "All Ions" acquisition mode using a collision energy of either 0 or 20 V (as to obtain the pseudomolecular ion and product ions), in both electrospray (ESI) positive and negative modes. Nitrogen was used as nebulizing (40 psi) and drying gas (300 °C, 5 L min^{-1}) in the dual ESI source and also as collision gas in the MS/MS and All Ions experiments. The QTOF instrument was operated in the high resolution 4 GHz mode. This mode provides a full width at half-maximum (FWHM) resolution of ca. 10 600 at *m/z* 118.0862 and ca. 16 900 at *m/z* 922.0097. A reference calibration solution, supplied by Agilent, was continuously sprayed in the source during the chromatographic run, providing the required accuracy of mass assignations, according to the manufacturer instructions.

The MS data treatment was made with the MassHunter B.07.01 software using the algorithm "Find by Formula" with fragment confirmation against the Agilent Forensic Toxicology, Veterinary Drugs, Pesticides and Water Pollutants Libraries, which all together contain ca. 11,000 chemicals, of which

about 3,500 have MS/MS spectra. Tentatively identified compounds were reinjected in MS/MS mode at 10, 20 and 40 V collision energies for further confirmation. Blank samples of the whole analytical process were also processed and chemicals were only reported if they were detected in at least two replicate analysis of the sample and its signal was 5 times higher than the blank.

The identified compounds are shown in Table S1, classified in confirmatory levels, according to Schymanski et al., 2014. Only those compounds at a high enough confirmation level (level 2b or higher) are reported. The presence of twelve out of 20 analytes was confirmed through their comparison with reference standards (Level 1), 5 analytes were confirmed by matching their structure with different commercial libraries (Level 2a) and finally 3 compounds were tentatively identified by checking the possible fragmentation pattern (Level 2b).

Table S1. Summary of chemicals detected in the wastewater sample.

Compound detected	Level*	Fórmula	Rt (min)	ESI mode	Uses	CAS
Metformin	1	C4 H11 N5	1.8	+	Hypoglicemic	657-24-9
Paracetamol	1	C8 H9 N O2	3.5	+	Analgesic	103-90-2
Dibutylphthalate	1	C16 H22 O4	18.9	+	Plasticizer	84-74-2
Benzophenone 8	1	C14 H12 O4	16.4	+	UV filter	131-53-3
TCPP	1	C9 H18 Cl3 O4 P	17.2	+	Flame retardant	13674-84-5
Piperine	2a	C17 H19 N O3	17.7	+	Food additive	94-62-2
Methyl paraben	1	C8H8O3	14.6	-	Antimicrobial agent	99-76-3
Triclosan	1	C12H7Cl3O2	18.5	-	Antiseptic	3380-34-5
Netilmicin	2b	C21 H41 N5 O7	12.6	+	Antibiotic	56391-56-1
Irbesartan	1	C25 H28 N6 O	16.1	+	Antihypertensive	138402-11-6
Valsartan	1	C24H29N5O3	16.9	-	Antihypertensive	137862-53-4
Diclofenac	1	C14 H11 Cl2 N O2	18.1	+	Antinflammatory	15307-86-5
Hippuric acid	2a	C9 H9 N O3	4.8	+	Bactericide	495-69-2
Fenofibric acid	2a	C17 H15 Cl O4	17.9	+	Antihyperlipidemic	42017-89-0
Acesulfame	1	C4H5NO4S	2.5	-	Sweetener	33665-90-6
4-Hydroxybenzoic acid	2a	C7H6O3	11.3	-	Chemical reagent / parabens metabolilte	99-96-7
Chenodiol	2a	C24H40O4	19.0	-	Anticholesterolemic	474-25-9
Carboxyibuprofen	2b	C13H16O4	14.8	-	Ibuprofen (NSAID) metabolite	15935-54-3
Saccharin	1	C7H5NO3S	3.8	-	Sweetener	128-44-9
Canbisol	2b	C24 H38 O3	19.3	+	Syntetic cannabinoid	56689-43-1

* Confirmation level according to Schymanski et al. (2014).

Table S1. Primer sequences and respective annealing temperature used in qPCR.

Gene	Genbank access number	Forward primer (5´- 3´)	Reverse primer (5´- 3´)	$T_{annealing}$ (°C)
RXRα	NM_001161551	ATTCAATGGCATCTCCTG	GCGGCTTAATATCCTCTG	60
PPARγ	NM_131467	GGTTTCATTACGGCGTTCAC	TGGTTCACGTCACTGGAGAA	60
SREBP1	NM_001105129.1	CAGAGGGTGGGCATGCTGGC	ATGTGACGGTGGTGCCGCTG	60
FASn	XM_682295	ATCTGTTCCTGTTCGATGGC	AGCATATCTCGGCTGACGTT	62
ACOX1	BC097101.1	GCACGGATGTGTGTACCGTGC	GCGTCCAGAGCCCCTTGACCT	62
C/EBPα	NM_131885	AACGGAGCGAGCTTGACTT	AAATCATGCCCATTAGCTGC	62
DGAT2	NM_001030196	TGGGGCTTTTTGTAACTTCG	TCTTCCTGGTGCACAGTCC	62
β-actin	NM_181601.3	ACTGTATTGTCTGGTGGTAC	TACTCCTGCTTGCTAATCC	60

Table S2. Primers designed to amplify the HR+LBD of the NRs. Forward primers contain the recognition sequence of XbaI; Reverse primer contains the sequence for KpnI.

NR	Forward Primer (5´- 3´)	Reverse Primer (5´- 3´)
RXRα	ATCGTCTAGACAGCGAGCCAA GGAACGC	AATTGGTACCTTATGTCATTTGG TGTGGAGCTT
PPARγ	ATCGTCTAGACTGGCCGAGTTC TCCAGT	AATTGGTACCTTAGTACAGGTCC CGCATGA

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