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Morphometric signatures of exposure to endocrine disrupting chemicals in zebrafish eleutheroembryos

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Highlights

- Morphological alterations of TBT and E2 were non-specific and mostly related to alterations in developmental rates
- BPA induced yolk sac malabsorption syndrome and craniovisual alterations
- Scoliosis and kyphosis were induced by PFOS exposures

Abstract

Understanding the mode of action of the different pollutants in human and wildlife health is a key step in environmental risk assessment. The aim of this study was to determine signatures that could link morphological phenotypes to the toxicity mechanisms of four Endocrine Disrupting Chemicals (EDCs): bisphenol A (BPA), perfluorooctanesulfonate potassium salt (PFOS), tributyltin chloride (TBT), and 17- β -estradiol (E2). Zebrafish (*Danio rerio*) eleutheroembryos were exposed from 2 to 5 dpf to a wide range of BPA, PFOS, TBT and E2 concentrations. At the end of the exposures several morphometric features were assessed. Common and non-specific effects on larvae pigmentation or swim bladder area were observed after exposures to all compounds. BPA specifically induced yolk sac malabsorption syndrome and altered craniofacial parameters, whereas PFOS had specific effects on the notochord formation presenting higher rates of scoliosis and kyphosis. The main effect of E2 was an increase in the body length of the exposed eleutheroembryos. In the case of TBT, main alterations on the morphological traits were related to developmental delays. When integrating all morphometrical parameters, BPA showed the highest rates of malformations in terms of equilethality, followed by PFOS and, distantly, by TBT and E2. In the case of BPA and PFOS, we were able to relate our results with effects on the transcriptome and metabolome, previously reported. We propose that methodized morphometric analyses in zebrafish embryo model can be used as an inexpensive and easy screening tool to predict modes of action of a wide-range number of contaminants.

Keywords: EDCs, dose-response, morphology, zebrafish, endocrine disruption, eleutheroembryo

1. Introduction

In the last decades contaminants of emerging concern have attracted the attention of many researchers due to their novelty and not yet fully understood effects (Diamanti-Kandarakis et al., 2009; Elliott et al., 2017; Jorgenson et al., 2018; Meador et al., 2018), publishing a wide spectrum of studies during the last years (Bieber et al., 2016; Shin and

Schideman, 2015; Sousa et al., 2019). This is the case of the Endocrine Disrupting Chemicals (EDCs), which are able to disrupt the hormonal status of exposed individuals, both in animals and humans (Diamanti-Kandarakis et al., 2009; Kinch et al., 2016; Waye and Trudeau, 2011). Nowadays, there is a need to further study the wide range of chemicals that integrate the EDCs (National Research Council (US), 1999; Okkerman P.C, 2000). These include many compounds with different structures and composition, such as polychlorinated organic compounds (*e.g.*, dioxins and PCBs), phthalates, organic solvents (*e.g.*, *xylene and toluene*), brominated flame retardants, parabens, biocides (*e.g.*, carbamates and tributyltin, TBT), perfluoroalkyls (*e.g.*, perfluorooctanoic (PFOA) and perfluorooctanesulfonic (PFOS) acids), alkyl- and bis- phenols (*e.g.*, bisphenol A (BPA) and bisphenol S (BPS)). Moreover other natural compounds, like estrone (E1), estradiol (E2) and estriol (E3), which are accidentally released into the environment (Adeel et al., 2017; Belhaj et al., 2015; Shore and Shemesh, 2016), are also considered EDCs.

EDCs have been linked to alterations of the reproductive success, fertility disruption (Fusani et al., 2007), feminization (Gross-Sorokin et al., 2006), neurodevelopmental problems (Kinch et al., 2015), behavior changes (Barrett and Patisaul, 2017; Ottinger et al., 2001) and even morphological abnormalities (Kinch et al., 2016; Sharma et al., 2016). The disruption of the steroidogenic pathway by EDCs is well-known and has been widely reported (Benninghoff et al., 2011; Carpenter, 2013; Lyssimachou et al., 2006; McGinnis and Crivello, 2011; Sanderson, 2006; Shi et al., 2008; Vandenberg et al., 2009). However, it has been shown that EDCs disrupt other hormonal pathways, pointing out the need of more complex and integrative interpretations (Martínez et al., 2018; Regnier and Sargis, 2014; Soto et al., 2009; Vandenberg et al., 2009). In the past, the study of EDCs has focused on lethality, effects at the phenotype level and/or identification and measurement of biomarkers of exposure (Kashiwada et al., 2002; Kinch et al., 2016; Scholz and Mayer, 2008). In the recent years the development of modern omics techniques allowed an advance in the study of EDCs identifying alterations at the molecular level studying the transcriptome, epigenome, proteome, metabolome or lipidome as a whole (Baker and Hardiman, 2014; Fuertes et al., 2018; Helsley and Zhou, 2017; Oliveira et al., 2016; Ortiz-Villanueva et al., 2018; Shrader et al., 2003).

Traditionally qualitative and quantitative observations of animal phenotypes had been related to genetic information contained in the genome. For example, this approach has been performed in human medicine not only to characterize several diseases (Baser et al., 2003; Beheshtian et al., 2008; Costantine et al., 2008; Trebo et al., 2008; Usta et al., 2014) but also to predict them (Listgarten et al., 2014; McCabe and McCabe, 2013; Si and Best, 2017; West et al., 2006; Weston and Hood, 2004). The development of next generation sequencing (NGS) technologies has provided new tools to establish genotype-phenotype correlations (Birtel et al., 2018; Huang et al., 2014; Lu et al., 2014; Tafazoli et al., 2018). On the other hand, morphological measures have been classically carried out, but recent studies have shown that the integration of several morphometric parameters permits a better description of different phenotypes (Lantz-Mcpeak et al., 2015; Teixidó et al., 2019). For that reason, it has recently been proposed to use these integrative morphometric analyses as a useful tool to screen possible pathways or modes of action of different pollutants (Teixidó et al., 2019). Recent efforts have been placed to describe and include all the related steps involved in the 'central dogma of the biology' (from DNA to protein) (Benfey and Mitchell-Olds, 2008; Biémont, 2010; Nassiri and McCall, 2018). In this regard, several studies have determined correlations between phenotypic alterations in animal models exposed to pollutants and the effects of those exposures at different biological levels. For example, it has been shown that transcriptomic and metabolomic alterations of exposures to some EDCs concur with the morphological alterations induced by them (Lee et al., 2017; Martínez et al., 2018; Orlando et al., 2004; Ortiz-Villanueva et al., 2018).

The aim of our study was to determine morphological signatures that could link phenotypes to the toxicity mechanisms of EDCs. We used the extensively studied zebrafish (*Danio rerio*), since it is considered a vertebrate model for human and environmental toxicology (Raldúa and Piña, 2014; Segner, 2009; Strähle et al., 2012), it has a highly conserved endocrine system compared to humans (Kimmel et al., 1995; Löhr and Hammerschmidt, 2011) and it has been pointed out as a model organism for the study of the endocrine disruptors in vertebrates (Scholz and Mayer, 2008; Stegeman et al., 2010). In addition, the development of zebrafish embryos (from fertilization up to

hatching) and eleutheroembryos (from hatching up to 5 days post-fertilization, dpf) is well known, they can easily be observed using a wide variety of optical methods and their transparency facilitates morphological measures to study possible malformations caused by toxicants (Kimmel et al., 1995; Parichy et al., 2009; Truong et al., 2011). In the present study, we have exposed zebrafish eleutheroembryos from 2 to 5 dpf to different EDCs that have shown estrogenic properties and effects in lipid metabolism/obesity even with a very different chemical structure: BPA, a plastic monomer widely used in bottles (vom Saal et al., 2012); TBT, an antifouling compound used in boats (Penza et al., 2011); perfluorooctanesulfonate potassium salt (PFOS), a fluorosurfactant used in frying pans and fire-fighting foams (Chen et al., 2016); and 17- β -estradiol (E2), one of the natural estrogenic hormones (Hao et al., 2013). The integration of morphometric parameters was used to analyze the effects of EDCs exposure identifying common and specific morphometric signatures of exposure to each EDC studied.

2. Materials and methods

2.1. Animals and rearing conditions

Adult wild-type zebrafish (*Danio rerio*, 12-18 months old) were maintained under standard conditions (28 ± 1 °C, 12L:12D photoperiod) and fed twice per day with dry flakes (TetraMin, Tetra, Germany). Fish water was composed by Instant Ocean (90 μ g/ml, Aquarium Systems, Sarrebourg, France) and $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ (100 μ g/ml, Sigma-Aldrich, St.Louis. MO- USA), dissolved in reverse-osmosis purified water. Zebrafish eggs were obtained in 4-L breeding tanks by natural mating of the adults (2:1 males:females). A bottom mesh was used to separate adults and eggs. Eggs were collected and rinsed at 2 hours post fertilization (hpf) and fertilized ones were placed in 6-well multiplates at a density of 3.3 embryos/ml (10 individuals per well in 3.0 ml of fish water; n=5-6 replicate wells per group distributed in different 6-well multiplates to account for possible “tank” effects). All procedures were carried out in accordance with the institutional guidelines under a license from the local government (DAMM 7669, 7964) and were approved by the Institutional Animal Care and Use Committees at the Research and Development Centre of the Spanish National Research Council (CID-CSIC).

2.2. Zebrafish larvae exposures

2.2.1. Stocks solutions preparation

Bisphenol A (BPA, CAS-RN: 80-05-7, ≥99.0% purity), perfluorooctanesulfonate potassium salt (PFOS, CAS-RN: 2795-39-3, ≥98.0% purity), tributyltin chloride (TBT, CAS-RN: 1461-22-9, ≥96.0% purity) and 17-β-estradiol (E2, CAS-RN: 50-28-2, ≥98.0% purity) were provided by Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of each compound covering a wide concentration range were prepared in dimethyl sulfoxide (DMSO) and stored at -20 °C. During the exposure period, fresh experimental solutions (**Supplemental Table ST1**) were prepared every day, dissolving the stock solutions in fish water (final DMSO concentration: 0.2% (v/v)). The pH of the working solutions was found to be between the tolerated range: 6.0-8.5 ensuring its stability after the compound dilution (Avdesh et al., 2012; Reed and Jennings, 2011). Moreover, measured pH after 24 h was found to be stable and there was no need to correct it. Final working solutions for each compound covered a wide concentration range, being the highest tested concentrations enough to achieve 100% mortality of the embryos and the lowest concentrations similar or at the same level as concentrations found for example in harbors waters in case of TBT (Antizar-Ladislao, 2008), human internal circulating fluids levels in case of BPA (Teeguarden et al., 2013), human serum or wild fish eggs concentrations in case of PFOS (Alexander et al., 2008; Kannan et al., 2005) or agricultural effluent and slurries discharges in case of E2 (Adeel et al., 2017; Lecomte et al., 2017).

2.2.2. Zebrafish eleutheroembryos EDC exposures

Zebrafish eleutheroembryos (n=50-60 individuals per group) were exposed from 2 to 5 days post-fertilization (dpf) to the above mentioned EDCs to the following concentrations: BPA (control (0.2% DMSO), 0.44, 4.4, 8.8, 17.5, 26.3, 35.0, 43.8, 219.0 and 438.0 µM); PFOS (control (0.2% DMSO), 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, 20.0 and 200.0 µM); TBT (control (0.2% DMSO), $3.0 \cdot 10^{-4}$, $3.0 \cdot 10^{-3}$, 0.03, 0.10, 0.15, 0.20, 0.25 and 0.30 µM); and E2 (control (0.2% DMSO), 0.1, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 100.0 µM). Exposures started at 2 dpf to avoid confounding factors due to the interference of the pollutants with the processes at very early embryonic stages, to focus in their effects in the already differentiated tissues of the larvae and to avoid the interaction factor due

to their different crossing through the chorion as the hatching of the zebrafish embryos take place between 48 and 72 hpf (Kimmel et al., 1995). Chemical analyses of the solutions were not required as BPA, PFOS, TBT and E2 have been shown to be stable and/or have half-lives of days in absence of catalyzed chemical degradation, specific bacteria or special conditions (Adeel et al., 2017; Cruz et al., 2015; Jordão et al., 2016; Jürgens et al., 2002; Kato et al., 2013; Meng et al., 2017). In any case, and due to the uptake and bioaccumulation of the compounds by the eleuthero/embryos (Souder and Gorelick, 2018) that could decrease the chemicals concentration in the water, the exposure medium was renewed every day to assure that zebrafish eleutheroembryos were exposed appropriately to each compound concentration.

2.2.3. Survival, hatching and swim bladder inflation rates

Survival, hatching and swim bladder inflation rates were assessed during the exposures by visual observation. Survival (at 3, 4 and 5 dpf), hatching rates (at 3, 4 and 5 dpf) and presence of inflated swim bladder (at 4 and 5 dpf) were recorded for each concentration with a dichotomous classification (live or death, hatched or not hatched, and SB inflated or not inflated).

2.2.4. Embryo collection, fixation and annotation of morphological traits

The annotation of morphological traits during normal development was determined to assess possible developmental delays due to the compound's exposure. Control eleutheroembryos (DMSO 0.2%) were collected and fixed at 3, 4, 5 and 6 dpf (30 individuals per day). At the end of the exposures (5 dpf), 50 eleutheroembryos per condition were also sampled and fixed to study the effects of the selected compounds on different morphological traits. Eleutheroembryos were fixed in 4% paraformaldehyde in PBS overnight at 4°C (in darkness, to avoid different pigmentations due to different light conditions during the fixation process) and then washed several times with PBS. Fixed embryos were gradually transferred to 90% glycerol for conservation and facilitation of embryo placement under the microscope (Raldúa et al., 2008). The eleutheroembryos were stored during few weeks at room temperature and all the conditions from the same tested compound (including its control) were photographed in less than 2-3 days. With the aim of comparing the effects

of the different tested compounds without any interfering morphometric alterations due to death processes, experimental conditions with statistical differences in survival rates at 5 dpf (compared to their respective control groups) were discarded for further morphological analysis. Furthermore, equilethal doses for each compound were calculated assigning the equilethal dose of 1.0 to the highest concentration that did not statistically affect survival at 5 dpf: the NOAEC (no observed adverse effect concentration) in terms of lethality. In the case of BPA, we observed a 24% of unhatched larvae and a 38% of larvae exhibiting gross malformations, such as the presence of semi-unrolled tail, at the highest BPA concentration sampled for morphometrics (43.8 μ M, 1.0 of equilethal dose). Thus, their positioning under the microscope was not appropriated to ensure the correct measurement of the different morphological parameters. For that reason, this experimental group was excluded from the morphometric analyses.

In order to report morphological effects, lateral and dorsal images of each fixed eleutheroembryo were taken using a stereomicroscope Nikon SMZ1500 (Nikon Co., Tokyo, Japan) coupled with a Nikon digital Sight DS-Ri1 camera. Dorsal overall pigmentation was measured by densitometry. The colour intensity of a parallelogram taken between the middle of the head and the end of the tail (in the dorsal images) was measured using the imaging free software GelAnalyzer 2010a (<http://www.gelalyzer.com/>). Background colour intensity was subtracted from each image and fold-changes were calculated respect each control group. In addition, the presence or the absence of scoliosis was annotated for each eleutheroembryo. Using dorsoventral images, a larva was considered to have scoliosis if it had spine malformations in, at least, two different directions presenting a 'zigzag' pattern. Finally, nine morphological traits were measured (**Supplementary Figure S1**) using the free graphical image analysis software ImageJ (National Institutes of Health, Bethesda, MD, USA) (Raldúa et al., 2008). These included: body length (BL), head-trunk angle (HTA), yolk sac area (YSA), swim bladder area (SBA), eye length (EL), eye width (EW), head width (HW), inter-ocular distance (IOD) and eye-snout distance (ESD). In the case of EL and EW, reported values reflect the mean of the size of both eyes. Craniofacial measurements (EL, EW, HW, IOD and ESD) were chosen because of the evidences that

BPA and TBT can alter visual and/or retinoic pathways (Martínez et al., 2018; Thayer et al., 2012); HTA, due to the possible effects of PFOS in the eleutheroembryos column (Hagenaars et al., 2014; Huang et al., 2010); YSA, because of the effects of the four compounds (BPA, PFOS, TBT and E2) over the lipid metabolism or obesity (Chen et al., 2016; Hao et al., 2013; Penza et al., 2011; vom Saal et al., 2012); and SBA, by virtue of its relationship with the YSA (Raldúa et al., 2008). To distinguish specific malformations caused by the pollutants from mere developmental delays, the length of the eleutheroembryos (BL) was measured as it has been defined as a good indicator of the developmental progress (Kimmel et al., 1995; Parichy et al., 2009; Teixidó et al., 2019).

2.3. Statistical analysis

2.3.1. Survival, hatching, swim bladder inflation rates and morphometric parameters

Differences between treatments in survival, hatching, swim bladder inflation, pigmentation and the nine morphological traits were analyzed using the non-parametric Kruskal-Wallis test with pairwise multiple comparisons (considering $p < 0.05$ as significance level). LC_{50} values were calculated by interpolation on the fitted curves (Hill equations with variable slope) over the survival data. All the raw morphometric measures except for the YSA were normalized to the mean control value ($measure_{Control} = 1.0$). Yolk sac area was expressed in mm^2 using a baseline correction subtracting the YSA mean of the control group to the rest of YSA individual values, avoiding mathematical artifacts due to the fact that YSA mean in the control group was near zero (most control larvae had reabsorbed their yolk sack at 5 dpf). Differences between treatments in the percentage of incidence of scoliosis was analyzed using a Fisher's exact test against the control group (considering $p < 0.05$ as significance level). Correlations between YSA and SBA, and between BL and YSA were performed with Pearson correlation tests ($p < 0.05$). All the statistical analyses were carried out with SPSS 24.0 (Armonk, NY: IBM Corp., 2016) and the associated graphs were performed using GraphPad Prism (v. 6.07, GraphPad Software, La Jolla, CA, USA).

2.3.2. PAM clustering and principal component analyses (PCAs)

With the aim of performing an integrative analysis of all variables and samples, hierarchical clustering and PAM (partition around medoids) clustering analyses were

performed using the BPA, PFOS, TBT and E2 exposure morphological traits (normalized and scaled data). After performing a principal component analysis (PCA), the PAM algorithm analyzes the covariance matrix (obtained from the variables) to search k representative objects (medoids) that explain the structure of the data. Each medoid will represent one cluster and is constructed by assigning the nearest observations to it. The number of k medoids is selected to minimize the dissimilarities of the observations to their nearest medoids and accordingly, each observation is assigned to a cluster that represents the general structure of all the variables of this specific observation (sample). Therefore, after the clustering and PAM analysis, the samples of our study were classified in groups (clusters), depending on the general pattern of all its nine measured parameters. Afterwards, we determined the number of larvae classified in each cluster for the different compound concentrations to establish a general degree of morphological impact produced by each condition (concentration and compound). Fisher's exact tests against each compound control group ($p < 0.05$) were used to establish statistical differences between groups in the percentage of affected individuals.

Two principal component analysis were also used as an unsupervised tool for exploratory data analysis using the nine measured morphological traits. Normalized data was also scaled before performing the PCAs. First PCA was carried out over the developmental data from a total of 128 non-exposed individuals at 3, 4, 5 and 6 dpf. Second PCA was performed over BPA, PFOS, TBT and E2 exposures data from a total of 1490 exposed individuals at different concentrations at 5 dpf. PCA and PAM clustering analyses were both performed and plotted with the packages *stats*, *factoextra*, *FactoMineR*, *gplots*, *ggplot2*, *corrplot*, *fpc*, *fmsb* and *cluster* in R (Hennig, 2014; Kassambara and Mundt, 2017; Lê et al., 2008; Maechler et al., 2019; Mevik and Wehrens, 2015; Nakazawa, 2018; R Development Core Team, 2008; Warnes et al., 2015; Wei et al., 2017; Wickham, 2011).

2.3.3. Polynomial regressions between morphological traits and body length

To study possible alterations on morphological traits due to changes on developmental rates, a polynomial regression was performed for each morphological trait using the

non-exposed individuals. Each morphological feature (“y”) and body length (“x”) of the age developmental controls (3, 4, 5 and 6 dpf) were used to build a second degree polynomial regression ($y = a + b \cdot x + c \cdot x^2$) which minimized the variance of the coefficients estimators in the model (Ostertagová, 2012; Ziegel et al., 2006). In the regression, an iterative method was used to assure that the multiple variable residuals minimization achieves the global and not a local minimum. Then, body length of each exposed individual was used to estimate predicted values for the different morphological traits of each larvae. Multiple t-test (Bonferroni correction) comparing predicted (using the polynomial prediction as a model) versus observed experimental (measured) values was performed for each group and parameter. All the statistical analyses were carried out with SPSS 24.0 (Armonk, NY: IBM Corp., 2016) and the associated graphs were performed using GraphPad Prism (v. 6.07, GraphPad Software, La Jolla, CA, USA).

2.3.4. PLS (Partial Least Squares) analyses

To explore and identify possible morphologic signatures that drive the differences between the ages (3, 4, 5 and 6 dpf) in the developmental controls and between the compounds (BPA, PFOS, TBT and E2) in the exposures, multivariate Partial Least Squares analyses were performed. A Partial Least Squares analysis is a supervised statistical method that finds a linear regression model by projecting observable variables (matrix X) and predicted variables (matrix Y) to a new space. The model is built to find the multidimensional direction that explains the maximum multidimensional variances. In case of the developmental age controls, a PLS1 was performed with 1D vector containing the age of the individuals used as the predicted variable. In case of the exposed data, a PLS2 was performed with a 2D matrix containing both the compound and its concentration as predicted variables (matrix Y). In both cases, the nine morphometric variables were used as predictors (matrix X). Normalized data was log-transformed after adding a negligible value (0.0001) to avoid zeros in the YSA data. In both cases, the features (variables) that best described the differences between groups were identified. Latent variable selection (number of selected components) was performed using cross validation (leave-one-out method) and based on cumulated R^2 (cumulative % of predictable variance), RMSE (Root Mean Squared Error) and cumulated Q^2 index (a statistic calculated from the cross validation that gives an estimate of the predictive

power of a PLS model). PLS1 and PLS2 analyses were both performed and plotted with the packages *factoextra*, *FactoMineR*, *gplots*, *ggplot2* and *pls* in R (Kassambara and Mundt, 2017; Lê et al., 2008; Mevik and Wehrens, 2015; R Development Core Team, 2008; Warnes et al., 2015; Wickham, 2011).

2.1 Open Data

Raw data as well as analyzed data were submitted to the open repository <https://digital.csic.es>. The DOI <http://dx.doi.org/10.20350/digitalCSIC/8645> was assigned.

3. Results

3.1 Survival, hatching and swim bladder inflation

Survival, hatching and swim bladder inflation rates during and after the exposure are shown as fold change respect the control group in **Supplemental Table ST2** (Kruskal-Wallis non-parametric test, $p < 0.05$). TBT was the tested compound that showed higher lethality at the end of the exposures (5 dpf), with lethal concentrations causing the death of 50% of tested animals (LC_{50}) at $0.17 \mu\text{M}$, followed by PFOS with LC_{50} of $18.2 \mu\text{M}$. E2 and BPA were the compounds showing higher survival rates, with LC_{50} of 62.4 and $79.9 \mu\text{M}$, respectively (**Supplemental Figure SF2**). To compare the effects of the different tested compounds, equilethal doses were calculated as explained in section 2.2.4. (**Supplemental Table ST1, Supplemental Figure SF2**). Equilethal doses of 1.0 were $43.8 \mu\text{M}$ for BPA, $15.0 \mu\text{M}$ in case of PFOS, $10.0 \mu\text{M}$ for E2 and $0.15 \mu\text{M}$ for TBT. A decrease in hatching rates was observed at 3, 4 and 5 dpf only at the BPA concentrations $\geq 35.0 \mu\text{M}$ and at $100 \mu\text{M}$ E2 (Kruskal-Wallis non-parametric test, $p < 0.05$). Effects on swim bladder inflation appeared at 4 dpf at lower concentrations than the ones affecting survival and hatching in all compounds. A significant decrease in swim bladder inflation was detected at concentrations $\geq 0.10 \mu\text{M}$ for TBT, $2.0 \mu\text{M}$ for E2, $5.0 \mu\text{M}$ for PFOS and $17.5 \mu\text{M}$ for BPA (Kruskal-Wallis non-parametric test, $p < 0.05$).

3.2. Analysis of the morphological traits

Visual inspection of the fixed larvae (**Figure 1**) revealed two major morphological effects. We observed that larvae exposed to high concentrations of all compounds presented darker skin phenotypes compared to their respective controls (**Supplementary Figure 3**). The same phenotype was also observed prior fixation and indicated an increase on the number and/or the area of the pigment cells. The densitometric results showed that exposed larvae presented a significant increase in pigmentation intensity than their control counterparts (between 12-29%, Kruskal-Wallis non-parametric test, $p < 0.05$). On the other hand, only the exposure to PFOS triggered the appearance of scoliosis at the two highest analyzed concentrations, i.e., 10.0 μM (18% of exposed individuals) and 15.0 μM (40%) (Fisher's exact test, $p < 0.05$; **Supplemental Table ST3**).

Figure 2 shows fold changes (or differences) in the measured morphological traits at different equilethal doses for all tested compounds. Mean measurements for body length (BL), yolk sac area (YSA), swim bladder area (SBA), head-trunk angle (HTA), eye-snout distance (ESD), eye length (EL), eye width (EW), head width (HW) and inter-ocular distance (IOD) of each compound concentration are shown in **Supplemental Table ST4**. We observed a significant decrease in BL between 3-9% after exposure to the highest analyzed concentrations of BPA, PFOS and TBT (Kruskal-Wallis non-parametric test with pairwise multiple comparisons, $p < 0.05$). Conversely, eleutheroembryos exposed to E2 were statistically bigger (~3 %) than controls at middle concentrations (Kruskal-Wallis non-parametric test with pairwise multiple comparisons, $p < 0.05$). YSA of the exposed eleutheroembryos increased significantly with increasing concentrations in all tested pollutants (Kruskal-Wallis non-parametric test with pairwise multiple comparisons, $p < 0.05$). BPA induced the highest effect with YSA reaching 0.125 mm² (a size of the YSA equivalent to a control eleutheroembryo at the age of 3.4 dpf) at the highest analyzed dose (35.0 μM). In the case of the E2 and TBT, treated eleutheroembryos at the highest concentrations increased their YSA to 0.048 and 0.039 mm², respectively (equivalent to a 4.1 and 4.2 dpf eleutheroembryo). PFOS was by far the compound with the least effect on YSA, reaching 0.011 mm² at its highest concentration (equivalent to a 4.6 dpf eleutheroembryo). On the other hand, all pollutants decreased the SBA in a similar dose-response pattern achieving a reduction of 50-64 % at the highest concentrations (Kruskal-Wallis non-parametric test with pairwise multiple comparisons, $p < 0.05$).

While PFOS at the two highest concentrations reduced HTA by 12%, the rest of the compounds only affected by approximately 4%. (Kruskal-Wallis non-parametric test with pairwise multiple comparisons, $p < 0.05$).

To establish possible alterations in the head and/or visual perception, craniofacial features were measured. A significant decrease in eye-snout distance (ESD) was observed in all tested pollutants, reaching a maximum difference of 12-17% between the controls and the highest concentration analyzed (Kruskal-Wallis non-parametric test with pairwise multiple comparisons, $p < 0.05$). Similarly, a decrease in eye length (EL), was observed in exposed individuals with reductions of up to 9%, 7%, 12% and 16% for PFOS, E2, TBT and BPA, respectively (Kruskal-Wallis non-parametric test with pairwise multiple comparisons, $p < 0.05$). From all tested compounds, PFOS and BPA presented up to 15 % decrease in eye width (EW), but interestingly, BPA significantly decrease of 9 % in EW was observed even at the lowest concentration tested (Kruskal-Wallis non-parametric test with pairwise multiple comparisons, $p < 0.05$). Compared to BPA and PFOS, the effects of E2 and TBT in EW was moderate showing a decrease of 7-8 % compared to their respective control groups (Kruskal-Wallis non-parametric test with pairwise multiple comparisons, $p < 0.05$). Similar to the effects on EW, PFOS exposure significantly decreased the head width of exposed eleutheroembryos, reaching a maximum of 20% at the highest concentrations. E2 and TBT only reduced HW significantly between 5-9% at their highest concentration. The response of HW after BPA exposures presented a hormetic shape with intermediate BPA concentrations decreasing HW up to 14%, while highest concentrations decreased it only by 7-8%. Finally, the inter-ocular distance (IOD), together with the YSA and SBA, was one of the traits that was most affected specially by exposure to PFOS and BPA showing opposite effects (Kruskal-Wallis non-parametric test with pairwise multiple comparisons, $p < 0.05$). Interestingly, the effects of exposure on IOD were different for each of the tested pollutants. Following a monotonic dose-response, PFOS exposure reduced IOD up to 35% at the two highest concentrations. Conversely, BPA exhibited a hormetic dose-response pattern with a decrease of the IOD at middle concentrations (up to 24%) and an increase at the high concentrations (up to 23%). TBT and E2 only exhibited significant changes at one of the tested concentrations, with a 7% decrease in IOD at the

highest TBT concentration (0.15 μM), and a 14% increase in IOD at intermediate E2 concentrations (6 μM).

3.3. Morphological traits integration analysis

With the aim to integrate the nine morphological parameters, a PCA linked to a partition around medoids (PAM) clustering analysis was performed. The first two components of the PCA explained more than 63% of the data variability and the PAM clustering defined two groups of samples (cluster A and B), including 897 and 593 larvae, respectively (**Figure 3A-B**). Cluster A is integrated by eleutheroembryos from control or low concentration groups with no changes or mild effects in the morphometric parameters compared to controls (**Figure 3B**). On the other hand, most of the individuals exposed to high concentrations and almost all BPA exposed individuals clustered together in cluster B (**Figure 3B**) and were characterized by moderate and high effects in the morphometric measurements. Accordingly, 91% of the eleutheroembryos of the control groups were classified in the cluster A and 98 % of the highest analyzed concentrations were classified in the cluster B. Therefore, we assigned individuals from cluster A as “unaffected” and the ones from cluster B as “affected” by the exposures. Afterwards, we determined the number of larvae classified in each cluster for all the different conditions (compound and concentration) to establish a general degree of morphological impact produced by them (**Figure 3C**). Considering equilethal concentrations, BPA and PFOS morphologically affected larvae at much lower concentrations than TBT and E2. In the case of BPA, the percentage of affected eleutheroembryos was statistically significant in all tested concentrations, reaching almost 75% of affected individuals at much lower concentrations than the rest of tested pollutants (equilethal concentration of 0.01 and 0.1, respectively, Fisher's exact test, $p < 0.05$). These concentrations were far away from the BPA doses where it starts to affect the larvae survival (morphometric $\text{EC}_{50} \sim 0.001$ % of the equilethal dose) indicating that the BPA is the most morphological-affecting compound regarding its lethality, compared with the other three pollutants. After BPA, we observed that PFOS was the pollutant with the second highest morphological impact with almost 70% of the individuals affected at 5 μM PFOS (equilethal concentration of 0.33, Fisher's exact test, $p < 0.05$). The EC_{50} of PFOS was approximately at 30 % of its equilethal dose, indicating

that it had a mild effect in the morphology. Much higher concentrations were needed for TBT and E2 to produce significant differences in the percentage of affected individuals (93 and 79%, respectively): only their highest equilethal doses (1.0) had significant differences (Fisher's exact test, $p < 0.05$). The EC_{50} of TBT and E2 were at 80 and 92 % of their equilethal doses, respectively. Therefore, TBT and E2 were the compounds with slighter effect in morphology, only showing morphometric changes at concentrations close to lethality.

3.4. Toxicity in development versus specific morphological effects of exposures

The developmental effects of the pollutants that triggered the morphological alterations that were observed could be caused by a mere effect of the EDCs in the developmental pace (growth and development) or by specific morphological alterations over a certain tissue/s (dysmorphogenesis). In order to distinguish toxicity in the development from specific morphological alterations caused by exposure to the different pollutants, a morphometric analysis was carried out in control samples at 3, 4, 5 and 6 dpf. As expected, we observed that during normal development: BL increased while HTA decreased; YSA decreased because larvae started to consume the yolk sac reservoir reaching a complete depletion at 5 dpf; SBA increased due to the start of swim bladder inflation at this age; ESD, EL and EW distances increased with age and IOD and HW presented different trends at different ages (**Supplementary Table ST4**). As a way to visualize how morphometric parameters changed with development, we carried out a PCA using morphometric data from 3, 4, 5 and 6 dpf non-exposed eleutheroembryos. Results indicate that although only two components had Eigenvalues higher than 1, three components were necessary to have a quality of representation of all variables between 60 and 95% (**Supplementary Figure SF4**). These 3 components explained up to 75.8% of the data variability. Representation of the selected components is showed in **Figure 4A, 4B and 4C**. We observed that component 1 reflected mainly the contribution of YSA, SBA, BL, ESD, EL and EW and differentiated samples with different ages. On the other hand, component 2 was mainly driven by IOD and HW while component 3 had a clear and unique influence of HTA contribution. With the aim to select the morphometric variables statistically relevant for the age development (drivers), a Partial Least Squares (PLS1) analysis was performed (**Figure 4D**). Three components were

selected which explained 70.1 and 83.1 % of the variability in the X (predictors, morphometric data) and y (responses, age of the individuals) matrices, respectively. It could be observed that BL, YSA, SBA, EL and ESD were the morphologic signatures (VIP score > 1.0) that mainly drove the changes in the development. Although EW had a VIP score higher than IOD, HW and HTA, and had a reasonably contribution in the first component of the PCA, its VIP score was lower than 1.0 so it couldn't be considered as a driver for the growth in these early developmental stages. Thus, these results indicate that IOD, EW, HW and HTA are parameters that are not directly related to changes in development and growth at the analyzed ages (3-6 dpf).

It is also well known that larvae body length is a trait that perfectly follows growth and developmental progress in zebrafish, in some cases even better than age (Kimmel et al., 1995; Parichy et al., 2009; Teixidó et al., 2019). **Figure 5** represents the mean measurements of the different morphological traits respect to the mean body length for all experimental groups at 5 dpf, as well as for the control groups at different developmental ages (3, 4, 5 and 6 dpf). Results indicated that some of the observed effects (at least at the low doses) could be due to a general effect of the pollutants on developmental rates and growth. Consequently, with the objective to differentiate the morphometrical changes that were driven by a compound specific effect and not related to a delay or advance in the development, the experimental morphological values of each larvae (all conditions considered) were compared with its predicted values (calculated using a polynomial regression performed using the developmental control individuals (3-6 dpf) and their individual body length). We observed that in most cases, when the effects of the pollutants on the different morphological traits were compared with those ones taking into account differences in body length (BL), results did not substantially change (**Figure 6A and 6B**). Although some could be attributed to changes in growth, these results indicate a prevalence of a specific mode of action of the pollutants over a general effect on the eleutheroembryos development.

One of the most distinct effects observed between the different compounds tested in the present study was the effect of the exposures on YSA. We observed that in some cases retention of yolk sac reservoir was partially attributable to the fact that

eleutheroembryos size was affected. For example, an increase in YSA was observed in medium doses of BPA and E2 (15.5 and 6.0-8.0 μM , respectively), but this was found to be related to eleutheroembryos size (**Figure 5; Figure 6B**, observed-predicted difference: $p > 0.01$ in both cases). Similarly, the YSA increase in embryos exposed to the highest TBT concentration could be attributable to the fact that eleutheroembryos were smaller in size (**Figure 5; Figure 6B**, observed-predicted difference: $p > 0.01$). Interestingly, different tendencies were observed between compounds after considering observed effects on development. Eleutheroembryos exposed to the highest BPA and E2 doses (26.3-35.0 and 10.0 μM , respectively) retained more yolk sac ($p < 0.001$ in both cases) than the predicted for individuals of their size (**Figure 6B**, observed-predicted difference: $p < 0.001-0.01$ for BPA and $p < 0.0001$ for E2). In contrast, results demonstrated that after considering eleutheroembryo size, disruption of yolk sac consumption by exposure to PFOS was affected in a completely different way than for BPA (and highest E2 dose). Although exposed eleutheroembryos to highest concentrations of PFOS presented higher YSA than their control counterparts at 5 dpf (**Figure 2, Figure 6B**, $p < 0.01$ and $p < 0.001$ at 10.0 and 15.0 μM , respectively), eleutheroembryos showed to have lower YSA values than the ones corresponding to their size (**Figure 6B**, observed-predicted difference: $p < 0.01$ for 5.0 μM and $p < 0.0001$ for 10.0 and 15.0 μM).

In relation to changes observed in SBA our results showed that those were mostly not correlated to changes in growth. Despite of the fact that BL and SBA were both decreased by BPA, PFOS and TBT exposures (**Figure 6A**, $p < 0.01$), differences between observed and predicted values on SBA were even observed when eleutheroembryo size was considered (**Figure 6B**, $p < 0.01$). In the case of E2, whereas an increase in size was observed at intermediate concentrations ($p < 0.05$), a decrease in SBA was detected ($p < 0.01$) with increasing concentrations (**Figure 6A**) and that observation was maintained after accounting for eleutheroembryo size (**Figure 6B**, $p < 0.001$). We also observed that effects on YSA and SBA seem to be related (**Figure 6**). Our results supported that observation and we found a significant inverse correlation between YSA and SBA (**Supplementary Figure SF5**, Pearson correlation: $p = 0.0003$). The decrease in HTA observed after exposures to increasing concentrations of BPA, PFOS and E2 (**Figure 6A**,

$p < 0.05$), was also observed when BL was considered. That indicates a specific effect of the exposures over this morphometric parameter, which barely exhibit any changes in control animals from different ages (and consequently different sizes) and was not considered as a driver for the development (**Figure 4C**). In the case of observed effects of exposures on eye-snout distance (ESD) we found that those were mostly related to changes in BL. The decrease on ESD observed at the highest TBT concentration was related to lower eleutheroembryo BL (**Figure 6B**, $p > 0.01$). In the case of BPA and PFOS, observed effects on ESD were mostly found to be related also to changes in BL, except for the highest tested concentrations, where animals showed lower and higher ESD, respectively, than what corresponded for their size (**Figure 6B**, $p < 0.001$ and $p < 0.0001$, respectively). Interestingly, E2 treated individuals presented significant lower ESD at relatively low concentrations (2 μM or higher, **Figure 6B**) that did not appear significant when body length was not considered (**Figure 6A**) due to the fact that E2 increased BL at moderate concentrations.

On the other hand, eye length (EL), eye width (EW) and head width (HW) were mostly not related to changes in body size during the 3-6 dpf stage and the significant alterations over them caused by the tested compounds corresponded to specific effects of the pollutants. In all cases, individuals exposed to increasing concentrations showed lower EL than the predicted value considering their size (**Figure 6B**, $p < 0.001$). Changes in eye width (EW) and head width (HW) responded in a similar way, with BPA and PFOS showing observed measures smaller than the predicted ones (considering the animal size). On the other hand, TBT and E2 were dependent on the concentration tested (**Figure 6B**) exhibiting generally highest EW and HW than their predicted, except for their highest concentration (15.0 and 10.0 μM , respectively) when they exhibit an opposite pattern. Finally, while TBT showed no specific effect on inter-ocular distance (IOD) (**Figure 6B**, $p > 0.01$), opposite trends were observed for PFOS and E2, where individuals exposed to increasing concentrations of PFOS presented smaller IOD (**Figure 6B**, $p < 0.0001$) and the ones exposed to intermediated concentrations of E2 presented bigger IOD (**Figure 6B**, $p < 0.0001$) than what was expected for their size, respectively. Interestingly, a pronounced hormetic effect in IOD was observed in exposed BPA eleutheroembryos (**Figure 6A**), characterized by a decrease of this parameter at low-

intermediated BPA concentrations and an increase at high BPA concentrations (Figure 2, **Supplementary Figure SF6**). This hormetic response was found to be unrelated to changes in BL, since same behavior was observed after considering BL (**Figure 6B**, $p < 0.0001$).

3.5. Morphological signatures of BPA, PFOS, TBT and E2 exposures

Unsupervised PCA analysis performed on the exposed individuals' morphometric data indicate that although only two components had Eigenvalues higher than 1, five components were necessary to include major contribution of all variables. Selecting 5 components the quality of representation of all variables were found to be higher than 75% (**Supplementary Figure SF7**). These 5 components explained up to 87.7% of the data variability. Representation of the selected components is showed in **Supplementary Figure SF8**. We observed that component 1 reflected mainly the contribution of HW, EW, EL, BL, SBA and ESD to differentiated individuals exposed to increasing concentrations. The rest of components helped to visualize differences between compounds. Component 2 was mainly driven by YSA and IOD differentiating individuals exposed to BPA and PFOS. Component 3 driven by BL, HTA and YSA differentiate TBT from the rest of compounds. Component 4 driven by HTA and SBA separated E2 and PFOS from TBT and BPA. Results were not evident in the case of component 5, mainly driven by SBA, suggesting that this component could include only biological variation.

Finally, we performed a multivariate partial least square regression (PLS2) with the aim to predict morphological features that characterizes each compound exposure and that differentiate it from the others. Based on cumulated RMSE and Q^2 coefficients we selected 4 components that explained up to 80.2 and 36.1% of the X-block (morphometric variables) and the Y-block (tested compounds and concentrations) variance, respectively (**Supplementary Figure SF9**). To explain the contribution of each morphological trait to differentiate between components in each of the model components, variables with VIP (Variable Importance in Projection) scores higher than 1 were selected and therefore are the only ones described below (**Figure 7**). The first component reflected the general dose-response effect of all tested compounds in HTA,

EL, EW and HW (**Figure 7**). In particular, it can be observed that component 1 explained the large decrease in EL, EW and HW produced by BPA and PFOS compared with those produced by TBT and E2 (**Figure 6B**). Moreover, it also described a higher decrease in HTA produced at the highest equilethal concentrations of PFOS and BPA (followed by E2). It is remarkable at this point that the nine morphological traits play a role over the compound loading so, for example, although E2 produced a reduction in the HTA, its loading have still the same sign (and not the opposite) due to its lack of effects on the EL, EW and HW. This principle that related loadings with the morphometric effects of each pollutant applies for all the components. Subsequently, component 2 helped us to explain the main differences between PFOS treated individuals and the rest. It was clear that loadings for PFOS in this component had opposite sign than for the other compounds (**Figure 7**). Meanwhile results showed a large increase in the YSA produced by BPA and followed by TBT, E2 and PFOS (which was slighter than the other three compounds, **Figure 2**), we observed that individuals exposed to PFOS presented less YSA than what corresponded to their size (the opposite behaviour that was found for BPA, and E2, with animals showing bigger YSA than their predicted value; **Figure 6B**). Furthermore, separation of PFOS from the rest of the compounds could be explained by the fact that it was the only compound that showed a high decrease in the IOD with increasing concentrations (**Figure 2, Figure 6A**). Although IOD showed a statistically hormetic behaviour only in BPA, it presented a similar pattern in TBT and E2 (albeit at different concentrations and opposite trend than observed for BPA), but not at PFOS, helping the separation of PFOS in this component. The contribution of a major decrease of HTA in PFOS exposed animals (compared to the other 3 compounds) is also present in this component (**Figure 7**). Component 2 also helped to explain differences that differentiate BPA from TBT and E2. For example, it can be also observed how EL and EW enhanced BPA loading due to the major effect of this compound on these morphological traits (**Figure 6A**). Both component 3 and 4 basically defined the effects of the four compounds over the HTA, YSA and SBA. In the case of component 3, major morphometric variables were HTA and SBA and the corresponding loadings for each compound point out that this component reflected differences between the effects of BPA from the ones caused by exposures to PFOS and E2. In particular, the compound with a higher negative loading in component 3 was E2 because of the fact that was the

compound with highest decreases on SBA (**Figure 2, Figure 6A**) at the same time that reduced HTA. PFOS showed a similar negative correlation, due to their decrease (**Figure 2, Figure 6A**) at both parameters at the same time. Conversely, BPA and TBT increases SBA and HTA, respectively, at some concentrations (although it is not statistically significant in the case of the BPA) which produces an annulment with the other parameter and made positive their loading at component 3. On the other hand, results showed that component 4 differentiate TBT (the only compound that significantly increased HTA) mainly from BPA. In this component, HTA, YSA and SBA presented VIP scores > 1 and their loadings are similar to each other so an explanation regarding all these three morphometrical data is needed: HTA (negative loading value) is increased in TBT (-), YSA (positive loading value) is slightly increased (+), and SBA (positive loading value) is decreased (-) so the final loading of TBT is negative. In case of BPA: YSA (+), HTA (+, double negative) and SBA (=, hormetic behaviour) so its compound loading is positive. In case of PFOS and E2, the final value is close to zero due to the annulment of the respective morphological traits.

4. Discussion

Zebrafish embryo morphologic malformations can be used to study the effects of exposure to different compounds and model the underlying etiological mechanisms involved in the response to pollutant exposures (Raghunath and Perumal, 2018; Truong et al., 2011; Zhang et al., 2017). Traditionally, researchers have focused in the study of singular morphological effects and/or effects on development (such as hatching delays or growth effects on embryos), demonstrating the potential of morphometric analysis to assess specific effects of some toxicants (Teixido et al., 2017; Teixidó et al., 2019). In the present study, we have not only evaluated and compared individual morphological effects of BPA, TBT, PFOS and E2, but also have integrated all the studied parameters to elucidate and determine general and specific morphometric signatures of exposure to BPA, TBT, PFOS and E2.

Our study determined that the compound that was most toxic in terms of lethality was the TBT followed by PFOS, E2 and BPA, with LC_{50} values of 0.17, 18.2, 62.4 and 79.9 μM ,

respectively. These results are very similar and in the same order of magnitude of previously reported LC_{50} for zebrafish larvae exposed to TBT (0.17-0.34 μ M) (Dong et al., 2006; Kalasekar et al., 2015), PFOS (25 μ M) (Jantzen et al., 2016) and BPA (12-25 ppm, i.e. 53-110 μ M) (Chen et al., 2011; Moreman et al., 2017; Saili and Tanguay, 2013). Although no specific LC_{50} values for E2 (17 β -estradiol) were found for other studies, its lethality in zebrafish embryos was found to be between 10 and 100 μ M (Chandrasekar et al., 2010; Kishida et al., 2001), a similar range to that found in this study. In contrast, we observed that in general the tested compounds affected most of the studied morphological parameters in an inverse manner regarding its lethal toxicity: for example, BPA was the compound with higher malformation rates at comparable equilethal concentrations but the one with lower toxicity (higher LC_{50}); and TBT presented the highest toxicity (lowest LC_{50}) but a low rate of morphological alterations. E2, the only natural compound (endogenous estrogen) studied, did not present the same trend. In terms of survival, hatching and swim bladder inflation rates, we established LOECs (lowest observed effect concentrations) of 0.1 μ M for TBT, 2.0 μ M for E2, 5.0 μ M for PFOS and 17.5 μ M for BPA, results that were very similar to other previous studies (Lam et al., 2011; Martínez et al., 2018; Ortiz-Villanueva et al., 2018).

Our results showed an increase in pigmentation intensity in the individuals exposed to increasing concentrations of the four pollutants (BPA, PFOS, TBT and E2). This general effect is well described and has been previously reported by other researchers when fish larvae are exposed to several contaminants (Fushimi et al., 2009; Huang et al., 2010; Lam et al., 2011; Ortiz-Villanueva et al., 2018), but also when those are reared under stress conditions (Backström and Winberg, 2017; Nilsson Sköld et al., 2013), indicating that the observed changes in pigmentation were a non-specific response to stress. In general, we also observed a dose-response pattern when looking at the effect of the exposures on animal body length (BL), with PFOS being the compound that most affected growth. Although PFOS caused scoliosis and kyphosis (reduced HTA), the measured effects on growth were not due to these phenomena, since BL was measured across the larvae notochord (**Supplementary Figure SF1**) and not from the snout to the tail following a straight line. We also observed a reduction of BL in eleutheroembryos exposed to BPA as well as for TBT (although only detected at the highest tested

concentration), suggesting that it could be caused by a general toxic effect and/or developmental delay, as shown in other studies (Qiu et al., 2018; Teraoka et al., 2002). On the other hand, our results showed that exposures to intermediate E2 concentrations significantly increased eleutheroembryos length. This is in concordance with other studies that have shown acceleration and increased growth (BL) in fish and amphibia exposed to estrogens (reviewed by Bulaeva et al., 2015). This effect has found to be related to the role of estrogens in growth hormone regulation (Cook, 2004; Fernández-Pérez et al., 2013; Leung et al., 2004).

Previous studies have proposed that GH also regulates lipid storage and control adipocyte lipolysis in other fish species (Albalat, 2005; Company et al., 1999; Mingarro et al., 2002) suggesting a possible role of the growth hormone in the yolk sac during the fish development (Di Prinzio et al., 2010), considering that the yolk sac is a main reservoir of lipids in the eleutheroembryo. One of the most remarkable findings of our study was the general effects on YSA after exposure to BPA, PFOS, TBT and E2, suggesting that lipid disruption may occur. This is in concordance with the fact that the tested compounds in this study are considered potential obesogens by the world health organization (WHO, (Bergman et al., 2013)). Interestingly, we found that there were differences in YSA response to the different compounds tested. At first, we observed that exposure to all tested compounds induced yolk sac retention with increasing concentrations, partially attributable to the fact that eleutheroembryos size was also affected by the exposures. However, our results also showed differences between compounds with 3 clear responses: 1) BPA specially, but also E2, exposures triggered eleutheroembryos to retain more yolk sac than what corresponded for their size; 2) PFOS exposed eleutheroembryos retained less yolk sac than the corresponded for their size; and 3) TBT exposed eleutheroembryos showing YSA that corresponded to their body size. We observed that E2 exposed eleutheroembryos accelerated growth at intermediated concentrations (2-4 μM) with complete consumption of the yolk sac, suggesting that eleutheroembryos were capable to use all the energy stored in the yolk sac. However, between 6-8 μM the YSA was increased and at 10 μM yolk malabsorption syndrome was observed (with larvae retaining more yolk sac than the one corresponding to animals of the same size (Raldúa et al., 2008)). Animals exposed to 26.3-35 μM BPA clearly showed

yolk sac malabsorption syndrome, as smaller individuals retained greater YSA. Several studies have reported that the obesogenic effects of BPA can be explained by its binding to ERs (estrogen receptors) (Heindel and Schug, 2014; Mu et al., 2018), PPAR- γ (peroxisome proliferator-activated receptor gamma) (Martínez et al., 2018), RXR (retinoid X receptor) (Martínez et al., 2018), and ERR- γ (estrogen-related receptor gamma) (Bergman et al., 2013; Tohmé et al., 2014), inducing adipogenesis and obesity. The fact that both E2 and BPA are known to disrupt estrogen receptor (Bhandari et al., 2015; Moreman et al., 2017; Zhao et al., 2017), together with the recently published results that demonstrate that exposures to BPA and E2 in zebrafish eleutheroembryo trigger similar transcriptomic effects on genes linked to lipid metabolism (Martínez et al., 2018), suggest that both compounds might have similar modes of action and that interact simultaneously with different cellular targets, causing the observed macroscopic effects on YSA. Conversely, retention of yolk sac in PFOS exposed eleutheroembryos was very low. Interestingly, and as mentioned previously, PFOS exposures decreased eleutheroembryo BL, but contrary to BPA, exposed animals had less yolk sac reservoir than what should be expected for their size. PFOS and other perfluorinated compounds interaction with different PPAR receptors have also been reported (Holtcamp, 2012; Martínez et al., 2019) and obesogenic effects assessed (Bergman et al., 2013). In this regard, we have recently published that exposure to PFOS at 0.03 mg/L (0.1 μ M), a concentration below the lowest dose tested in the present study, significantly increased transcripts related to lipid transport and metabolism (Martínez et al., 2019). Therefore, we conclude that observed effects on YSA are due to disruption of lipid transport and metabolism pathways. Finally, we observed that changes in yolk sac area in TBT exposed eleutheroembryos were associated mostly to changes in growth, demonstrating that the observed increase in YSA was mainly due to delays in growth, probably associated to a decrease in energy needs, and in consequence, to yolk sac consumption. It has been proposed that the effects of TBT in lipid metabolism (Bergman et al., 2013) are related to its interaction with the PPAR- γ and the RXR receptors (Heindel and Schug, 2014; Thayer et al., 2012; Zang et al., 2018). Nevertheless, we suggest that in our study, the relationship between observed increases in YSA by TBT exposures and any possible observed effect at the transcriptomic level, could be more related to a delay in development rather than a direct disruption of the

lipid metabolism pathway related to yolk sac reabsorption. From all the exposed above, we conclude that observed differences on the eleutheroembryo YSA response after exposures to BPA, PFOS, TBT and E2 are mostly due to the differences in their mode of action. Other researchers have linked EDCs dysregulation of energy consumption and metabolism with obesity (reviewed by Petrakis et al., 2017). Therefore, considering that the YSA is the principal energy reservoir of the zebrafish larvae up to 5 dpf, from which the nutrient uptake occurs, we suggest that the observed effects in this study on YSA could also be related to dysregulation of energy consumption and metabolism.

Results from our study showed that exposures to BPA, PFOS, TBT and E2 to increasing concentrations affected eleutheroembryos swim bladder area (SBA) indicating an impairment of swim bladder inflation. To elucidate if this impairment was related to developmental delays, eleutheroembryo body size was considered. We observed that in general, SBA experimental values of exposed eleutheroembryos were lower than the predicted ones by their body size, indicating that the observed effects in SBA were not due to developmental delays caused by the exposures. During development, inflation of the swim bladder naturally occurs after the depletion of the yolk sac (Robertson et al., 2007) and it has been suggested that swim bladder inflation delays can be a consequence of the mechanical pressure carried out by voluminous yolk sac remnants (Raldúa et al., 2008). Our results showed a strong relation between YSA and SBA suggesting that impairment of swim bladder inflation was related to mechanical pressure from the yolk ball and not directly related to effects on development and growth. Other negative effects in the SB inflation have been proposed as the thyroid disruption (Stinckens et al., 2018), which could be the case of all our four compounds (Sharan et al., 2014; Šošić-Jurjevic et al., 2015; Stinckens et al., 2018). Moreover decreases in the heart rate related to SB inflation have been also described by others (Yue et al., 2015). Particularly, previous studies showed that exposures to BPA and TBT reduced heart rates (Mu et al., 2018) while E2 increased them (Romano et al., 2017). In the case of PFOS, other studies showed contradictory results (Dang et al., 2018; Ding et al., 2011). For that reason, although effects in SB inflation in BPA and TBT exposed animals could be related to heart rate alterations in our study, opposite (for E2) or

contradictory (for PFOS) effects suggest that this generalized effect on SB inflation could be more related to the observed alterations in yolk sac absorption.

One of the most specific effects of the exposures to PFOS was the high impact of this compound in the notochord formation and body length (Huang et al., 2010; Shi et al., 2008). Our results showed that eleutheroembryos exposed to PFOS presented much higher rates of scoliosis and also a much higher reduction of head-trunk angle (HTA), a.k.a. kyphosis, than the other compounds. This specificity was also denoted by the fact that HTA barely changed in control animals at these developmental stages (3 to 6 dpf), pointing out that any observable effects in this morphological trait was completely unrelated to developmental or growth delays. Similar spinal deformities in zebrafish larvae exposed to PFOS have been hypothesized to be related to dysregulations in myosin and others muscle fibers (Huang et al., 2010). That is coincident in our studies, with transcriptomic dysregulations in myosin, actin and tropomyosin transcripts showing a dose-response pattern according to increasing PFOS concentrations (Martínez et al., 2019).

Interestingly, another result that drew our attention was the observed alterations of craniofacial features caused more specifically by BPA (even at the lowest tested concentrations). Since the observed effects of head width was a combination of eye width and inter-ocular distance, we will only discuss these two craniofacial features. From one side, our results demonstrated that increasing concentrations of BPA provoked a decrease in eye size (microphthalmia) that was not found to be directly related to developmental delays. Similar or related effects in visual functions as visual behavior alterations, dysruption in opsin transcripts expression or craniofacial abnormalities were also observed in zebrafish exposed to BPA and its derivatives (Kinch et al., 2016; Liu et al., 2018; Pelayo et al., 2012). Moreover, we have recently shown that exposing eleutheroembryos to BPA during the same concentrations and developmental period caused dysregulation of genes related to vision (Martínez et al., 2018). However, whether gene disruption is a cause or a consequence of a reduction of eye size remains unknown, since both effects were observed at the same BPA concentrations. We therefore suggest that alterations in visual functions could be related to the eye

malformations observed in the present study, but further studies that include BPA doses that do not affect eye size are needed to understand the underlying mechanism. On the other hand, several studies had proposed the interaction of BPA with the thyroid and retinoid pathways, as one mode of action of this compound not only for zebrafish but also in other species, via binding with the TR and RXR receptors (Boucher et al., 2014; Iwamuro et al., 2006; Martínez et al., 2018; Pelayo et al., 2012). Retinoid pathway plays a key role during the eye development and microphthalmia has been also reported during thyroid hormone T3 exposures (Pelayo et al., 2012). In this regard, we also previously reported dysregulation of the retinoid pathway in eleutheroembryos after BPA exposures, but not for the thyroid pathway (Martínez et al., 2018). Although further studies are necessary to make any conclusions, we suggest that is probable that the disruption of the retinoid pathway by exposure to BPA might be altering eye formation and development. Another remarkable result from our study was the hormetic response of the zebrafish eleutheroembryo inter-ocular distance (IOD) after BPA exposures, with intermediated concentrations decreasing IOD and higher concentrations triggering its increase. Other studies reported an increase in IOD after 15.0 μ M BPA exposures during 7 days (McCormick et al., 2010). Although at the same concentration eleutheroembryos exposed in our study presented similar IODs than their control counterparts and higher concentrations were required to observe increased IODs, these results aren't contradictory since the previous study exposed the larvae for a longer period. It has been proved that BPA exposures affected the development of the midbrain structure of developing zebrafish larvae via *otx* (orthodenticle homeobox) genes (Mercier et al., 1995; Tse et al., 2013). Furthermore, microphthalmia is a disease that has been associated with alterations in the *otx* human orthologues ((Howe et al., 2012), <http://zfin.org>), indicating that *otx* disruption by BPA could be not be only responsible for the effects on IOD but also on eye size, in conjunction with the via RXR, above explained. Another study demonstrated that BPA altered zebrafish larvae behavior by reducing their movements (Olsvik et al., 2019). With all the exposed above in mind, another question that remains unknown is whether alterations in behavior by BPA could be due to vision impairment caused by BPA-induced microphthalmia and cranial malformations. This could explain BPA specific effects in yolk sac consumption and lipid metabolism already discussed since a reduced movement phenotype could be linked to

less energy requirements and consequently, less lipid reservoir expenditure. Accordingly with previous results (Jantzen et al., 2016), we observed that PFOS exposures also affected craniofacial features of exposed zebrafish eleutheroembryos (shown as decreases in EW and IOD at highest concentrations, even when considering body length reduction). Although others have found alterations of the thyroid system (Ghassabian and Trasande, 2018; Lee and Choi, 2017; Wang et al., 2014) by PFOS exposures, we haven't found in our recent study any alterations at the molecular level associated to vision, thyroid or retinoic pathways (Martínez et al., 2019), suggesting that exposures to PFOS might not alter vision function at the tested concentrations.

In summary, we have found that TBT, with higher lethality (lower LC_{50}) than PFOS, BPA and E2, produced less morphological effects (and those were mainly observed at the highest equilethal doses) and that BPA, with less toxicity (highest LC_{50}), induced higher morphological effects (even at the lowest doses). We can also conclude that morphological effects of both TBT and E2 were mostly related to alterations in developmental rates and can be assigned to non-specific effects of the pollutant. This was particularly true for the case of TBT. In the case of E2, we also reported a clear effect on growth, with exposed animals showing higher body lengths. This was contrary to what we observed for the rest of the compounds, where exposed individuals were smaller than their control counterparts. On the other hand, the higher degree of morphological malformations observed in eleutheroembryos exposed to BPA (even at its lowest concentrations) and in the ones exposed to the medium-high PFOS concentrations were mostly related to specific effects and not to general alterations in developmental rates. Exposure to BPA induced the yolk sac malabsorption syndrome and also characterized by alterations on craniofacial traits. On the other hand, it was remarkable that malformations of eleutheroembryo notochord (appearance of scoliosis and kyphosis) was one of the main alterations produced in PFOS exposures. In general, the results indicate that the four studied compounds, with different chemical structure but common related effects in the estrogenic and lipid pathways (according to the bibliography), trigger different specific and non-estrogenic related morphological alterations most likely due to different ligand-receptor interactions. Results obtained in the present study will be very useful to establish linkages between effects at the

transcriptome (Martínez et al., 2018) and metabolome (Ortiz-Villanueva et al., 2018) levels with observed morphological effects after exposures to the tested compounds. Further studies involving gene expression and gain and loss of functions (i.e. knockout experiments (Cornet et al., 2018; Housden et al., 2016; Varshney et al., 2013)) should be performed to establish correlations between the above mentioned phenotypic and transcriptomic changes. The combination of all available information at the different organizational levels will help us to understand and better characterize modes of action of the studied compounds in an integrative and a holistic manner.

5. Conclusion

It is known that the toxicity of the different pollutants does not underlie only in a general oxidative stress mechanism by reactive oxygen species (ROS), but also in specific ligand-receptor interactions, which are different in each case and explain the distinctive effects of each toxicant. In this scenario, we think that integration analysis of morphometric data can be used as a useful tool to study specific morphological effects in zebrafish embryo model which can be related to the underlying mode of action of the compound and, thereby, be an inexpensive and easy screening to predict modes of action of a wide-range number of contaminants. Integration of these morphometrics analyses with further biomolecular *omics* studies will allow us to elucidate and better understand pollutants modes of action, a main component of the toxicity characterization, one of the central elements of the environmental risk assessment, together with the environmental exposure characterization.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1. Lateral (A) and dorsal (B) images of 5 dpf zebrafish eleutheroembryos exposed to BPA, PFOS, TBT and E2. Morphologically-representative images of the control, low observed effect concentration (LOEC, in terms of survival, hatching and swim bladder inflation) and maximum concentration used for the morphometric analysis are shown. Scale bar = 1 mm. Yolk sacs are encircled by a yellow line for easier viewing.

Figure 1

Figure 2. Morphometric measurements of 5 dpf eleutheroembryos exposed from 2 to 5 dpf to different concentrations of BPA (red), PFOS (green), TBT (blue) and E2 (purple). Represented values per condition are relative to the average of the control group for each of the tested compounds (control average = 1.0), except for YSA which value was corrected by the control baseline (YSA of the control = 0.0). In order to compare the effects of the different tested pollutants, equilethal doses were calculated assigning to each compound the equilethal dose of 1.0 to the highest concentration that did not affected survival at 5 dpf. For each group, the mean (of the relative values) $\pm$ SEM (standard error of the mean) is shown (n=50-60 individuals per group). Non-parametric test (Kruskal-Wallis with all possible pairwise multiple comparisons, $p < 0.05$) was performed. Colored asterisks indicate statistical differences respect each compound

control. The morphometric parameters at the BPA equilethal dose = 1.0 were not determined because of the presence of high rates of malformations at this BPA concentration. Data represents a total of 1490 eleutheroembryos. In each sub-graph, control mean value (1.0) is shown at the x-axis with a black dotted line, except for the YSA where it represents the mean value of the 4 dpf control group (0.039 mm²).

Figure 2

Figure 3. A) Medoid PAM clustering of the measured morphometric traits from all eleutheroembryos exposed to BPA, PFOS, TBT and E2 ($n=50-60$ individuals per group) by PCA analysis. The first two components of the PCA explained 63.45% of the data variability and defined two groups of individuals: cluster A with 897 individuals assigned as “non-affected” eleutheroembryos (cyan) and cluster B with 593 individuals assigned as “affected” eleutheroembryos (orange). **B)** Heatmaps of measurements of morphometric traits of “non-affected” and “affected” eleutheroembryos after exposure to BPA, PFOS, TBT and E2. Values were normalized by each control group parameter (control average = 1.0). Exceptionally, for the yolk sac area and to avoid the bias due to the proximity of its values to zero, data was base-corrected and centered to 1.0 respect the control group and used as input value. Color scale ranges from red to blue (increase or decrease, respectively, in the measure of the parameter, respect the mean value of the control group). Both rows (individuals) and columns (parameters) were grouped by

hierarchical clustering and the corresponding dendrograms are shown at the left and the top of the panel, respectively. Color legends indicate both the equilethal dose (in grey scale) and the compound (yellow: control; red: BPA; green: PFOS; blue: TBT; purple: E2). **C)** Percentage of eleutheroembryos morphologically affected by the exposures (assigned to cluster B) for each condition (type of pollutant and equilethal dose). Experimental groups are represented in different colors: BPA in red, PFOS in green, TBT in blue and E2 in purple. Dose-response patterns were adjusted by a sigmoidal model.

Figure 3

Figure 4. A, B, C) Representation of the selected components from a Principal Component Analysis (PCA) using morphometric data from 3 (cyan), 4 (orange), 5 (green) and 6 dpf (pink) non-exposed eleutheroembryos ($n=29-35$ individuals per group). Standardized values (normalized by the average of the control group and centered to zero) were used as input data, except for the YSA, which was corrected by the control baseline (being also zero the average of the control group). The three first components represented up to 75.8% of the data variability. Arrows represent variable (morphological traits) loadings indicating contribution of each variable to differentiate between individuals. The centroid of each group is shown as a color sphere to avoid oversaturation of the graph. **D)** First two components of a Partial Least Squares (PLS1) analysis using the control developmental age morphometric data (3, 4, 5 and 6 dpf). The nine morphometric variables were used as predictors (matrix X), normalized and log-

transformed after adding a negligible value (0.0001) to avoid the zeros in the YSA. Unidimensional vector containing the age of the individuals was used as responses (matrix y). Three components were selected to construct the model which explain 70.1 and 83.1 % of the variability in the X- and y-matrix, respectively. First two graphed components explain 57.8 and 82.1 % of the variability in the X- and y-matrix, respectively. Blue and orange arrows represent the loadings of X-matrix (morphological traits) and y-matrix (age) variables. The morphologic signatures that drive the changes in the development (BL, YSA, SBA, EL and ESD) are marked with an asterisk and were selected based on their VIP score values (VIP score > 1.0).

Figure 5. Graphic representation of the relative body length (BL) versus the relative values of the different morphometric parameters measured in control eleutheroembryos at different ages (3, 4, 5 and 6 dpf) and exposed eleutheroembryos to different concentrations of BPA, PFOS, TBT and E2 at 5 dpf. Represented morphological traits are the following: yolk sac area (YSA), swim bladder area (SBA), head-trunk angle (HTA), eye-snout distance (ESD), eye length (EL), eye width (EW), head width (HW) and inter-ocular distance (IOD). Represented values for control eleutheroembryos at different ages (3 dpf, triangles; 4 dpf, squares; 5 dpf, dots; and 6 dpf, rhombus) are displayed in yellow. Dashed lines represent a polynomial regression

performed for each morphological trait using non-exposed individuals measured from 3 to 6 dpf (regression parameters are indicated in the figure). Represented values for exposed eleutheroembryos are relative to the average of the control groups at 5 dpf for each of the tested compounds (control average = 1.0), except for YSA which value was corrected by the control baseline (YSA of the control = 0.0). Exposures to different pollutants are displayed in different colors: BPA in red, PFOS in green, TBT in blue and E2 in purple. Dotted lines ($x=1.0$, $y=1.0$) represents control averages at 5 dpf. The color scale for each individual compound increase in intensity/darkness with the increasing concentrations. For each experimental condition, the mean (of the relative values) $\pm$ SEM (standard error of the mean) is shown (data represent $n=29-35$ individuals per group for the age controls and 50-60 individuals for the exposed groups).

Figure 6. A) Heatmap corresponding to relative average measurements of all morphological traits at 5 dpf for each of the tested compounds ($n=50-60$ individuals per group). Values represent relative average values with respect to the average of the control groups at 5 dpf. Represented morphological traits are the following: yolk sac area (YSA), swim bladder area (SBA), head-trunk angle (HTA), eye-snout distance (ESD), eye length (EL), eye width (EW), head width (HW) and inter-ocular distance (IOD). Color scale ranges from red (increase respect to controls) to blue (decrease respect to controls).

White represents values similar to controls (control average = 1.0). Non-parametric Kruskal-Wallis test was performed for each group and parameter to determine significant differences from the control group. Asterisks mark groups statistically different from the control: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). **B)** Heatmap corresponding to the difference between the observed experimental values of the morphometric parameters and their predicted values when considering eleutheroembryo body length. Predicted values for each morphometric trait were estimated from individuals body length using the polynomial regressions indicated in figure 5. Then, predicted values were subtracted from observed values (observed - predicted) and averages calculated and represented for each experimental group. Color scale ranges from red (higher than the expected value) to blue (lower than the expected value). White represents no differences between observed and predicted. Multiple t-test (Bonferroni correction) was performed for each group and parameter using each predicted value as a reference group to determine significant differences. Asterisks mark groups where the experimental value was statistically different from its predicted one: * ($p < 0.01$), ** ($p < 0.001$), *** ($p < 0.0001$).

Figure 6

Figure 7. Multivariate Partial Least Squares (PLS2) model performed with the exposed eleutheroembryo morphometric data using morphometric variables as predictors (X-

block matrix) and a 2D matrix containing both the compound and its concentration as responses (Y-block matrix). A total of 9 morphological traits measured in 1490 eleutheroembryos ($n=50-60$ individuals per condition) were analyzed from eleutheroembryos exposed to BPA, PFOS, TBT and E2. Normalized data were log-transformed after adding a negligible value (0.0001) to avoid the zeros in the YSA. **A)** PLS loadings of the compounds and the morphological traits for the four first components (C1, 2, 3 and 4) of the PLS2 analysis, which explained up to 80.2 and 36.1% of the X-block (morphometric variables) and the Y-block (tested compounds and concentrations), respectively. Asterisks mark the variables with a VIP score > 1.0 . **B)** Loadings representation for each pair of components. Compounds and morphological traits loadings are colored in orange and blue, respectively.

Figure 7