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Combined western diet and bisphenol A exposure induces an oxidative stress-based paraoxonase 1 response in larval zebrafish

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ABSTRACT

Paraoxonase 1 (PON1) is an antioxidant enzyme linked to metabolic disorders by genome-wide association studies in humans. Exposure to metabolic disrupting chemicals (MDCs) such as bisphenol A (BPA), together with genetic and dietary factors, can increase the risk of metabolic disorders. The objective of this study was to investigate how PON1 responds to the metabolic changes and oxidative stress caused by a western diet, and whether exposure to BPA alters the metabolic and PON1 responses. Zebrafish larvae at 14 days post fertilization were fed a custom-made western diet with and without aquatic exposure to two concentrations of BPA for 5 days. A combination of western diet and 150 μg/L BPA exposure resulted in a stepwise increase in weight, length and oxidative stress, suggesting that BPA amplifies the western diet-induced metabolic shift. PON1 arylesterase activity was increased in all western diet and BPA exposure groups and PON1 lactonase activity was increased when western diet was combined with exposure to 1800 μg/L BPA. Both PON1 activities were positively correlated to oxidative stress. Based on our observations we hypothesize that a western diet caused a shift towards fatty acid-based metabolism, which was increased by BPA exposure. This shift resulted in increased oxidative stress, which in turn was associated with a PON1 activity increase as an antioxidant response. This is the first exploration of PON1 responses to metabolic challenges in zebrafish, and the first study of PON1 in the context of MDC exposure in vertebrates.

1. Introduction

Paraoxonase 1 (PON1) is a high density lipoprotein (HDL)-bound serum enzyme with lactonase, arylesterase and phosphotriesterase activities. It was first discovered due to its ability to hydrolyze organophosphate pesticides such as paraoxon (Costa et al., 2020). Its physiological role, however, is situated in lipid metabolism (Meneses et al., 2019). For example, PON1 protects against the oxidation of low density lipoproteins (LDLs) (Mackness et al., 1991). Because oxidized LDL particles contribute to atherosclerosis, PON1 has a known atheroprotective function (Kotur-Stevuljević et al., 2020). Because of this protective function of PON1, it is generally known as an antioxidant enzyme (Meneses et al., 2019).

Polymorphisms in the PON1 gene typically result in changes in PON1 activities towards various substrates (Deakin and James, 2004). Interestingly, some of these polymorphisms have been associated to obesity by genome-wide association studies in humans (Huen et al., 2013). In addition to genetic associations, changes in PON1 enzyme activities have also previously been observed in metabolic disorders. In obesity patients, serum PON1 activities were lowered, correlating negatively to body mass index (BMI) and oxidative stress (Bajnok et al., 2008; Ferretti et al., 2005; Martinelli et al., 2012; Sentí et al., 2003). Decreased serum PON1 activities were also observed in hepatosteatosis patients (Atamer et al., 2008). In rats, a fructose-enriched diet decreased hepatic PON1 activity and increased oxidative stress (Ackerman et al., 2010) while another study observed no significant changes in serum PON1 activities in rats after a high-fat diet (Thomas-Moya et al., 2008). The protective capacity of PON1 against metabolic disorders has also been studied. Koren-Gluzer et al. (2011) administered recombinant PON1 to mice before using streptozocin to induce diabetes (Koren-Gluzer et al., 2011) and showed that recombinant PON1 decreased the incidence of streptozocin-induced diabetes. Garcia-Heredia et al. (2013) observed a

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higher incidence of liver steatosis in PON1-deficient mice fed a high-fat-high-cholesterol diet. In summary, it is known that PON1 plays a protective role in the development of metabolic disorders. At the same time, serum PON1 activities were shown to decrease in patients suffering from the same metabolic disorders it is protective against. The underlying mechanisms of this interaction are not yet fully understood.

Metabolic disorders such as obesity are caused by an imbalance of energy intake versus expenditure. This imbalance can be induced by multiple factors. The western diet, which is characterized by a high intake of carbohydrates and fats, is considered one of the main factors causing obesity in humans (Kopp, 2019). On top of this western diet comes the issue of exposure to environmental pollutants. Some of these environmental pollutants have the ability to disrupt energy metabolism and to contribute to the development of metabolic disorders (Heindel et al., 2017). For example, exposure to polychlorinated biphenyls, tributyltin and bisphenol A (BPA) has been shown to alter adipose tissue or body weight in vertebrate models and humans, and is associated to metabolic disorders (Heindel et al., 2017). These compounds are generally known as metabolic disrupting chemicals (MDCs) and are considered a subgroup of endocrine disrupting chemicals (EDCs).

While both MDCs and PON1 are known to play a role in metabolic disorders individually in mammals including humans, the interaction between MDCs and PON1 remains poorly investigated. One could envision three aspects to such a PON1-MDC interaction. First, PON1 activity levels are known to change in metabolic disorders, often correlating negatively with oxidative stress. Since MDCs contribute to oxidative stress and metabolic disorders, exposure to these compounds can be expected to further alter PON1 activities. Second, some MDCs may directly cause increased oxidative stress, potentially triggering an antioxidant response involving PON1 (Gassman, 2017). Lastly, PON1 has the ability to metabolize a number of xenobiotic compounds (Rosenblat et al., 2006) including organophosphates such as parathion, for which metabolic disrupting properties have been reported (Costa et al., 2020; Czajka et al., 2019; Slotkin, 2011), and aryl esters such as phenyl acetate (Taler-Verčič et al., 2020).

This study reports on the first investigation of the threefold interaction of diet, toxicant exposure, and PON1. The central objective of this study was to explore the PON1 response to the metabolic shift and oxidative stress caused by a western diet, and to investigate whether exposure to an MDC alters the metabolic and PON1 responses. The zebrafish is a highly suitable animal model for studying these interactions as it is often used for both dietary metabolic studies and toxicological studies (Bambino and Chu, 2017; Zang et al., 2018). In zebrafish, the onset of exogenous feeding occurs at 5 days post fertilization (dpf) and lipid deposition has been observed from around 12–15 dpf (Imrie and Sadler, 2010). The larval life stage has been shown to be responsive to dietary treatments and MDCs (Tingaud-Sequeira et al., 2011). Therefore, this life stage was selected to address the central objective of the present study. The larvae were fed either a nutritionally balanced diet or a western diet to investigate the consequences of different nutritional compositions. In addition to the western diet, the larvae were exposed to BPA, a known MDC that is also capable of inducing oxidative stress and is known to contribute to metabolic disorders in fish and mammals, including humans (Canesi and Fabbri, 2015; Gassman, 2017; Wu et al., 2020). BPA is used in the manufacturing of polycarbonate plastics and epoxy resins, which are often used for plastic food containers, an important exposure route for humans (EFSA Panel on Food Contact Materials and Aids, 2015; MacKay and Abizaid, 2018), and in a large number of other consumer products. BPA is primarily known as a xenoestrogen and interacts with several nuclear receptors including the peroxisome proliferator-activated receptor gamma (PPARγ) (MacKay and Abizaid, 2018). The present study is the first exploration of PON1 responses to metabolic challenges, both dietary and toxicological, in the zebrafish. In addition to improving the understanding of metabolic disruption in fish, our study provides new insights for extrapolating results from the zebrafish model to mammals regarding the behavior of PON1 in different metabolic and toxicological scenarios.

2. Materials & methods

2.1. Ethics statement

The experimental work was approved by the Ethical Committee for Animals of the University of Antwerp (project ID 2022-01) and was carried out in strict accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.2. Zebrafish husbandry and treatments

Non-exposed adult wild type AB zebrafish were housed in a recirculating freshwater (28 ± 1 °C, pH 7.5 ± 0.3, 500 ± 25 μS/cm) ZebTec active blue stand-alone system (Tecniplast, Buguggiate, Italy) at a density of 17 animals per 3.5 L tank with a 14/10 h light/dark cycle and continuous mechanical, biological, carbon and UV filtration. Ammonia, nitrite and nitrate concentrations were measured twice a week to ensure concentrations below 0.25, 0.3 and 25 mg/L respectively. These fish were placed in breeding nets for the production of embryos. Reconstituted freshwater was prepared based on reverse osmosis (RO) water, brought to a conductivity of 500 μS/cm and a pH of 7.5 using Instant Ocean® Sea Salt (Blacksburg, VA, USA) and $NaHCO_3$ (VWR International, Radnor, PA, USA) respectively. The larvae were raised in a 28.5 °C incubator in plastic containers. From 5 dpf to 9 dpf, the larvae were fed dry feed (<100 μm Zebrafeed, Sparos, Olhão, Portugal) four times daily. From 10 dpf to 14 dpf, the larvae were fed the same dry feed (Zebrafeed) twice daily, supplemented by freshly hatched *Artemia* spp. nauplii twice daily.

At 14 dpf, the larvae were measured on 1 mm grid paper. Fish with lengths 0.5–0.8 cm were separated into 4 treatment groups of 36 fish each and transferred to 4 glass aquaria filled with 15 L reconstituted freshwater. In each aquarium 4 water-permeable breeding tanks (Domus fish 3, Prodac International, Citadella, Italy) were placed that each contained 9 larvae, resulting in 4 pseudo replicates per group (Fig. S1). These aquaria were outfitted with heaters and a water pump (Magic Pump 200, Prodac International) to allow for sufficient temperature control and water circulation. The temperature of each aquarium was kept at 28 ± 1 °C.

Four treatment groups were selected. A balanced diet group was given a custom-made nutritionally balanced dry feed, and 3 groups were fed a custom-made western zebrafish dry feed (increased lipid, cholesterol, and fructose content, resulting in a higher estimated calorie content). Custom feeds were produced by Sparos using Zebrafeed composition as a reference (Table 1) (Jobling, 1995). Energy and lipid content of both diets were analytically measured (Supplementary file 1, Tables S1, S2). The 3 western diet groups consisted of (1) a western diet group without BPA exposure, (2) a western diet group exposed to a nominal concentration of 200 μg/L BPA and a (3) western diet group exposed to a nominal concentration of 2000 μg/L BPA (Fig. S1). Concentrations around 200 μg/L have previously been shown to cause subtle

Table 1
Nutritional components of experimental diets. Values in bold indicate modifications made to the balanced diet to produce the western diet.

Rough components (%)	Balanced	Western
Fat	12.5	**25.1**
Of which cholesterol	0.23	**0.51**
Protein	50.6	50.6
Carbohydrates	5.0	**7.5**
Of which fructose	0.0	**2.5**
Fibers	14.8	0.1
Ash	10.7	10.7
Total energy (kcal/100 g)	429	**559**

effects in lipid metabolism and estrogen receptor signaling (Sun et al., 2020; Sun et al., 2019). The BPA concentration of 2000 μg/L was chosen based on effects previously observed at a physiological level, such as changes in weight (Martínez et al., 2020). Data are reported as a function of actual, analytically confirmed exposure concentrations (i.e., 150 and 1800 μg/L) in the results and discussion section. For determining the feeding rate, gradually increasing body weight was estimated based on an average larval wet weight of 1.8 mg at 14 dpf and 6.5 mg at 21 dpf obtained from a previous experiment. Each group of 36 larvae was fed 10 % of their estimated body weight in dry feed (balanced or western) across 2 feeding moments (12:00 and 17:00), supplemented by an average portion of 60 mg (wet weight) freshly hatched *Artemia* spp. twice daily (9:00 and 15:00), acquired by allowing 4.8 g of frozen *Artemia* cysts to incubate for 24 h at 28 °C in 2.5 L RO water containing 30 g/L Instant Ocean Sea Salt in a Fleuren & Nooijen (Nederweert, The Netherlands) system.

BPA (Tokyo Chemical Industry Europe, Zwijndrecht, Belgium) stock solutions of 20 and 2 g/L in pure dimethyl sulfoxide (DMSO) (Acros Organics B.V.B.A., Geel, Belgium) were prepared, aliquoted and stored at −20 °C for the two BPA exposure groups. The exposure medium of each group was refreshed daily. The final DMSO concentrations of all media was 0.01 % DMSO.

At 19 dpf, the larval length was measured on 1 mm grid paper by taking pictures of the larvae in a petri dish without exposure to air, after which the larvae were sacrificed by overdose of 1 g/L tricaine methanesulfonate (MS222; pH 7.5; Sigma-Aldrich, St. Louis, MO, USA), rinsed using medium, pooled together per replicate, weighed and snap-frozen in liquid nitrogen. This resulted in 16 pooled whole-body samples (4 replicates per treatment, 4 treatments) which were homogenized using a liquid nitrogen cooled mini mortar (SP Bel-Art, Wayne, NJ, USA) and aliquoted for biochemical and molecular analyses, resulting in paired measurements for each endpoint. Condition factors were determined based on weight (W) and average length (L). Parameters a and b were determined by the function of $W = aL^b$ of the balanced diet group and used for condition factor calculations using the formula $K = W/aL^b$.

2.3. PON1 activity measurements

PON1 lactonase (LACase) activity was measured using 5-thiobutyl butyrolactone (TBBL) as substrate. For PON1 arylesterase (AREase) activity measurements, the Arylesterase/Paraoxonase assay kit of Zeptometrix (Buffalo, NY, USA) was used. The full protocols can be found in Supplementary file 1 (Section S1.3). Extinction coefficients used for calculations were 7000 M-1 cm-1 and 1310 M-1 cm-1 for LACase and AREase activities respectively. One unit of activity at pH 8 amounted to the hydrolysis of 1 μmole of TBBL per minute at 37 °C for LACase activity and 1 μmole phenylacetate per minute at 25 °C for AREase activity. PON1 activities were normalized to protein content using the Bradford method.

2.4. Malondialdehyde and triglyceride level measurements

Malondialdehyde (MDA) concentration was measured as a marker for lipid peroxidation, a form of oxidative stress, using the Malondialdehyde (MDA) Assay Kit (Northwest Life Science Specialties LLC., Vancouver, WA, USA) with some alterations for use with a 96-well plate. Lipids were extracted using 4:1 isopropanol:hexane and 1:1 hexane: diethylether mixtures. Triglyceride levels were measured in extracted lipids using the Triglycerides GPO Method Kit (Biolabo S.A.S., Maizy, France). The full protocols can be found in Supplementary file 1 (Sections S1.4-S1.5).

2.5. BPA concentration measurements

Water samples of 100 mL were taken on the first day and the fourth day of the treatment 1 h after renewal of the exposure medium, as well as 24 h later (i.e., before the next medium renewal).

For the analytical measurements of BPA concentration in the exposure media, a defined volume (50 μL) of $^{13}C_{12}$-BPA internal standard (IS) in methanol was added in a LC injection vial to a defined volume of medium (50, 100 or 200 μL medium depending on the concentration), and the mixture was vortexed for 1 min. Afterward, the mixture was analyzed using a Agilent 1290 liquid chromatograph coupled to a 6460 tandem mass spectrometer (LC-MS/MS) with electrospray ionization in negative mode. Detailed information on the LC-MS/MS analysis of BPA and applied QA/QC procedures is given in Supplementary file 1 (Section S1.6).

One aliquot per replicate of homogenized tissue collected at 19 dpf was used for internal BPA measurement. This resulted in a total of 16 BPA concentration measurements (4 samples per treatment, 4 treatments).

For the measurement of the internal BPA concentrations in 19 dpf larvae, a defined volume (50 μL) of $^{13}C_{12}$-BPA internal standard (IS) in methanol was added in a LC injection vial to zebrafish whole-body samples (typically 30 mg), together with 1 mL methanol and the mixture was vortexed for 1 min, followed by sonication for 5 min. After filtration to a 0.22 μm centrifugal filter, the mixture was analyzed using an LC-MS/MS system with electrospray ionization in negative mode. Detailed information on the LC-MS/MS analysis of BPA and applied QA/QC procedures is given in Supplementary file 1 (Section S1.5).

2.6. RNA extraction and cDNA synthesis

RNA was extracted using the NucleoSpin RNA Isolation Kit (Macherey-Nagel, Düren, Germany) according to the provider's instructions. Around 5 mg of homogenate was transferred to a sterile microcentrifuge tube containing 550 μL lysis buffer and 15 mM dithiothreitol. RNA integrity was analyzed using a 12 capillary Fragment analyzer (Agilent, Santa Clara, CA, USA) and an RNA Kit (15NT) (Agilent). All resulting RNA quality number (RQN) values were 9.9 or above. RNA purity was confirmed using absorption ratios of 260/280 and 260/230 measured in a BioDrop μLite+ (Biochrom Ltd., Cambridge, UK), ranging between 2.1 and 2.2, or 1.8 and 2.2 respectively, except for three samples with 260/230 ratio of 1.3–1.4. cDNA synthesis was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc.). Again, absorption ratios of 260/280 and 260/230 were measured using a BioDrop μLite+ (Biochrom Ltd.), and ranged between 1.8 and 1.9 across all samples.

2.7. RT-qPCR

Primers for qPCR analysis (Table S3) were obtained from Eurogentec (Seraing, Belgium) and diluted to a concentration of 10 μM. Wells of a 96-well plate (AmpliStar-II, semi-skirted, FAST type, Westburg, Leusden, The Netherlands) were filled with 15 μL of a mix containing 1 μL forward primer, 1 μL reverse primer, 8 μL diethyl pyrocarbonate (Sigma-Aldrich) treated water and 5 μL CAPITAL qPCR Green Master Mix LROX (Biotechrabbit, Henningsdorf, Germany), followed by addition of 5 μL of 70 ng/μL cDNA sample. Each sample was run in duplicate. The ROX dye included in the master mix served as a passive reference dye for well-to-well normalization. Each 96-well plate contained two blanks and a calibration curve of a reference sample (a mix of an equal amount of each cDNA sample) in a serial dilution (1×, 0.67×, 0.44×, 0.3×, 0.2×, 0.13×) in duplo. Amplification and SYBR Green measurement occurred in a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific Inc.). Each program consisted of 3 min 95 °C – 45× (20 s 95 °C – 15 s annealing temperature (Table S3) – 30 s 72 °C). A melting curve was generated by heating to 95 °C for 60 s followed by 60 °C for 30 s and a 0.15 °C/s increase to 95 °C. Melting curves were evaluated and primer efficiencies were determined in each run for use in the calculations. From the eukaryotic translation elongation factor 1 alpha (*ef1a*), 18S rRNA (*18S*), hypoxanthine guanine phosphoribosyl transferase 1

(*hprt1*), ribophorin 2 (*rpn2*) and beta actin (*actb*), two reference genes (*ef1a*, *18S*, GeNorm score of 0.372) were selected using the method of Vandesompele et al. (2002) in the Thermo Fisher Cloud's Applied Biosystems Relative Quantitation Analysis Module. The ΔΔCt method (Pfaffl, 2001) was used to calculate fold changes for statistical analysis. Values were expressed as fold changes relative to the average of the balanced diet samples.

2.8. Statistical analysis

Excluding larval length analysis and principal component analysis (PCA), all statistical tests were performed using Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) tests were performed for embryo weights, condition factors, triglyceride levels, MDA, PON1 activities and gene transcription results with Tukey's multiple comparisons test for post hoc analyses. Whole-body BPA concentration was analyzed using Welch's ANOVA with Dunnett's T3 multiple comparisons test, medium BPA concentration was analyzed with a two-way ANOVA and bioconcentration factors (BCF) were analyzed using an unpaired *t*-test. Because length data have been collected on an individual basis, embryo lengths were analyzed using a linear mixed model in Rstudio (R version 1.3.1093) including the replicate as a source of random effects, also using Tukey's multiple testing correction for post hoc analyses. Correlations between all endpoints were determined using Spearman's correlation test. PCA was performed using RStudio using the 'prcomp' function with scaling to have unit variance. The PCA plot was made using the 'autoplot' function of the 'ggplot2' package. All endpoints measured in fish or tissue were included in the PCA. Results were considered statistically significant if $p < 0.05$.

3. Results

3.1. BPA exposure was stable over time and resulted in BPA uptake

Measurement of medium BPA concentrations showed that for the western diet 200 μg/L BPA exposure group, actual concentrations were 72–83 % of the nominal concentration, and for the group exposed to 2000 μg/L BPA, actual concentrations were 89–92 % of the nominal concentration (Fig. 1A). Therefore, the groups have been defined as the balanced diet group (BD), western diet group without BPA exposure (WD0), western diet group with 150 μg/L BPA (WD150) and western diet with 1800 μg/L BPA (WD1800). The concentration in the medium was highly stable over time with very little BPA (3 % in the WD1800 group) lost from the medium in the 24 h time frame between medium renewals. Whole-body BPA concentrations were significantly increased as a function of (but not proportional to) BPA exposure concentrations (Fig. 1B). The WD1800 group had a significantly higher BCF of 6.2 compared to the BCF of 4.4 for the WD150 group (Fig. 1C).

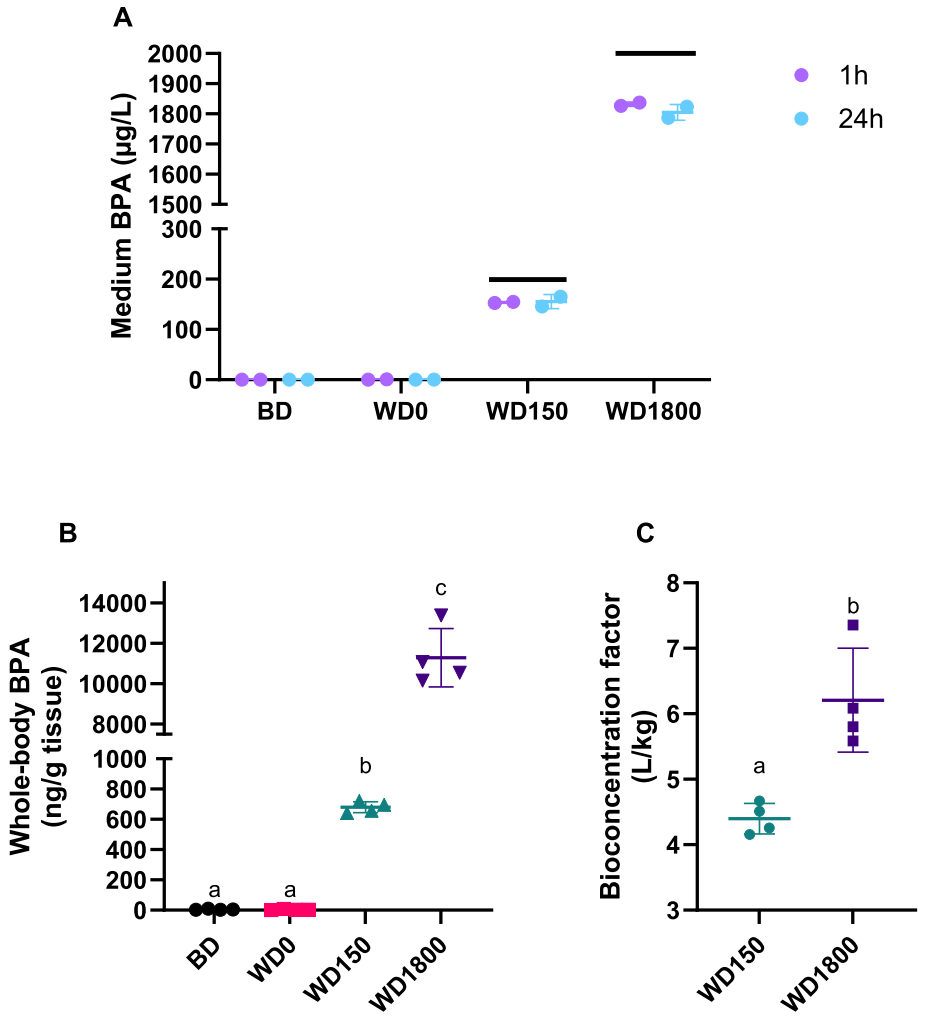

Fig. 1. BPA measurements in medium and whole-body homogenate and resulting bioconcentration factor. Data represent the mean ± SD of BPA concentration in medium 1 h and 24 h after renewal (A, $n = 2$, 15 and 18 dpf), concentration of BPA in whole-body homogenates at the end of the exposure (19 dpf) (B, n = 4) and bioconcentration factors (C, $n = 4$). Colored lines in (A) indicate nominal exposure concentrations. BD: balanced diet, WD: western diet. 0, 150, and 1800 indicate BPA concentration in μg/L.

3.2. Combination of BPA exposure and western diet caused weight gain and increased growth

Larval weight, length and condition factor at 19 dpf are shown in Fig. 2. A significant increase in weight was observed in the WD150 group compared to the BD group (Fig. 2A). At the same time, the WD150 larvae were significantly longer than all other experimental groups (Fig. 2B). Condition factors (Fig. 2C) were not significantly different between any of the experimental groups.

3.3. Combination of BPA exposure and western diet resulted in increased oxidative stress

Whole-body MDA levels and triglyceride levels are shown in Fig. 3. MDA levels were significantly higher in both BPA-exposed groups when compared to the BD group, indicating increased oxidative stress (Fig. 3A). There were no significant differences in whole-body triglyceride contents (Fig. 3B).

3.4. PON1 activities increased in response to western diet and BPA exposure

Whole-body PON1 LACase and AREase activities of the different experimental groups are shown in Fig. 4. PON1 LACase activity (Fig. 4A) was significantly higher in the WD1800 group compared to the BD group. On the other hand, PON1 AREase activity (Fig. 4B) was significantly increased in each group receiving western diet when compared to the BD group.

3.5. BPA exposure altered gene transcription

Relative mRNA expression of all tested genes is shown in Fig. 5. A significant increase in acetyl-CoA carboxylase alpha (*acaca*) transcription was observed in the western diet BPA exposed groups compared to the WD0 group. An increase was also observed in estrogen receptor 1 (*esr1*) mRNA expression in the WD1800 group compared to all other groups. No significant changes were observed in the transcription of paraoxonase 1 (*pon1*), peroxisome proliferator-activated receptor gamma (*pparg*), and hydroxyacyl-CoA dehydrogenase (*hadh*) across the different treatments.

3.6. PON1 activities and mRNA expression correlate to metabolic parameters

Because all measurements were performed on aliquots of the same 9 pooled fish, correlations between all endpoints could be analyzed for each replicate. These correlations are shown in Fig. 6. Significant correlations based on Spearman's correlation test were observed between

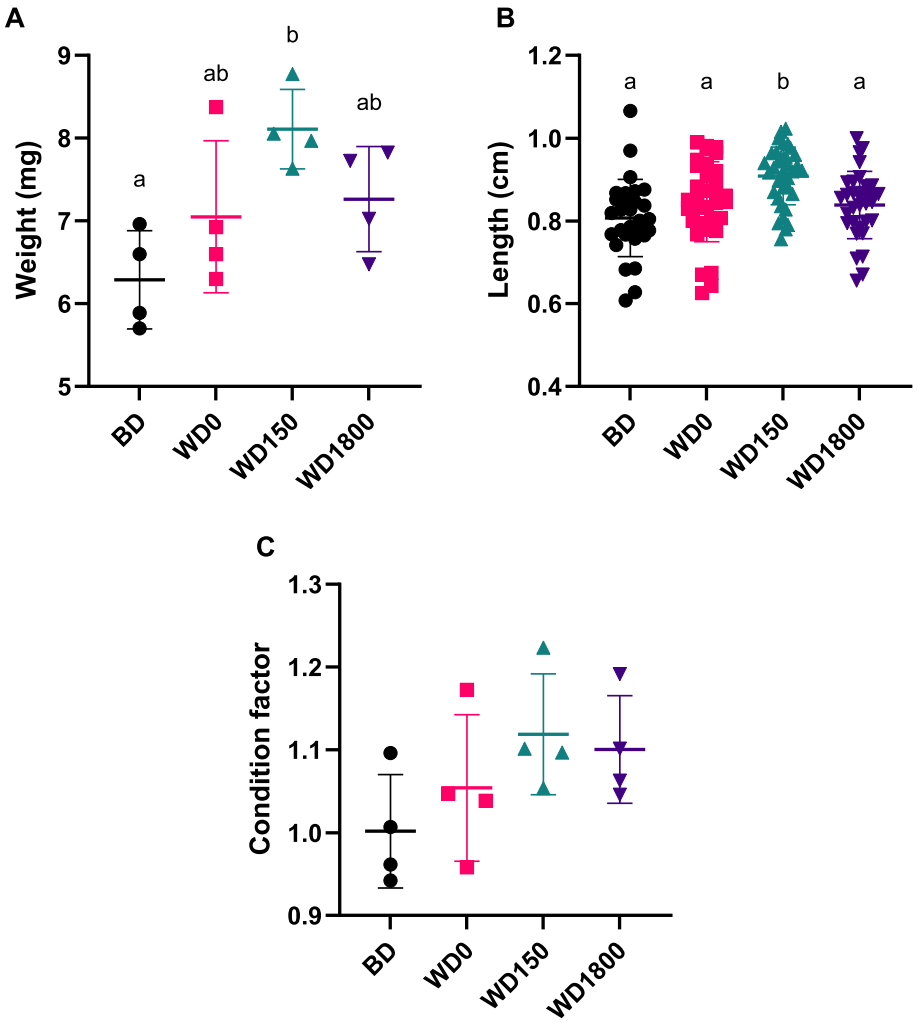

Fig. 2. Weight, length and condition factors. Data represent the mean ± SD of larval weights (A, n = 4), lengths (B, *n* = 36) and condition factors (C, n = 4) of each group at the end of the exposure (19 dpf). Different letters indicate a statistically significant difference based on the ANOVA post hoc analyses with Tukey's multiple comparisons test. BD: balanced diet, WD: western diet. 0, 150, and 1800 indicate BPA concentration in μg/L.

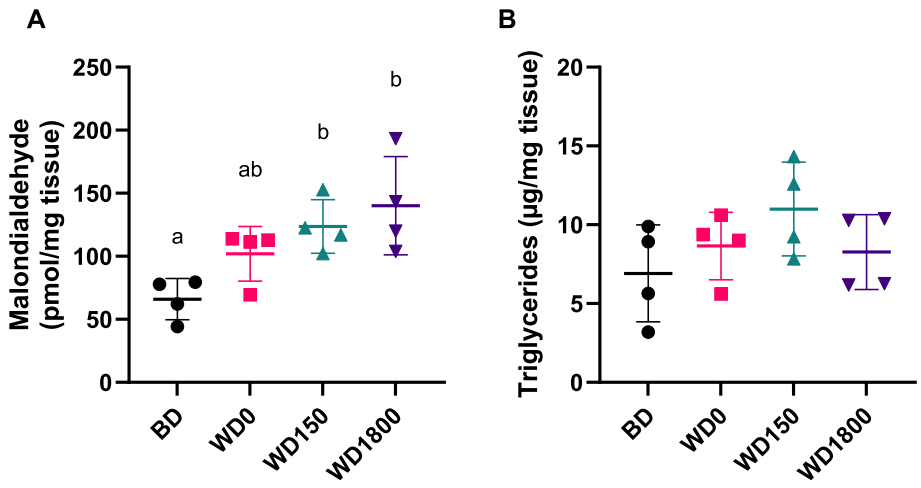

Fig. 3. Whole-body malondialdehyde (MDA) and triglyceride content at the end of the exposure (19 dpf). Data represent the mean ± SD of MDA (A, n = 4) and triglyceride levels (B, n = 4). Different letters indicate a statistically significant difference based on the ANOVA post hoc analyses with Tukey's multiple comparisons test. BD: balanced diet, WD: western diet. 0, 150, and 1800 indicate BPA concentration in µg/L.

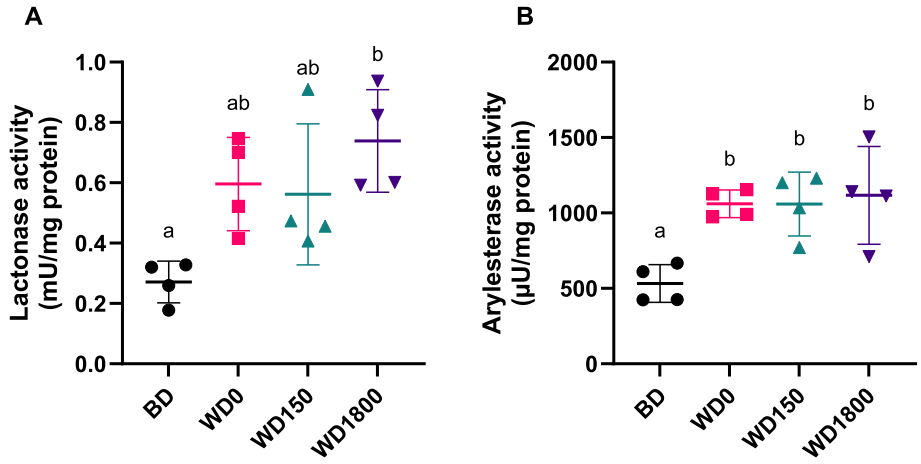

Fig. 4. Whole-body PON1 LACase and AREase activities at the end of the exposure period (19 dpf). Data represent the mean ± SD of LACase (A, n = 4) and AREase (B, n = 4) activities. Different letters indicate a statistically significant difference based on the ANOVA post hoc analyses with Tukey's multiple comparisons test. BD: balanced diet, WD: western diet. 0, 150, and 1800 indicate BPA concentration in µg/L.

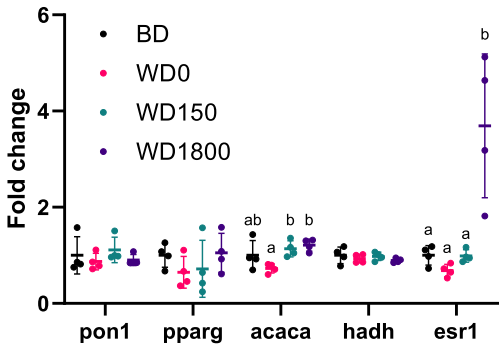

Fig. 5. Transcript levels of *pon1*, *esr1* and genes involved in lipid metabolism. Data represent the mean ± SD of paraoxonase 1 (*pon1*), peroxisome proliferator-activated receptor gamma (*pparg*), acetyl-CoA carboxylase (*acaca*), hydroxyacyl-coA dehydrogenase (*hadh*) and estrogen receptor 1 (*esr1*) fold change normalized to the BD group. Different letters indicate a significant difference based on the ANOVA post hoc analyses with Tukey's multiple comparisons test. BD: balanced diet, WD: western diet. 0, 150, and 1800 indicate BPA concentration in µg/L.

internal BPA concentration and *esr1* transcription (Fig. 6A), PON1 LACase activity and MDA levels (Fig. 6B), PON1 AREase activity and MDA levels (Fig. 6C), PON1 AREase activity and triglyceride content (Fig. 6D), condition factor and *pon1* transcription (Fig. 6E), and whole-body BPA concentration and *acaca* transcription (Fig. 6F).

3.7. Principal component analysis shows stepwise metabolic shift across treatments

A PCA was performed based on the full dataset (Fig. 7). The first two principal components shown in Fig. 7 describe 53.02 % of the variation in the dataset, with PC1 accounting for 31.88 % of the variation in the dataset while PC2 accounts for another 21.9 % of the variation. Along the first principal component (PC1), the BD group is clustered on the negative side of the axis. The WD0 group is clustered in the middle with some overlap with the BPA exposed western diet groups on the positive side of PC1. The two BPA exposed groups are separated along the second principal component (PC2), with the WD150 group on the positive side of the axis, and the WD1800 group on the negative side. Most endpoints are clustered along the positive side of PC1, including weight, length, condition factor, triglyceride levels, MDA, LACase activity and AREase activity, which were generally shown to be higher in the western diet

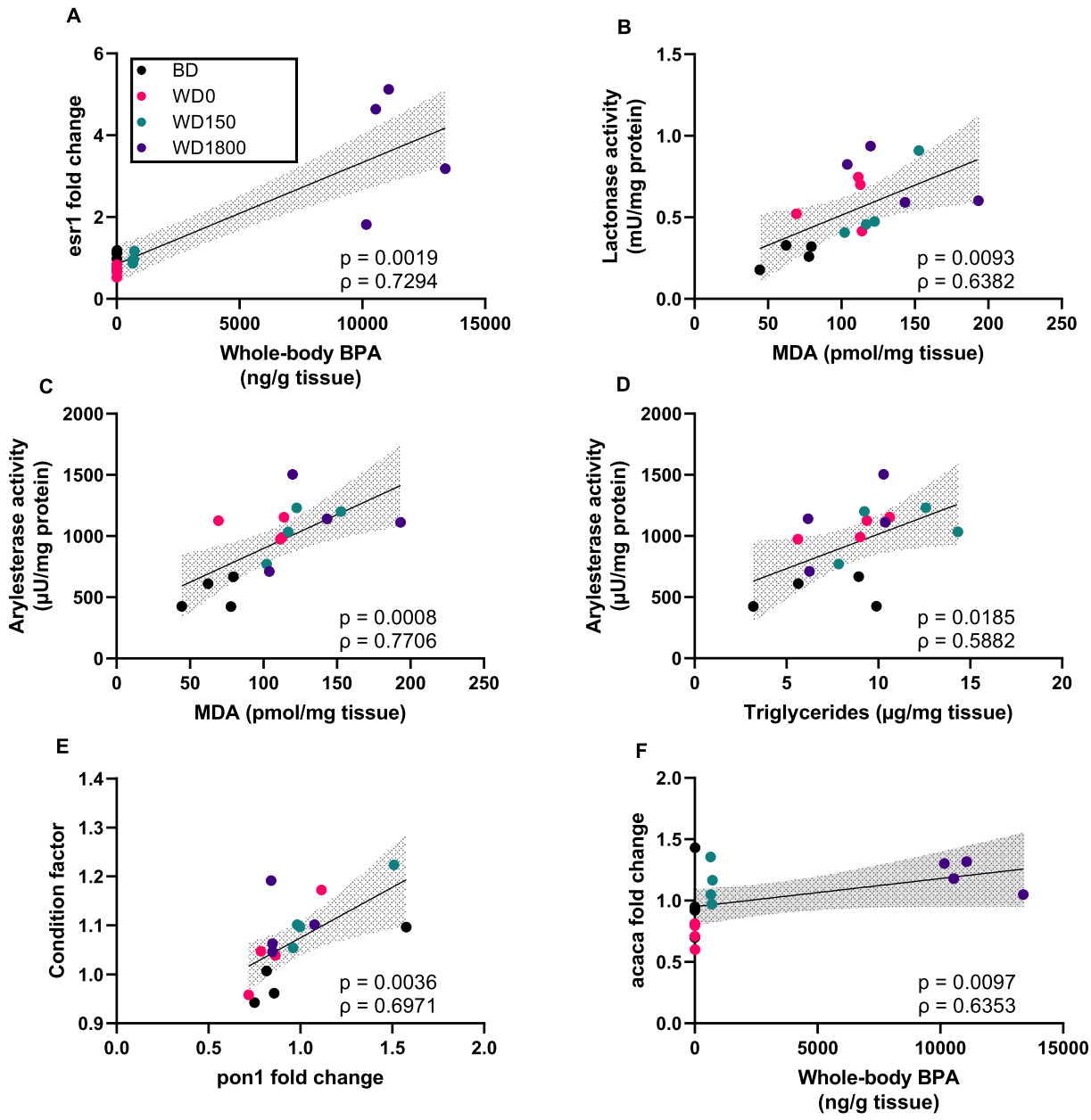

Fig. 6. Significant correlations between pairs of endpoints. Each datapoint represents the (average) measurement of one replicate. Black lines show simple linear regressions and gray area shows 95 % confidence interval. (A) Correlation of tissue BPA concentration and *esr1* transcription. (B) Correlation of PON1 LACase activity and MDA levels. The BD group datapoints are mostly concealed beneath the WD0 datapoints. (C) Correlation of PON1 AREase activity and MDA levels. (D) Correlation of PON1 AREase activity and triglyceride content. (E) Correlation of condition factor and *pon1* mRNA expression. (F) Correlation of tissue BPA concentration and *acaca* transcription. BD: balanced diet, WD: western diet. 0, 150, and 1800 indicate BPA concentration in μg/L.

groups with BPA exposure compared to the western diet group without BPA exposure.

4. Discussion

PON1 has previously been shown to play a role in metabolic disorders, conditions that are associated to imbalances in energy metabolism, in mammals including humans. Exposure to MDCs is known to contribute to the development of metabolic disorders, but the interaction between these factors has not been sufficiently studied. The central objective of this study was to investigate how PON1 responds to the metabolic shift and oxidative stress caused by a western diet, and whether exposure to BPA alters the metabolic and PON1 responses. We used a zebrafish larval feeding trial to study the combination of a western diet and BPA exposure.

4.1. Combined western diet and BPA exposure causes increased oxidative stress

Studies have previously shown that zebrafish fed high-calorie diets showed signs of metabolic disturbances, including increased body weight, length, condition factor and lipid accumulation (Landgraf et al., 2017). Here we combined a custom-made western diet with BPA exposure. BPA was taken up by the larvae in the 5 day exposure time frame until 19 dpf and the whole-body BPA concentration increased with increasing exposure concentration (Fig. 1A). The bioconcentration factors for the WD150 group and the WD1800 group (4.4 and 6.2 respectively) agreed with previously reported BCFs in the range of 2–6 after

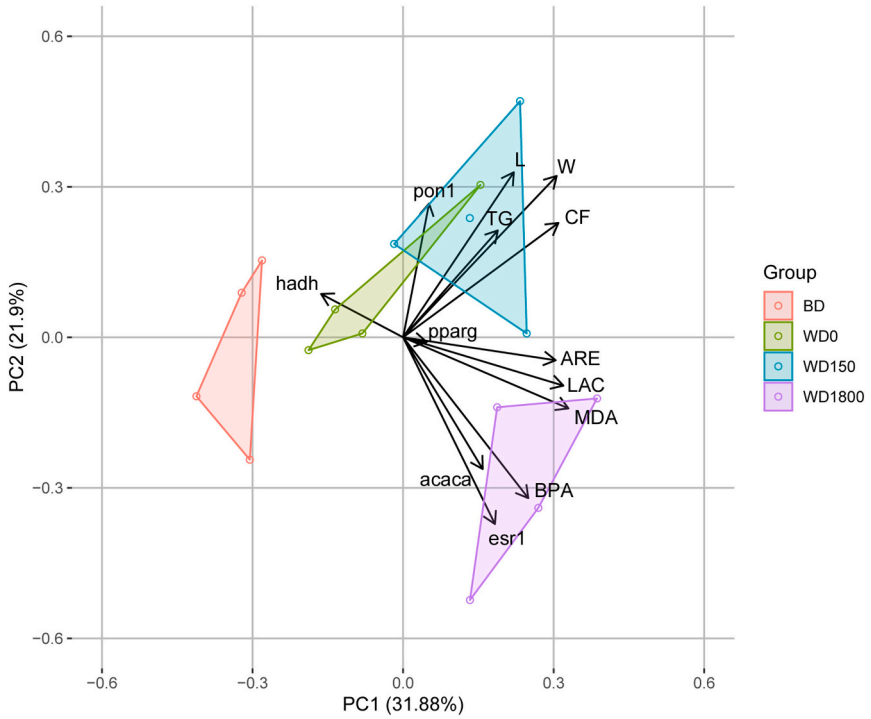

Fig. 7. Principal component analysis. Biological replicates are shown as separate dots in the PCA. Different endpoints are shown as arrows, including weight (W), length (L), condition factor (CF), whole-body BPA concentration (BPA), MDA levels (MDA), triglyceride levels (TG), LACase activity (LAC), AREase activity (ARE) and mRNA expression of the measured genes. BD: balanced diet, WD: western diet. 0, 150, and 1800 indicate BPA concentration in μg/L.

exposure of zebrafish adults and embryos to 100–1000 μg/L BPA for 5 days (Brown et al., 2019; Lindholst et al., 2003; Moreman et al., 2017). Similar internal concentrations have previously been shown to activate estrogen response elements in embryos (Brown et al., 2019; Moreman et al., 2017).

We observed a significant increase of weight and length in the WD150 group compared to the BD group (Fig. 2A, B). Taken together, increased weight and length indicated increased growth in the WD150 group, which has previously been observed in human children with metabolic disorders (Chung, 2017). Previous studies have shown that high-fat diets can upregulate fatty acid oxidation as well as stimulate lipogenesis in mice and rats respectively (Sikder et al., 2018; Zhukova et al., 2014). This indicates that a diet high in lipids may result in a metabolic shift towards increased fatty acid metabolism. The combination of a western diet with BPA exposure resulted in specific transcriptional changes (Fig. 5). In both the WD150 and WD1800 groups, the

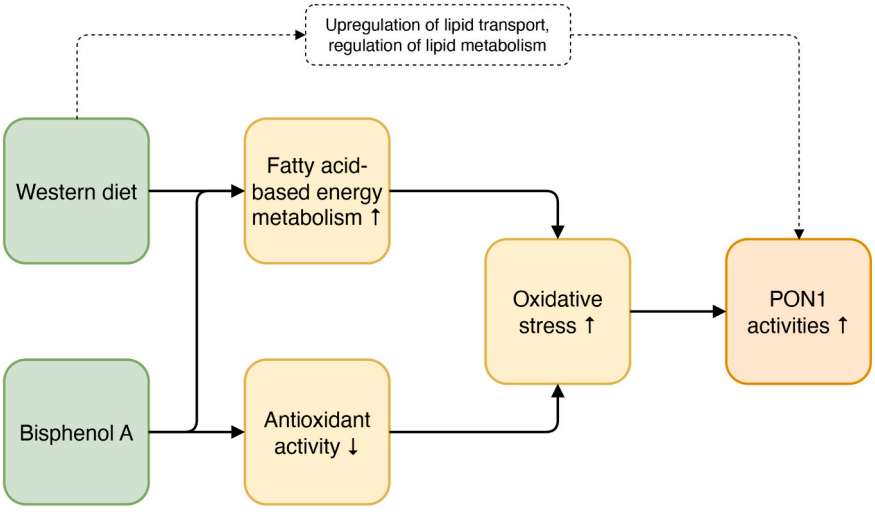

Fig. 8. Hypothesized mechanisms linking western diet and BPA exposure to oxidative stress and PON1 activity changes based on the observations in this study and supported by mechanistic knowledge from existing literature (Rani et al., 2016; Tan et al., 2018; Gassman, 2017). The western diet likely caused a metabolic shift towards a fatty acid-based energy metabolism, which was increased by BPA exposure. The increased fatty acid-based energy metabolism caused increased oxidative stress. Additionally, BPA independently contributed to oxidative stress by lowering antioxidant activity and producing reactive oxygen species. The increased oxidative stress likely triggered the increased PON1 activity as an antioxidant response. Although we have not direct evidence for a link with lipid transport, PON1 is known to be associated to lipid transport through HDL and is influenced by regulators of lipid metabolism. These factors are therefore also likely to contribute to the PON1 response caused by the western diet (Blatter Garin et al., 2006; Camps et al., 2012; Escolà-Gil et al., 2011).

transcript levels of *acaca*, a gene involved in fatty acid synthesis, were increased compared to the WD0 group, but not the BD group. In addition, the whole-body BPA concentration was positively correlated with the *acaca* fold change (Fig. 6F). This suggests that BPA exposure affects fatty acid synthesis. Fig. 8 summarizes the current mechanistic understanding based on our observations in combination with evidence available in the literature. Taken together, our data suggest that a western diet caused a metabolic shift towards increased fatty acid-based metabolism, and that BPA exposure amplifies this shift (Fig. 8 top left side). As a known estrogen receptor agonist, BPA exposure caused increased *esr1* mRNA expression in the WD1800 group compared to all other groups (Mu et al., 2018). BPA also affects many other nuclear receptors including PPARγ, the thyroid hormone receptor and glucocorticoid receptor, among others (MacKay and Abizaid, 2018). It is currently unclear which of these pathways, or most likely combinations thereof, lie at the basis of the observed effects and what the exact consequences are on a biochemical and physiological level. Neither whole body triglyceride content (Fig. 3B) nor condition factor (Fig. 2C) showed any significant differences among treatments. As condition factor is also typically used as an indicator for metabolic disturbances similar to BMI in humans, this suggests that severe metabolic disturbances did not occur (Gurka et al., 2018). Instead, this study was able to identify subtle, sublethal changes in the metabolic state and PON1 response.

The differences in metabolic state are further supported by the PCA (Fig. 7), where the western diet groups are separated from the BD group along PC1. The analysis shows that endpoints such as weight, length, condition factor, whole-body triglyceride content and whole-body MDA levels were the most important contributors to this separation along PC1 with these endpoints generally showing higher levels in the groups fed a western diet. A further shift of the BPA exposed groups compared to the WD0 group, a shift to which both principal components contribute, confirms that the western diet had metabolic effects, and BPA exposure further increased these effects. In summary, the following four metabolic states were observed. The BD group was considered to be in a balanced metabolic state, with the lowest weight and length. The WD0 group showed very little significant metabolic changes compared to the other groups, but did represent an intermediary state between the BD and BPA exposed groups. The WD150 group experienced a more pronounced metabolic shift compared to the WD0 group, indicating that the metabolic changes were amplified by BPA. Lastly, the WD1800 group experienced metabolic disruption on a biochemical level, but showed no differences on a physiological level.

Oxidative stress, measured as MDA levels, were increased in the WD150 and WD1800 groups compared to the BD group (Fig. 3A). Such an increase in oxidative stress is typical for many metabolic disorders (Marseglia et al., 2014). In addition, in the PCA, MDA was positioned to the positive side of PC1, along with weight, length and triglyceride content (Fig. 7), suggesting a link between the shift towards fatty acid metabolism and increased oxidative stress (central hypothesized mechanism in Fig. 8). Oxidative phosphorylation and fatty acid oxidation are important metabolic sources of oxidative stress (Rani et al., 2016; Tan et al., 2018), and these processes would both be highly active in a fatty acid-based metabolism. Experimentally, diets high in fat and carbohydrates, such as the western diet, have indeed been shown to promote oxidative stress (Rani et al., 2016).

BPA exposure was shown to exacerbate the oxidative stress-inducing effect of a western diet (Fig. 3A). One contributor to this effect was its contribution to the metabolic shift induced by the western diet, as observed in the PCA (Figs. 7, 8). In addition, BPA has also been shown to directly induce oxidative stress through the enzymatic and non-enzymatic formation of phenoxyl radicals. Furthermore, BPA was also shown to reduce the total antioxidant capacity by decreasing the activities of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase in various tissues in rodents. Taken together, BPA can directly increase oxidative stress in an organism in addition to its indirect contribution to oxidative stress through metabolic effects (Gassman, 2017, bottom hypothesized mechanism in Fig. 8).

The WD1800 group did not show the same pattern for most of the metabolic endpoints compared to the WD150 group. While length and weight significantly increased in the WD150 group (Fig. 2), no significant difference was observed in the WD1800 group. Only MDA was significantly higher in the WD1800 group compared to the BD group (Fig. 3A). These results could imply that the metabolic effects of BPA followed a non-monotonic dose response curve where the expected dose-response relationship is not maintained. This is not unusual for an endocrine disruptor (Lagarde et al., 2015). Alternatively, the highest BPA dose might be close to concentrations causing systemic toxicity. In previous studies, a decreased body length was observed in zebrafish embryos at 4 mg/L BPA and higher, likely caused by systemic toxicity, although no changes in mortality were observed until 10 mg/L (Martínez et al., 2018; Martínez et al., 2019; Scopel et al., 2021). A previous study determined the 120-hpf LC50 of BPA for zebrafish to be around 9.5 mg/L, which is similar to 96-h LC50 values of other fish species (Alexander et al., 1988; Scopel et al., 2021). Although this is the same order of magnitude as the exposure in the WD1800 group, this concentration is below the LC10 value of 8.2 mg/L obtained from the same data, indicating that the exposure in the WD1800 group was sublethal. Given the sublethal dose, the effects observed in MDA (Fig. 3A), *acaca* transcription (Fig. 5), and the positive positioning along PC1 in the PCA (Fig. 7) for the WD1800 group, which indicates the metabolic shift, the metabolism of this group was disrupted, but the physiological consequences were different compared to the WD150 group.

4.2. PON1 responds to western diet and BPA-induced oxidative stress

PON1 was selected as a gene of interest in the development of metabolic disorders due to its link with obesity determined by genome-wide association studies (Huen et al., 2013). While the zebrafish is a recognized model for human metabolic disease, no studies currently exist regarding the relation between PON1 and metabolism in zebrafish. In the present study, whole-body PON1 AREase activity significantly increased in all groups receiving western diet irrespective of BPA exposure compared to the BD group (Fig. 4B) and PON1 LACase activity significantly increased in the WD1800 group compared to the BD group (Fig. 4A). These increases in PON1 activities coincided with increases in oxidative stress (Fig. 3A), which was likely a result of the shift towards fatty acid-based energy metabolism. Both PON1 activities were correlated with MDA levels (Fig. 6B, C). The indirect relation between the shift towards a fatty acid-based metabolism and increased PON1 activity can also be observed in the correlations between triglyceride levels and PON1 AREase activity (Fig. 6D), as well as between the condition factor and *pon1* transcription (Fig. 6E). These results strongly indicate a link between oxidative stress and PON1 activities (hypothesized mechanism on the right in Fig. 8). Indeed, PON1 is known to fulfill the role of an HDL-bound antioxidant (Kunachowicz et al., 2023; Mackness et al., 1991). More specifically, PON1 LACase activity is thought to be predominantly responsible for its direct antioxidant functions (Rosenblat et al., 2006). In summary, a shift towards fatty acid-based energy metabolism through the combination of a western diet and BPA exposure caused increased oxidative stress, which likely triggered the PON1 antioxidant response and increased PON1 activities (Fig. 8).

Additional mechanisms may have contributed to the PON1 response observed in the present study. Although we have not directly provided evidence for this link, previous studies have shown that PON1 is responsive to various dietary factors through changes in lipid transport and regulation of lipid metabolism. For example, mono-unsaturated fatty acids have been shown to increase PON1 activities in humans (Kunachowicz et al., 2023). As PON1 is directly bound to HDL in serum, it might have been directly affected by changes in lipid transport and metabolism in larval zebrafish. A higher dietary lipid and cholesterol content has been shown to increase HDL content (Escolà-Gil et al., 2011; Fernandez and Murillo, 2022). Indeed, HDL concentration and PON1

concentration and activities have been shown to be tightly correlated in humans (Blatter Garin et al., 2006). PON1 activity is also stimulated by the activation of PPARγ, a master regulator of adipogenesis, independent of HDL levels (Camps et al., 2012). These findings suggest that a western diet may increase PON1 activities via changes in lipid transport and the regulation of lipid metabolism (top hypothesized mechanism in Fig. 8). For the first time, these results provide biochemical and transcriptional evidence for a role of PON1 in metabolic disruption in zebrafish larvae.

Relations between PON1 activities and metabolic disorders have previously been studied in mammals, but this is the first report of such relations in zebrafish. When extrapolating PON1 responses from zebrafish to mammals, biological as well as methodological differences should be considered. First, there are uncertainties related to the extrapolation of exposure of zebrafish via water to exposure of humans via food as well as to the extrapolation of whole-body internal doses in fish to human biomonitoring data mostly presented as concentrations in serum and urine and to tolerable daily intakes. Second, obese patients tend to have decreased serum PON1 activities (Bajnok et al., 2008; Ferretti et al., 2005; Martinelli et al., 2012; Sentí et al., 2003). This pattern of decreased serum activity persists in patients with obesity-associated disorders such as non-alcoholic fatty liver disease (Kotani et al., 2021). The latter decreases in mammalian PON1 activities have been measured in serum, whereas the present study measured increased whole-body PON1 activities in zebrafish larvae. The whole-body PON1 activity increases observed here might indicate a more important role of local PON1 activities in affected tissues, where it can specifically attenuate lipid peroxidation, rather than in serum. Indeed, this hypothesis of a local PON1 antioxidant response has been proposed by previous studies, where an increased PON1 protein content in human steatotic livers with elevated oxidative stress was observed without a change in serum PON1 activities (Desai et al., 2014). Furthermore, because PON1 activities were measured in whole larvae, other esterases may also have contributed to the measured activity levels. The conditions proposed by Ceron et al. (2014) to reduce the activities of these esterases relative to PON1 activities, including the use of the correct buffer, pH, temperature, substrates and substrate concentrations were followed in the current study.

5. Conclusion

Our study presents the first exploration of PON1 responses to metabolic challenges in zebrafish, and the first study of PON1 in the context of MDC exposure in a vertebrate. We hypothesize the following mechanisms based on the observations in this study and supported by mechanistic knowledge from existing literature: The western diet likely caused a metabolic shift towards a fatty acid-based metabolism, which was increased by BPA. This shift resulted in increased oxidative stress, with additional oxidative stress induced directly by BPA. The increased oxidative stress likely triggered the increased PON1 activity as an antioxidant response. Additionally a direct impact of altered lipid transport (increased HDL) and regulation of lipid metabolism may have contributed to the PON1 response. Together, these results show that a combined western diet and BPA exposure results in an oxidative stress-based PON1 response in larval zebrafish.

These data could serve as a stepping stone for future research to further uncover the mechanisms underlying metabolic disruption-induced PON1 responses. For example, the hypothesis of local PON1 responses in tissues could be investigated by measuring protein abundance in tissues with high oxidative stress and comparing them to serum levels. Another topic of interest could be a less-studied antioxidant mechanism of PON1, namely its peroxidase-like activity, which has been put forward based on the ability of PON1 to hydrolyze hydrogen peroxide in vitro (Aviram et al., 1998). PON1 inhibition studies could also make use of animal models to investigate the cause-effect link between PON1 and oxidative stress. This could be done by generating a knock-out line, silencing by siRNAs or by chemical inhibition such as NF-κB/PON1-IN-1 inhibiting the NF-κB/PON1 pathway, which may be involved in a PON1 inflammation response (Cheng et al., 2013). The present study can serve as a point of comparison, where PON1 responded to both a dietary and toxicological metabolic challenge, as well as provide a new basis for extrapolation from the zebrafish model to other vertebrates.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw data are available in Supplementary file 2.

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Appendix A. Supplementary data

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