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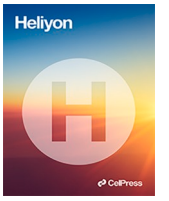

Research article

Gene expression patterns and DNA methylation of neuron and pancreatic β-cell developments in zebrafish embryos treated with bisphenol F and AF

Eric Gyimah [a,*], Hai Xu [b], Shadrack Fosu [a], John Kenneth Mensah [c], Xing Dong [b], Osei Akoto [c], Eliasu Issaka [b], Zhen Zhang [b]

[a] Department of Environmental and Safety Engineering, University of Mines and Technology, Tarkwa, Ghana
[b] Institute of Environmental Health and Ecological Security, School of Environment and Safety Engineering, Jiangsu University, Zhenjiang, 212013, China
[c] Department of Chemistry, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

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ABSTRACT

Bisphenol F (BPF) and bisphenol AF (BPAF) are structural analogues of bisphenol A (BPA) that are used in the manufacture of a myriad of BPA-free products; however, there is a paucity of information regarding their developmental effects. The present study investigates the effects of BPF and BPAF on neurodevelopment and pancreatic β-cell differentiation via altering DNA methylation and gene expression patterns using the zebrafish model. BPF and BPAF induced behavioral perturbations: increased average speed, increased maximum acceleration, increased mania time and decreased static time, in 0.3 and 1.0 μM groups in zebrafish embryos. Glucose level was significantly increased in 1.0 μM BPF (28 %); while a monotonic increase of 29 %, 55 %, and 74 % were observed in 0.1, 0.3, and 1.0 μM BPAF, respectively. Consistent with a decreased insulin mRNA level, the expression of two critical transcription factors (*pdx-1* and *foxa2*) essential for the development and functioning of beta-cells decreased following the bisphenols exposure. In addition, embryonic exposure to BPF and BPAF upregulated the transcription of developmental genes (*vegfa, wnt8a,* and *mstn1)* and neuron-related genes (*mbp, elavl3, gap43, gfap*). Also, the expressions of DNA methyltransferases (*dnmt1, dnmt3, dnmt4, dnmt5, dnmt6, dnmt7,* and *dnmt8*) were significantly aberrant compared with the control group. The Bisulfite PCR results indicate increased DNA methylation at promoter regions of *pdx-1* in BPF (8.2 %) and BPAF (7.6 %); *α1-tubulin* in BPF (5.3 %) and in BPAF (4.1 %), congruous with the increased *dnmt1* and *dnmt3* transcription, at early stage of zebrafish development. The present study indicates that zebrafish embryonic exposure to BPF and BPAF elicits islet dysfunction and neuron perturbations resulting in increased DNA methylation levels.

1. Introduction

Bisphenol A (BPA), a raw material used to manufacture polycarbonate plastics epoxy resins and lacquer coatings [1], has frequently been detected in human tissues, serum, urine; and aquatic environments [2]. The ubiquitous presence of BPA, as well as the weighty

* Corresponding author.
E-mail address: egyimah@umat.edu.gh (E. Gyimah).

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data sets reporting the potential health risks associated with its exposure, has caused the European Commission and the US Food and Drugs Administration (USFDA) to restrict BPA usage in infant-related products [3]. This restriction, consequently, has resulted in the manufacturing of BPA-free products. The production and usage of bisphenol F (BPF) and bisphenol AF (BPAF) purported to be safer alternatives to BPA has increased [4]. This supposition has led to the environmental accumulation of BPF and BPAF reaching staggering increasing levels [2]. Therefore, BPF and BPAF levels of the same order of magnitude or even higher than those of BPA have been reported [2,4]. For example, the levels of BPAF (0.9–246 ng/L) similar to that of BPA (6.6–74.6 ng/L) in river water from Moat in Beijing, China, were reported [2]. Moreover, in drinking water, BPAF (n.d-4.7 ng/L), BPF (n.d-0.9 ng/L), and BPA (n.d-6.5 ng/L) were detected in China [5]. Environmental monitoring data have shown that BPF and BPAF are increasingly distributed in several surface water bodies in China [6]. Hence, the adverse effects of BPF and BPAF exposure on aquatic organisms have gradually gained worldwide attention.

Intriguingly, it still needs clarification whether BPF and BPAF exposures especially during early developmental stages result in neurobehavioral perturbations. Limited studies have reported the neurotoxic potency of BPF [6,7]; and BPAF [8,9] in animal studies including vertebrates. Increased prevalence of metabolic disorders such as diabetes mellitus, especially type 2 diabetes (T2D) has been associated with elevated risk of exposure to environmental contaminants, including bisphenols [10]. Pancreatic β-cells dysfunction is a known etiology of T2D [11]. Insulin deficiency in later life could result from the dysregulation of key transcription factors in beta-cell differentiation during early development, thereby predisposing the individual to susceptibility to pancreatic β-cell destruction [12]. Although not a direct cause and effect, available data have indicated that disruption in insulin regulation induced by exogenous chemicals could contribute to neurodegenerative diseases [13]. However, to date, data on the pancreatic fate as a sensitive target for BPF and BPAF exposure is by far lacking. Therefore, the present study used zebrafish as a model organism to evaluate the pancreatic fate of BPF and BPAF exposure. In vertebrates, the pancreas is fully developed by 96 hpf (hour post fertilization) after which it begins to secret insulin directly into the bloodstream for glucose homeostasis [14].

The role of epigenetics as molecular sensors for environmental toxicants has gained attention in recent decades [15,16]. Of interest, aberrations in DNA methylation patterns may induce alterations in gene expression [17]. Basically, DNA methylation involves the activities of a group of enzymes, DNA methyltransferases (DNMTs), classified as *de novo* or *maintenance* methylation. Among the functional DNMTs, DNMT1 maintains methylation patterns while DNMT3 (DNMT3a and DNMT3b) mediates the *de novo* methylation [18]. In teleost, including zebrafish, six subtypes of the DNMT family (*dnmt3* to *dnmt8*) have been identified [19]. BPA-induced DNA methylated aberrations have been demonstrated at some toxicity endpoints [20]. However, it remains unclear whether BPF and BPAF could induce aberration in DNA methylation.

This exploratory study aimed to investigate the neurotoxicity and pancreatic toxicity of embryonic exposure to BPF and BPAF using a zebrafish embryo model. In addition, we provide the molecular underpinning to evaluate the plausible epigenetic mechanism for BPF/BPAF developmental effects; which reflects the environmental risks posed by BPF and BPAF.

2. Materials and methods

2.1. Zebrafish Husbandry

Wild-type adult zebrafish (AB strain) were purchased from the China Zebrafish Resources Center (Wuhan, China). Zebrafish maintenance, the preparation and collection of zebrafish embryos are reported in our previous work [21](Gyimah et al., 2021a). Briefly, adult zebrafish were maintained in a recirculating water system under 14 h light:10 h dark photo-cycle. The experimental procedures with zebrafish were performed in accordance with the China National Institute of Health's recommendations [22].

2.2. Bisphenol exposures

Analytical grades of BPF (>99 %; CAS: 620-92-8) and BPAF (>99 %; CAS: 1478-61-1) were purchased from Sigma-Aldrich (St. Louis, MO). The stock solutions of concentration 10 g/L, each for BPF and BAF were prepared in dimethyl sulfoxide (DMSO) and then kept at 4 °C. The exposure solutions of BPF and BPA (0.01, 0.03, 0.1, 0.3, and 1.0 μM) were prepared by diluting the stock solution using freshly prepared embryo medium. Following the OECD's fish embryo toxicity protocols [23], embryos were treated with bisphenol concentrations in a 90 mm Petri dish (n = 200 embryos/per dish) at 4-hpf. A 0.01 % DMSO v/v was used as vehicle control. All exposure groups were set in triplicates. Exposure solutions were changed daily until the 120 h (5-d). At 24 h post-exposures, the analytical verification of BPF/BPAF concentrations was performed using liquid chromatography-mass spectrometry (LC-MS/MS; Aligent 1260 Infinity, Santa Clara, CA) according to our previous works [24,25].

2.3. Morphological observations

Using 30 embryos for each treatment, the developmental effects such as mortality of eggs, hatching rate, body length, malformation (curved spine) and heartbeat were observed using a light microscope (Olympus, Japan). Video recordings and images were taken daily. The heartbeat of larvae (n = 5) were quantified using the DanioVision (Noldus, Wageningen, Netherlands) and DanioScope (Version 1.0; Noldus) software. All experiments were set in triplicates.

2.4. Larvae behavioral experiment

Detailed procedure for larvae behavior analyses are described in our previous studies [21]. Briefly, normally developed (appear healthy microscopically) larvae (n = 24) from each exposure group were selected for the behavioral analyses. Larvae were washed with embryo culture and placed in a 96-well plate (1 larva per well) containing 300 μL embryo culture. The loaded plate was placed in a dark box (for 10 min acclimatization, at 28 °C) in the Noldus DanioScope Observation Chamber (Viewpoint Life Science). The acclimatized larvae were allowed free swimming activity observed under 10 min light stimulation. All experiments were set in triplicates.

2.5. Glucose assay

Colorimetric determination of glucose levels in larvae (at 120 hpf) were performed using commercially available kit (Catalogue number: F006-1-1, Nanjing Jiancheng Bioengineering Institute, China). In brief, 300 μL of 0.9 % NaCl was added to the larvae of zebrafish (30 larvae), homogenized and centrifuged at 5000×*g* for 10 min at 4 °C in a 1.5 mL micro-centrifuge tube. In 96-well plates, a 5 μL supernatant solution was transferred into a 250 μL reagent, and allowed to react at room temperature for 10 min. Accordingly, normal glucose and blank samples were prepared. The glucose content of the mixtures was measured at a wavelength of 505 nm using a microplate reader (Infinite® M1000 PRO, Tecan, Switzerland), and on each replicate sample, the reactions were carried out four times. All experiments were triplicate-set (n = 3).

2.6. RNA extraction and real-time RT-qPCR

At each developmental time point, total RNA was extracted by using the TAKARA reagent in accordance with the manufacturer's protocol. Total RNA concentration and cDNA synthesis procedures are detailed in our previous work [21]. Real-Time quantitative PCR (RT-qPCR) was performed using the LightCycler® 96 system (Roche, USA) according to the kit instructions provided by BIO-RAD SsoFast™ EvaGreen® Supermix (Bio-Rad Inc., CA). The primer sequences for genes related to normal development (*vegfa, wnt8a,* and *mstn1*); neurodevelopment (*ngn 1, α1-tubulin, mbp, elavl3, gap43, gfap*); pancreatic development (*pdx-1, foxa2, ptf1a, isl1, and ins*); and DNA methyltransferases (*dnmt1, dnmt3, dnmt4, dnmt5, dnmt6, dnmt7,* and *dnmt8*) are depicted in Table S1. Quantification of transcripts was according to the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008), using *β-actin* as an internal control. All RT-qPCR reactions were performed in three technical replicates for each independent replicated exposure group.

2.7. Bisulfite polymerase chain reaction (BS-PCR)

The methylation analysis of CpG islands was performed with BS-PCR, at 24 hpf embryonic exposure. Concisely, a 50 μL PCR reaction was performed on a 50 ng bisulfite-treated genomic DNA according to the manufacturer's instructions. Using the MethPrimer online software, we predicted the DNA methylated CpG islands of *pdx-1* and *α1-tubulin* (Su et al., 2020), and selected the suitable CpG islands as target fragments. Bisulfite sequencing primers for pdx-1 were obtained from literature: forward, 5′-GTGGTTAGTTTTGTTTAATTATTGTTAAAGC-3'; reverse, 5′-AACACAAAAATCCTTATACAAATAATTAAAC-3'. That of *α1-tubulin* bisulfite sequencing was: forward, 5′- GTTTAAAGATTATTATTAGTTAATAGGGTGTTAT-3'; reverse, 5′-TAACACAAATCTCAAAACACCTATTCTTAATA-3'. The PCR cycling conditions included denaturation at 98 °C for 10 s, followed by a two-step amplification; 35 cycles at 55 °C, and 72 °C each for 30 s. Bisulfite amplified PCR products were refined using the AccuPrep PCR purification kit (Bioneer) according to the manufacturer's protocol. The amplification was detected by 1 % agarose gel electrophoresis with ethidium bromide visualization. Finally, the methylation levels in *pdx-1* and *α1-tubulin* were visualized using the pairwise sequence alignment online software (https://www.ebi.ac.uk/Tools/psa/).

2.8. Statistical analysis

All statistical analyses were carried out using SPSS version 22.0 (IBM, Chicago, IL, USA). Differences between treatments and control were determined by one-way ANOVA and followed by Fisher's LSD post hoc test, where differences are significant. A significant difference was set at $p < 0.05$. For all, data are presented as mean ± standard error (S.E.).

3. Results

3.1. Chemical confirmation of bisphenol exposure

The measured ranges of BPF and BPAF for the five nominal concentrations are presented in Tables S2 and S3. The analyzed results indicated that the measured concentrations confirmed the nominal BPF and BPAF concentrations with a deviation of less than 20 %. Therefore, the nominal dose is able to represent the actual content in this work. It is worth mentioning that the selected concentrations are higher at environmentally relevant levels but proven to be sublethal using the zebrafish model [7,26].

3.2. Alterations in embryos/larvae development

Mortality of the embryos (from 24 to 48 hpf), following the BPF and BPAF exposure, was not statistically significant compared to the untreated groups (Fig. S1). Morphological abnormalities of zebrafish larvae after treatments with BPF and BPAF are presented in Fig. S2. In addition, no significant changes in body length were observed in larvae following the bisphenol treatments (Fig. 1A & B). However, BPF significantly inhibited hatch rate by 62 % and 78 % in 0.3 and 1.0 μM concentration, respectively, at 48 hpf; while reducing hatch rate of 8 % and 9 % for 0.3 and 1.0 μM BPF were respectively observed at 72 hpf (Table 1). Hatch rates of 88 % and 89 % were observed in 0.3 and 1.0 μM BPAF concentrations at 48 hpf, respectively; and 8 % and 11 % were observed at 72 hpf in 0.3 and 1.0 μM BPAF, respectively, compared with the control group. Furthermore, both BPF and BPAF (0.3 and 1.0 μM) reduced larvae heartbeat at 72 and 96 hpf (Fig. 1C & D). The ratio and extent of malformation (curved spine) increased in a concentration-dependent manner for BPF and BPAF (Fig. 1E), with the highest percentage of 15.34 % observed in the 1.0 μM BPAF groups. In summary, our findings indicate that the tested BPF and BPAF concentrations had low lethal potency, but high restriction of early development of zebrafish embryos.

3.3. Effect of BPF and BPAF on larvae behavior

Table 2 presents behavioral endpoints: the average speed, maximum acceleration, and mania time of bisphenol-treated zebrafish larvae were normalized to DMSO group (variation of <20 %; data not shown). Results show increased behavioral changes in a concentration-dependent manner; with significant effects observed in 0.3 μM and 1 μM treatments for both BPF and BPAF, relative to the untreated group. Also, a marked increase of 65 % in maximum acceleration was observed in the 0.01 μM BPAF group. However, a significant decline in static time was observed in both the 0.3 μM and 1.0 μM BPF (52 % and 64 %, respectively) and BPAF (70 % and 69 %, respectively) compared with the control group.

3.4. Glucose levels and insulin expression

The glucose level of larvae was measured at 120 hpf and results are presented in Fig. 2A. A distinct increase in glucose level was

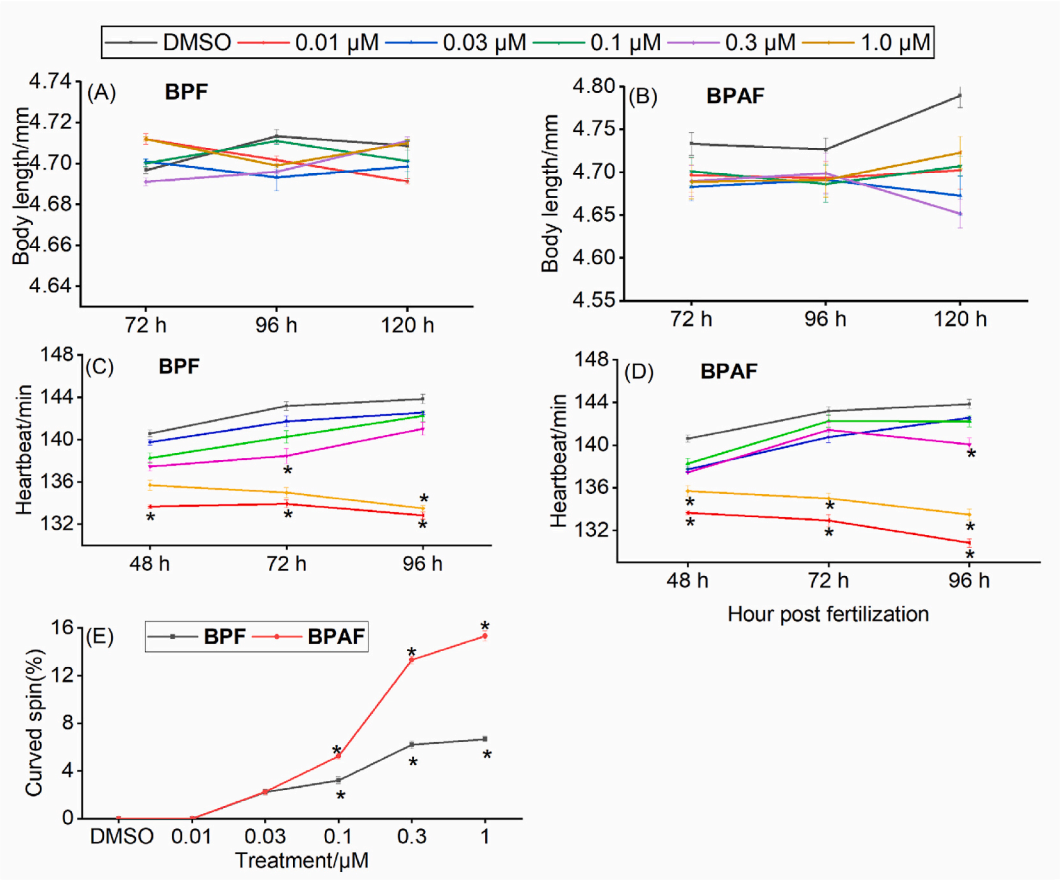

Fig. 1. Developmental effects of BPF and BPAF on zebrafish embryo/larvae. Data presented as mean ± S.E (n = 3). Asterisk (*) indicates significant differences with the control at $p < 0.05$.

Table 1
Percent cumulative hatching (%) of eggs following the exposure to differing concentrations of BPF and BPAF at 48, 72, and 96 hpf. Data presented as mean ± S.E (n = 3). Asterisk (*) indicates significant differences with the control at $p < 0.05$.

	BPF			BPAF		
	48 hpf	72 hpf	96hpf	48 hpf	72 hpf	96hpf
DMSO	8.89 ± 0.02	97.11 ± 1.21	98.92 ± 1.12	8.89 ± 0.02	97.11 ± 1.21	98.92 ± 1.12
0.01 μM	7.54 ± 0.11	91.46 ± 2.05	98.91 ± 1.02	6.42 ± 0.02	90.44 ± 2.62	98.89 ± 2.02
0.03 μM	7.50 ± 1.71	86.42 ± 1.21*	98.95 ± 2.01	5.67 ± 1.02	91.23 ± 2.21	98.77 ± 2.02
0.1 μM	8.21 ± 1.09	93.70 ± 1.09	97.88 ± 1.22	4.43 ± 1.22*	93.03 ± 1.88	97.56 ± 1.89
0.3 μM	3.42 ± 0.88*	89.56 ± 2.01*	96.22 ± 2.04	1.04 ± 1.03*	88.78 ± 2.10*	93.10 ± 1.09*
1.0 μM	1.94 ± 0.09*	88.21 ± 2.07*	95.11 ± 1.99*	0.94 ± 0.01*	86.60 ± 2.13*	92.33 ± 2.11*

Table 2
Larvae locomotor behavior after developmental BPF and BPAF exposure. Larvae behavioral changes after embryonic exposure to BPF and BPAF performed at 120 hpf. Data shown as the fold change after normalization to the respective DMSO values (mean ± S.E; n = 3). Asterisk (*) indicates significant differences between treatments and the control at $p < 0.05$.

		Average speed (mm/s)	*Maximum acceleration (mm/s²)*	*Mania Time (s)*	*Static time (s)*
	DMSO	**1.00**	**1.00**	**1.00**	**1.00**
BPF	0.01 μM	0.90 ± 0.08	1.22 ± 0.29	1.11 ± 0.11	0.96 ± 0.09
	0.03 μM	1.12 ± 0.06	1.28 ± 0.18	1.37 ± 0.21	0.86 ± 0.11
	0.1 μM	1.13 ± 0.10	1.18 ± 0.15	0.98 ± 0.11	0.86 ± 0.10
	0.3 μM	1.38 ± 0.10*	1.52 ± 0.21*	1.26 ± 0.18	0.52 ± 0.11*
	1.0 μM	1.40 ± 0.09*	1.83 ± 0.37*	1.55 ± 0.24*	0.64 ± 0.09*
BPAF	0.01 μM	1.25 ± 0.18	1.65 ± 0.26*	1.20 ± 0.24	0.88 ± 0.11
	0.03 μM	1.11 ± 0.13	1.37 ± 0.18	1.26 ± 0.15	0.90 ± 0.29
	0.1 μM	1.12 ± 0.14	1.30 ± 0.08	1.48 ± 0.21	0.94 ± 0.07
	0.3 μM	1.62 ± 0.16*	1.74 ± 0.10*	1.59 ± 0.29*	0.70 ± 0.07*
	1.0 μM	1.90 ± 0.18*	1.85 ± 0.13*	1.75 ± 0.20*	0.69 ± 0.04*

elicited by 1 μM BPF (28 %) while a monotonic increase of 29 %, 55 %, and 74 % occurred in 0.1, 0.3, and 1.0 μM BPAF respectively, compared with the control groups. Significantly increased folds of 1.42 (for 0.3 μM BPF) and 1.82 (for 1.0 μM BPF) at 24 hpf; and 2.11 (for 0.3 μM BPF) and 2.29-folds (for 1.0 μM BPF) at 48 hpf of *ins* mRNA levels were observed (Fig. 2B). Similarly, all BPAF treatments significantly increased *ins* mRNA levels at 24 hpf, while respective folds of 1.52-, 1.67-, and 1.25- were observed in 0.01, 0.03, and 1.0 μM BPAF at 48 hpf (Fig. 2C). Further, a significant decrease in *ins* transcript of 0.89-fold was observed in 1.0 μM BPF at 96 hpf, while a significantly monotonic decrease in *ins* expression was observed in BPAF treatments at 96 hpf. Thus, our study highlighted that BPF and BPAF significantly suppressed *ins* expression at 120 hpf.

3.5. Gene expression

Fig. 3 depicts the transcription of development-related genes (*vegfa, wnt8a,* and *mstn1*). Suppressed expression at 0.48 and 0.40-folds of *vegfa* were respectively elicited by 0.3 and 1.0 μM BPF (Fig. 3A) at 48 hpf. All BPAF concentrations downregulated *vegfa* mRNA at 48 hpf (Fig. 3B). Significant upregulation by 1.99-, 1.83-, 1.62-, 1.87-folds of *vegfa* expression were induced in embryos exposed to 0.01, 0.03, 0.1, and 1.0 μM BPAF respectively at 72 hpf. The 1.0 μM BPF significantly induced a 1.21-fold increase whereas BPAF concentrations elicited markedly downregulation *vegfa* expressions: 0.67-fold (0.01 μM), 0.76-fold (0.1 μM), 0.75-fold (0.3 μM), and 0.65-fold (1.0 μM) at 96 hpf. Both bisphenols significantly upregulated the expression of *vegfa* at 120 hpf. The expression of *wnt8a* was significantly increased in folds of 2.82- and 3.12- in 0.3 and 1.0 μM BPF respectively at 24 hpf (Fig. 3C), while *wnt8a* mRNA levels remained constant in the BPAF groups at both 0.3 and 1.0 μM concentrations (Fig. 3D). A 1.51- and 2.27-fold increase in *wnt8a* expression at 72 and 96 hpf were respectively observed in the 1.0 μM BPF group, whereas BPAF concentrations monotonically downregulated *wnt8a* expression at 96 hpf. Also, *wnt8a* expressions were upregulated in BPF (0.3 and 1 μM) and BPAF (0.1, 0.3 and 1.0 μM) at 120 hpf. BPF concentrations upregulated *mstn1* throughout the developmental stages 24–120 hpf (Fig. 3E). BPAF concentrations (0.3 and 1.0 μM) markedly upregulated *mstn1* by 1.86- and 1.89-fold at 48 hpf, respectively. Also, a decrease *mstn1* expression; 0.96- and 0.73-fold, respectively at 96 hpf was recorded. However, the mstn1 expression increased at 120 hpf in 0.3 and 1.0 BPAF groups (Fig. 3F).

The transcription of genes related to the development and functioning of the neuron (*ngn1, elavl3, gfap*, *α1-tubulin*, *mbp*, and *gap43*), the pancreatic β-cell (*pdx-1*, *foxa2*, *isl1,* and *ptf1a*); and DNA methylation (*dnmt1, dnmt3, dnmt4, dnmt5, dnmt6, dnmt7,* and *dnmt8*) measured (from 24 to 120 hpf) for BPF and BPAF exposure are depicted in Fig. 4 & Table S4; and Fig. 5 &Table S5, respectively.

BPF induced *ngn1* expression in 0.3 μM (1.73-fold) and 1.0 μM(1.91-fold) groups at 24 hpf, but remained constant in BPAF treatments, compared with the control group. BPF (0.1, 0.3, 1.0 μM) significantly upregulated *ngn1* mRNA level in folds of 1.56-, 1.69-, and 2.01-, respectively; while a suppressed expression of 0.62-fold was observed in 1.0 μM BPAF at 48 hpf. BPAF significantly

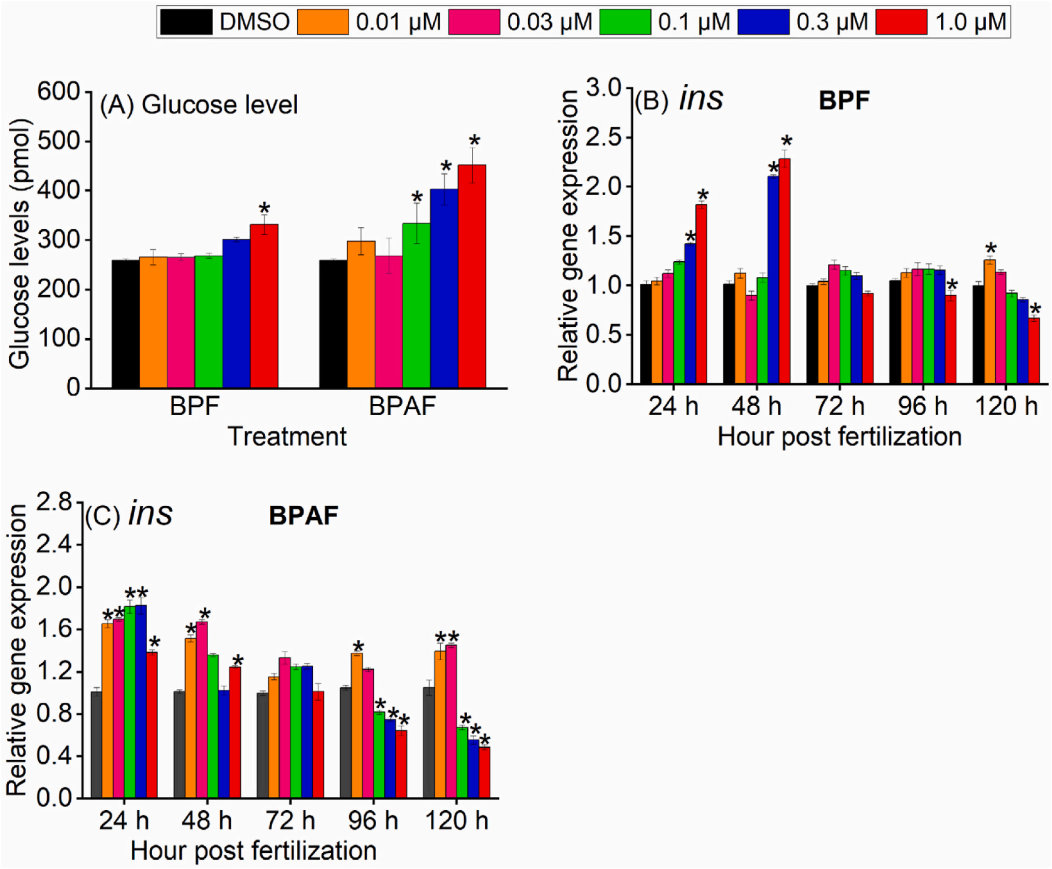

Fig. 2. BPF and BPAF altered glucose levels in zebrafish embryos (A), insulin mRNA transcription upon exposures to BPF (B) and BPAF (C). Data presented as mean ± S.E (n = 3). Asterisk (*) indicates significant differences with the control at $p < 0.05$.

increased *ngn1* expressions (>10-folds) at 96 hpf; both BPF and BPAF significantly upregulated *ngn1* at 120 hpf. *elavl3* expression remained constant at 24 hpf for both BPF and BPAF groups, but increased expression was observed at 48 hpf in both treatments. Both BPF and BPAF significantly upregulated *elavl3* expression at 120 hpf (>2-fold change). Significant upregulation of *gfap* expression was observed in BPF concentrations, but *gfap* expression was not restrained in BPAF groups, at 48 hpf. Both BPF and BPAF increased *gfap* expression (>2-fold change) during embryonic late developmental stages (72–120 hpf) at 120 hpf. Decreased *α1-tubulin* mRNA level (<0.5-fold change) was observed in BPF (0.1, 0.3 and 1.0 μM), while 0.67-fold was observed 1.0 μM BPAF, at 24 hpf. *α1-tubulin* mRNA level remained constant during 48–96 hpf stages of development, but significantly decreased in BPF (0.1, 0.3 and 1.0 μM) and BPAF (0.3 and 1.0 μM) at 120 hpf compared with the untreated group. *Mbp* transcription was not detected at 24 hpf for all groups, including the control group. *mbp* expression did not change in BPF, but increased *mbp* expression was detected in 1.0 μM BPAF (2.77-fold), at 48 hpf. Both BPF and BPAF induced *mbp* transcription throughout later stages of embryonic development (72–120 hpf). Expression of *gap43* remained constant in the bisphenol groups at 24–96 hpf with the exception of its significant expression of 2.45-fold (1.0 μM BPF) at 24 hpf. Nonetheless, gap*43* expression was significantly enhanced (>7-fold change) in both BPF and BPAF groups at 120 hpf, compared with the control (Figs. 4 and 5). Noteworthily, fold changes observed in BPF treatments were of greater magnitudes relative to those observed in BPAF, at 120 hpf.

With regards to the pancreatic-related genes; *pdx-1* expression was remarkably suppressed after treatments with 0.3 μM and 1.0 μM BPF/BPAF at 24 and 48 hpf compared with the control. *Pdx-1* was significantly upregulated in 0.1, 0.3, and 1.0 μM BPF in respective folds of 1.68-, 1.87-, and 1.97- at 72 hpf. Decreased *Pdx-1* expression was observed at 120 hpf, however, significant expression was observed in the highest treatment, BPF (0.46-fold) and BPAF (0.52-fold). *Foxa2* was aberrantly expressed during the developmental exposure to BPF and BPAF. *Foxa2* was upregulated until 72 hpf (except for marked downregulation, 0.56-fold observed in 1.0 μM BPF at 72 hpf). *Foxa2* expression was downregulated at 120 hpf in both bisphenols, compared with the untreated group. Significantly enhanced *ptf1a* expression (>2.0-fold change) was observed in 0.3 and 1.0 μM for both BPF and BPAF treatments at 48 hpf. BPAF (1.0 μM) increased *ptf1a* expression by 2.07-fold at 96 hpf. However, the transcription of *ptf1a* increased in both BPF and BPAF, at 120 hpf. *Isl* showed a decreased expression (0.58-fold) in 1.0 μM BPF, but remained constant in BPAF, at 24 hpf. At 48 hpf, *isl* mRNA transcript increased gradually in both BPF and BPAF; however, significant fold change was observed in BPAF groups; 0.03 μM (2.27-fold) and 0.1 μM (2.32-fold). Although the expression of isl did not significantly change at 72 hpf, a decreasing trend was observed in the BPF group.

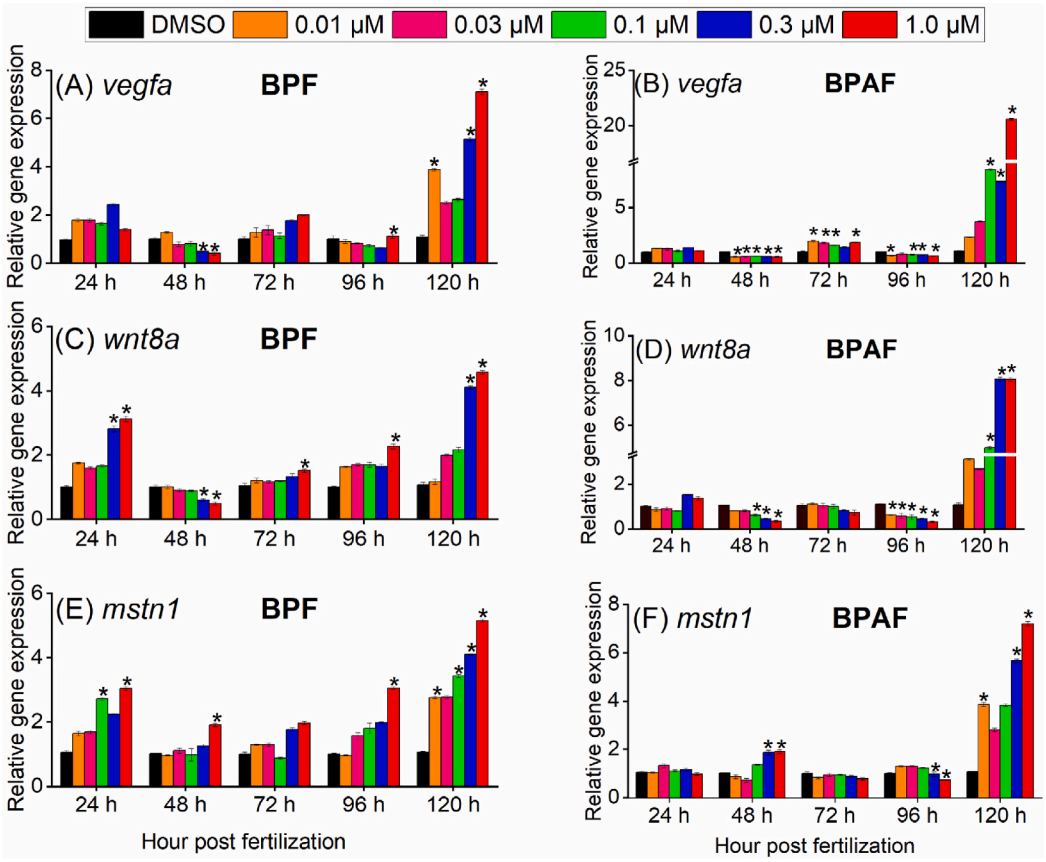

Fig. 3. BPF and BPAF affected the relative expression of development-related genes in zebrafish embryos. The data are expressed as mean ± S. E (n = 3). * indicates significant differences at $p < 0.05$ with the control.

Isl expression decreased in 1.0 μM BPF and BPAF in folds of 0.63- and 0.68-, respectively, at 120 hpf (Figs. 4 and 5). Overall, data indicated that BPF and BPAF could restrain the expression of key genes involved in pancreatic organogenesis.

The *dnmt1* was significantly upregulated in 1.0 μM BPF (1.97-fold), and 1.93-, 2.15-, 2.89-fold in BPAF concentrations (0.1, 0.3, and 1.0 μM, respectively), at 24 hpf. Significant downregulation of *dnmt1* by folds of 0.63- and 0.60- were observed in 0.3 and 1.0 μM BPF, respective at 48 hpf. Both BPF and BPAF induced *dnmt1*expression at 120 hpf compared with the control groups. BPF concentrations increased the expression of *dnmt3* by folds of 1.95- and 2.45- in 0.3 μM and 1.0 μM BPF, respectively, at 24 hpf. BPAF concentrations (0.03, 0.1, 0.3, and 1.0 μM) significantly increased *dnmt3* expression by respective folds of 1.54-, 1.70-, 1.98-, and 2.15- at 24 hpf. Both bisphenols, at concentrations of 0.1, 0.3, and 1.0 μM, distinctively upregulated *dnmt3* expression (>2-fold change) at 120 hpf. BPF and BPAF concentrations (0.3 and 1.0 μM) decreased the expression of *dnmt4* and *dnmt5* at early stages of zebrafish development, 24 hpf. However, *dnmt4* and *dnmt5* transcripts generally increased throughout (48–120 hpf) developmental stages. The transcription of *dnmt6* enhanced until the 72 hpf in both bisphenols, except for the 0.90-fold change observed in 0.03 μM BPF at 48 hpf. At 96 hpf, mRNA levels of *dnmt6* decreased in higher BPF and BPAF concentrations which was accompanied by a monotonic increase in *dnmt6* expression at 120 hpf. The *dnmt7* expression was significantly suppressed by 0.67-fold in 1.0 μM BPAF treated zebrafish at 24 hpf. Both BPF and BPAF showed a decreasing trend of *dnmt7* expressions at later zebrafish developmental stages, 96–120 hpf (Figs. 4 and 5). BPF and BPAF induced *dnmt8* transcription at 48 hpf (>2.0-fold change) and 120 hpf (>4.0-fold change, except for 1.78-fold in 0.01 μM BPF).

3.6. BS-PCR analysis

Bisulfite DNA sequencing was employed to identify the methylation levels of the CpGs in the *pdx-1* and *α1-tubulin* promoter genes. Noteworthily, *pdx-1* and *α1-tubulin* were selected as promoter genes among the candidate genes for the pancreas and neuron development because of their suppressed expression at 24 hpf. The genomic DNA sequence for both promoter genes was 3010 bp in length. The *pdx-1* promoter gene had two CpG islands; 192 bp (1933–2124) and 275 bp (2181–2455). However, the *α1-tubulin* had only one CpG island of 109 bp in length with a transcription factor binding site at 1880–1988. The predicted results for CpG islands in *pdx-1* and *α1-tubulin* genes are shown in Fig. 6A & Fig. 6E, respectively. Methylation levels were further analyzed in the highest BPF and BPAF treatments (1 μM) at 24 hpf. Data revealed variations of methylations at different CpG site of *pdx-1* and *α1-tubulin* for both bisphenol

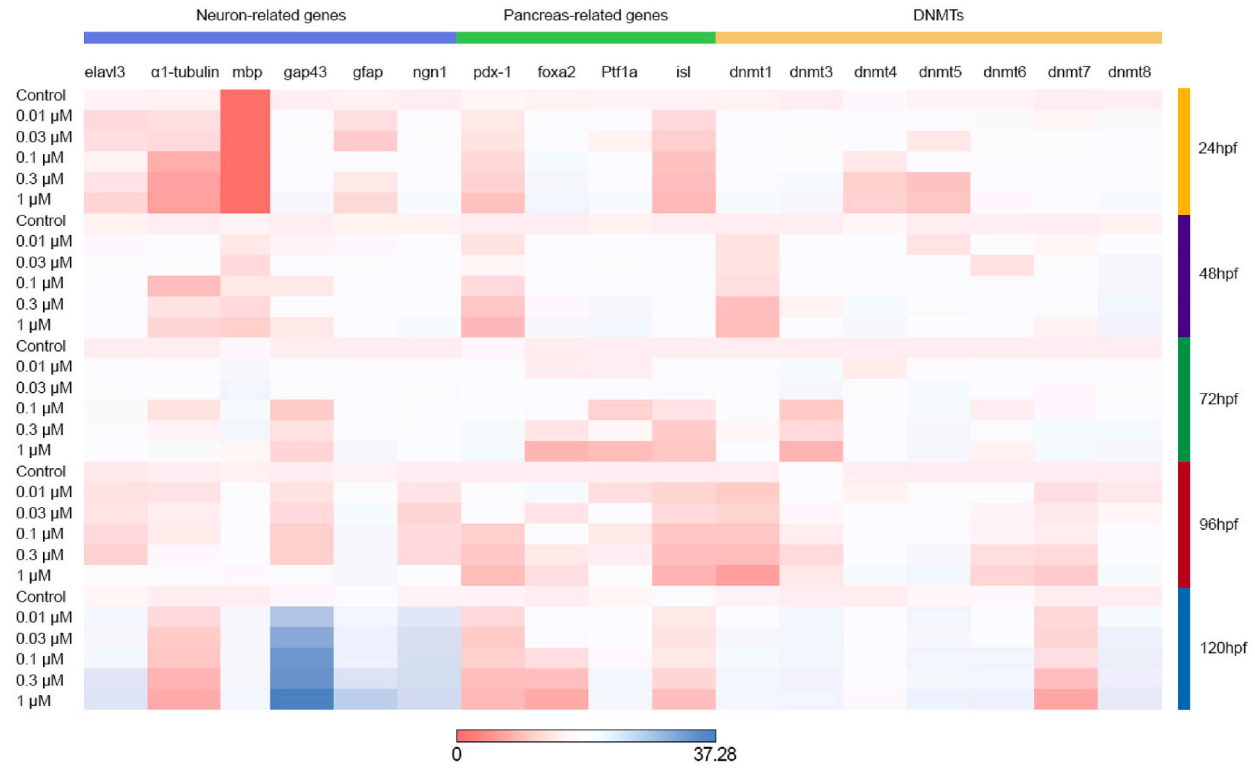

Fig. 4. Heat map of gene expression after zebrafish embryonic exposure to BPF. Gene transcription assayed at 24–120 hpf during zebrafish BPF exposure are represented by Log_2(fold-change) value using freeware (https://software.broadinstitute.org/morpheus/). Up-regulated genes are shown in red, and down-regulated genes are indicated in blue.

compared with the control group. Methylation levels of *pdx-1* increased in both BPF (8.2 %) and BPAF (7.6 %), compared with the control (6.5 %, Fig. 6B–D). For the *α1-tubulin*, increased methylation levels of 5.3 % and 4.1 % were observed in both BPF and BPAF treatments, respectively, relative to the control group (0.9 %, Fig. 6F–H).

4. Discussion

Regarding the predominant use of BPF and BPAF as BPA alternatives, their risks towards human and the environment are gaining more attention especially at environmentally relevance levels. In our study, we found that the tested BPF and BPAF concentrations caused no mortality to the zebrafish embryos. However, BPF and BPAF influenced zebrafish embryo development which led to delayed hatching, and a decreased heart rate. Moreover, the tested BPF and BPAF concentrations elicited spine malformation in zebrafish larvae, with the intensity of the malformation increasing with increasing BPF and BPAF concentrations [27]. reported similar developmental defects in zebrafish embryos treated with 2.5–50 mg/L BPF and BPAF, albeit the marked differences with our tested concentrations (0.2 mg/L for BPF and 0.34 mg/L for BPAF). In addition, at a concentration of 200 μg/L, BPAF reduced zebrafish larvae heart rate (by 87.5 %) at 72 hpf [7]. In zebrafish embryonic development, the transcriptions of genes, such as *vegfa, wnt8a,* and *mstn1,* associated with normal development have been used as biomarkers for the assessment of developmental defects. *vegfa* is known to induce endothelial cell proliferation, enhance cell migration as well as trigger permeability of blood vessels [28]. *Wnt8a* is crucial in inducing the expression of dorsal-specific genes responsible for the formation of dorsal axis in teleost, including zebrafish [29]. Moreover, *mstn1* gene is widely transcribed in the brain and eyes in teleost development [30]. Strikingly, the tested BPF and BPAF concentrations dysregulated the transcription of *vegfa, wnt8a*, and *mstn1* of zebrafish development. Data indicated that the tested BPF and BPAF concentrations had low lethal potency but elicited aberrant development in zebrafish.

In addition, locomotor behavior depicts the activities regulated by the nervous system and it is known to be 10–100 times more sensitive compared with the lethal effects of neurotoxicants [31,32]. Toward this end, we explored the effects of BPF and BPAF treatments on behavioral parameters. Our results report a markedly increased average speed, maximum acceleration, and mania time in 0.3 μM and 1.0 μM BPF and BPAF; while larvae static time obviously decreased accordingly. Increased mania time and decreased static time indicate that BPF and BPAF evoked hyperactivity in zebrafish embryonic exposure and could mediate neurotoxic effects in the early life of zebrafish. A recent study has indicated that at 200 μg/L of BPAF and BPF, the former exerts a greater atypical locomotor behavior in zebrafish early development [33]. Induced hyperactivity could result in the attenuation of energy necessary for essential biological activities such as growth and development [34]. Therefore, the delayed hatching rate as well as the decreased heartbeat in

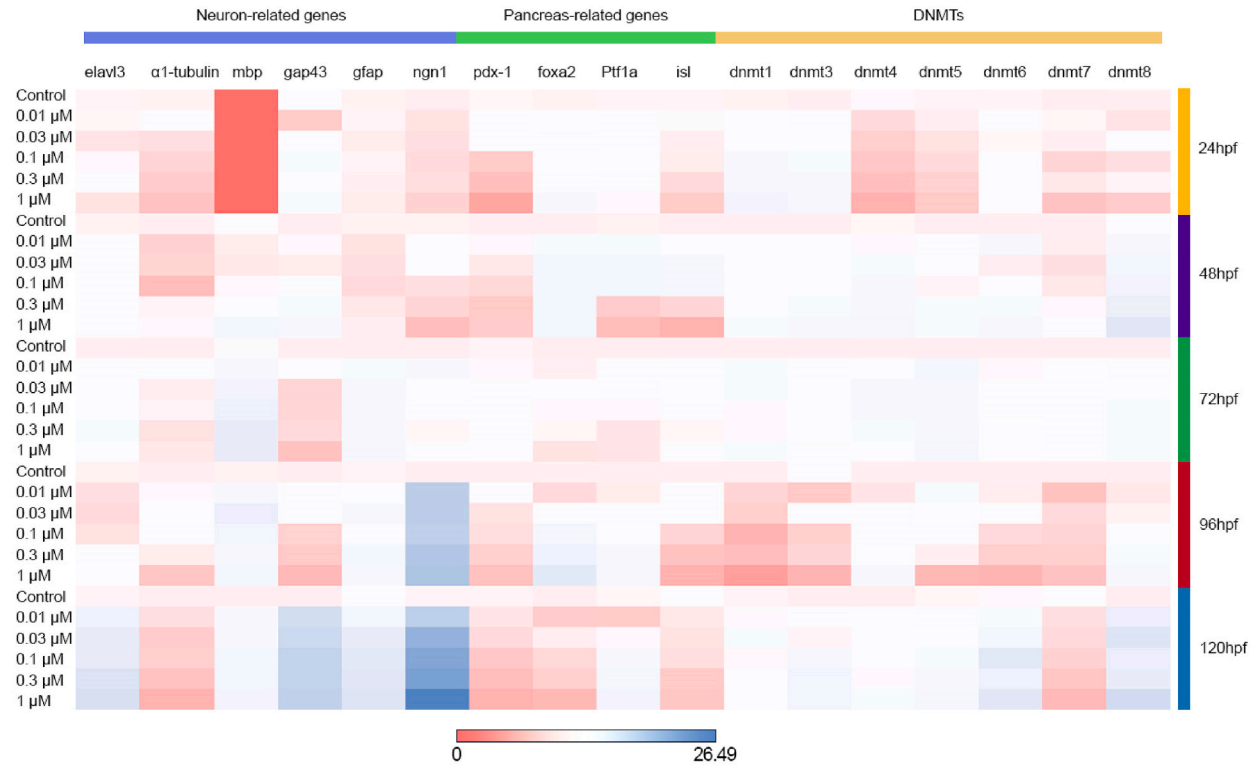

Fig. 5. Heat map of gene expression after zebrafish embryonic exposure to BPAF. Gene transcription assayed at 24–120 hpf during zebrafish BPF exposure are represented by Log_2(fold-change) value using freeware (https://software.broadinstitute.org/morpheus/). Up-regulated genes are shown in red, and down-regulated genes are indicated in blue.

zebrafish early development might in part be due to the induced hyperactivity which could result in a plausible cause of ecological death within the aquatic ecosystem following exposure to BPF and BPAF.

Perturbations in locomotor behavior are early indicators of neurotoxicity, the transcriptional profiles of neurodevelopment-related genes were assayed for zebrafish embryonic BPF and BPAF exposure. *Ngn1* is responsible for neuronal differentiation, and its role in growth and development relays the initial developmental stage of neuronal assembly (Lu et al., 2019). *Elavl3* is a neuronal protein which has RNA binding activity, and it plays a critical role in neurogenesis [35,36]. Specifically, the function of *gfap* is unclear albeit several studies have revealed its significant function in astrocyte-neuron interactions [37]. In addition, *α1-tubulin* is a microtubule protein which is responsible for the regeneration of the retina [38]. In CNS development, *mbp* is the second most abundant protein; its transcription offers a unique function in the myelin sheath (Fan et al., 2010). During axonal growth and development, *gap43* is a useful biomarker with an important role in proliferating neural progenitor cells [39]. A previous study by Ref. [7] reported a remarkable inhibition of the same candidate genes (*α1-tubulin*, *elavl3, mbp,* and *gfap*) for BPF neurotoxicity in zebrafish after 96 hpf at a concentration of 700 μg/L (3.5 μM). The developmental expression of neuron-related genes was examined in the present study. Consistent with the behavioral perturbations reported for zebrafish exposure to BPF and BPAF, the expression of ngn1, elavl3, gfap, and gap43 distinctively increased at later stages of zebrafish development, after significant dynamic expression in early life stages (24–96 hpf). However, the expression of α1-tubulin was inhibited in BPF/BPAF-treated embryos. Moreover, our findings reveal that BPF and BPAF markedly dysregulate the expressions of neurodevelopment-related genes during zebrafish early development. Taken together, results of the extant literature and the present study, despite differences in exposure concentrations, corroborate that BPF/BPAF impair the development of the central nervous system in zebrafish. Therefore, evoking neurodevelopmental and behavioral perturbations likely occur. However, future studies in deciphering the underlying mechanisms are still needed.

The central nervous system (CNS) is involved in energy (glucose) balance and pancreatic islet development. Elevated glucose level in animal models, including zebrafish, has been reported as an early pathological indicator of diabetes induced by environmental insults. The present study measures glucose levels in zebrafish larvae at 120 hpf following BPF/BPAF treatments. Our results report significant increase in glucose levels in BPF/BPAF-treated fish. Consistently, expression of the insulin gene (*ins*) which is fundamental for regulating blood glucose level significantly decreased in both BPF and BPAF treatments. The low *ins* mRNA levels in the bisphenol-treated groups observed could be a toxicological effect, predisposing the exposed progeny to future development of type 2 diabetes [25]. Similarly, BPS has been reported to induce elevated glucose level via decreasing plasma insulin levels in zebrafish [40]. In addition, increased insulin gene expression accompanied with decreased mRNA of genes encoding insulin receptor substrates in zebrafish larvae treated with BPF have been reported [41]. Hyperglycemia in type 2 diabetes is first complemented by compensatory

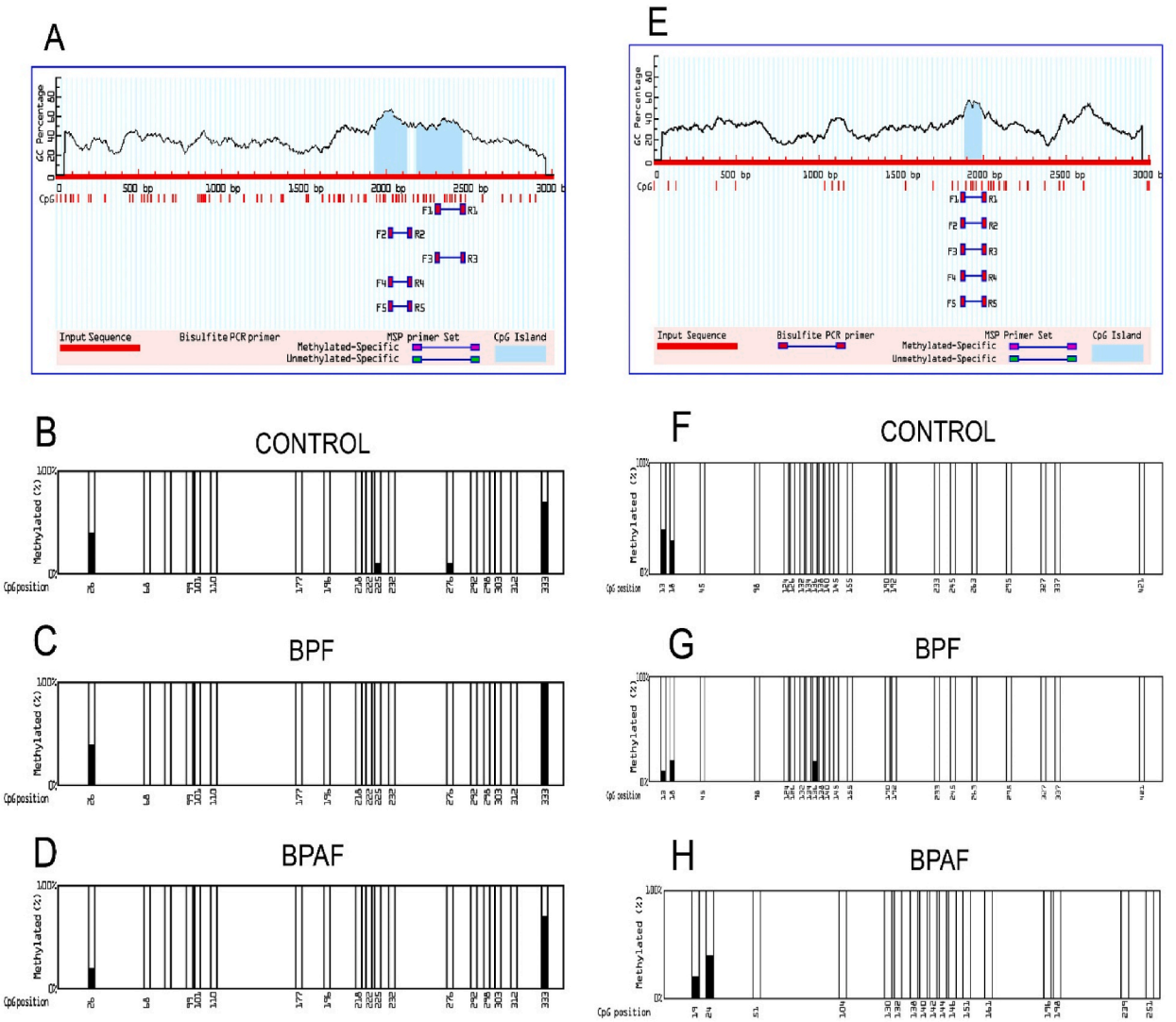

Fig. 6. The CpG islands of the *pdx-1* cDNA predicted by Methprimer software (A) and methylation levels in the control (B), BPF (C), and BPAF (D) groups; The CpG islands of the *α1-tubulin* cDNA predicted by Methprimer software (E) and methylation levels in the control (F), BPF (G), and BPAF (H) groups.

hyperinsulinemia. Nonetheless, as the disorder progresses, oxidative stress triggers apoptosis of the beta cells eliciting a decreasing amount of insulin secreted by the pancreas [42]. Therefore, findings of our study could infer that the decreased *ins* mRNA levels observed in the BPF/BPAF-treated groups could in part be due to a toxicological response that might translate into low insulin level. However, in-depth studies of this hypothesis are needed. The decreased insulin level and increased glucose level were congruous with a decreased mRNA levels of *pdx-1* (glucose-dependent insulin transcription factor), indicating that the BPF/BPAF exposure modulate β-cell formation in zebrafish early development. The foxa2 is an early transcription factor essential for the formation of pancreatic islet and maturation of the beta cells (Kaestner, 2015). Both the foxa2 and pdx-1 are key genes responsible for regulating insulin to maintain the hormone-producing phenotype of the β-cells [43]. We found that the decreased pdx-1 expression induces the expression of foxa2 until the 96 hpf. Increased expression of foxa2 could help restore inhibition of pdx-1 which is critical for islet development and beta-cell functionality [44]. Furthermore, in vivo and in vitro studies have indicated that foxa2 positively regulates pdx-1 transcription [45]. Foxa2 knockout in mice model (at embryonic stage) elicits developmental deficiencies in endocrine pancreas and neural tube [44]. Using the zebrafish model, our results indicate that the expression of foxa2 decreased in similar trend with pdx-1 transcription at 120 hpf, following BPF/BPAF treatments. This interesting result suggest that embryonic BPF/BPAF exposure might interfere with pancreatic fate; therefore, in-depth studies on the use of morpholino to elucidate the effect of bisphenol on *foxa2* and *pdx-1* transcription in zebrafish development is needed in future studies. Transcriptional factors isl1 and ptf1a are key regulators of pancreatogenesis which contribute to the identity and maintenance of the exocrine pancreas. Interestingly isl1 and ptf1a expressions were aberrant following BPF and BPAF embryonic exposure, a result that establish their known role in the development of the pancreas. In

summary, our study revealed that BPF/BPAF treatments induced dysregulation of the pancreas-associated genes during zebrafish early development, an indication that the endocrine and exocrine pancreas might be perturbed upon embryonic BPF and BPAF exposure.

Several lines of studies have highlighted the potential link between behavior and development of the CNS [46], and pancreatic β-cell development [45] with DNA methylation levels and gene expression. To understand the epigenetic impact of BPF/BPAF, we explored the developmental transcription patterns of DNA methyltransferase as well as the methylation in neurodevelopment and pancreatic β-cell development promoter genes. The methylation patterns during cell division is maintained by the maintenance methyltransferase, *dnmt1* [15]. During early development, *de novo* methyltransferases (*dnmt3-dnmt8*) are essential for establishing DNA methylation [45,47]. A study conducted by Ref. [20] revealed increased mRNA levels of *dnmt3* and *dnmt7*, and a declined *dnmt6* and *dnmt8* expression following zebrafish exposure to BPA. Similarly, our study revealed that the mRNA expressions of the maintenance and *de novo* DNMTs were significantly and distinctively dysregulated at the developmental stages of zebrafish embryonic exposure to BPF and BPAF. BPF and BPAF exposure induced the transcription of *dnmt1* and *dnmt3* (key enzymes involved in DNA methylation) at the early stages of zebrafish development, before 48 hpf and at 120 hpf. Studies report that methylation levels of promoter CpG islands might influence the specific binding of transcription factors to promoters, and subsequently regulate gene expression [16]. Inspired by this notion, we measured the methylation levels of *pdx-1* and *α1-tubulin* as promoter genes for the pancreatic β-cell and neuron development, respectively. Generally, the methylation levels in promotor regions correlates inversely with gene expression of downstream genes in most organism, including zebrafish (Yun et al., 2019). The selection of *pdx-1* and *α1-tubulin* as promoter genes is based on their significantly suppressed mRNA expressions at 24 hpf following the embryonic exposure to BPF and BPAF. Herein, the bisulfite conversion sequencing data reveal increased expression of cytosine methylation in promoter regions of *pdx-1* and *α1-tubulin*; which are consistent with the upregulation of *dnmt1* and *dnmt 3* expression during the early stages of zebrafish development, at 24 hpf. These findings indicate that DNA methylation may contribute to interrupted pancreatic and neuronal cell differentiation and functions during embryonic development. Increased cytosine methylation in *pdx-1* promotor region, as a contributor to pancreatic β-cell differentiation, in zebrafish embryonic exposure to polycyclic aromatic hydrocarbons has been reported [10]. Considering the vital role of DNA methylation in regulating gene expression, our data suggest the possible epigenetic-mediated toxicity mechanisms for BPF and BPAF-induced embryonic neurodevelopment and pancreatic β-cell development. Therefore, future studies in exploring all CpG islands sequences employing integrated transcriptomic and epigenetic profiling are recommended.

5. Conclusion

Embryonic exposure of zebrafish to environmental concentrations of BPF and BPAF triggered early developmental defects including impaired locomotor behavior, elevated glucose levels. Dysregulated neurodevelopmental gene expression levels, aberrant pancreatic β-cell development as well as the alteration in DNA methylation levels in the promoter of downstream developmental genes were observed. In view of the essential role of DNA methylation in gene regulations, our findings could suggest an epigenetic-mediated toxicity mechanism for BPF/BPAF-induced neurotoxicity and β-cell toxicity. Our study also reinforces the need for future research on BPF/BPAF-induced epigenetic modification which aims at finding the underlying mechanisms involved in islet dysfunction and neurotoxicological effects.

Ethics statement

The animals used in this experiment were cared for and utilized in accordance with the National Institutes of Health's laboratory animal care and use standards (no. 85e23, revised in 1996). Jiangsu University's Animal Protection and Utilization Committee approved the experiment.

Data availability

Data included in article/supp. material/referenced in the article.

Consent for publication

All authors have read and approved the content of the work.

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Informed consent

Not Applicable.

CRediT authorship contribution statement

Eric Gyimah: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Hai Xu:** Supervision, Conceptualization. **Shadrack Fosu:** Methodology, Data curation. **John Kenneth Mensah:** Writing – review & editing, Validation. **Xing Dong:** Supervision, Investigation. **Osei Akoto:** Writing – review & editing, Supervision. **Eliasu Issaka:** Software, Investigation. **Zhen Zhang:** Supervision, Project administration.

Declaration of competing interest

The authors declare no competing financial interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33805.

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