



# Exposure of embryos to phenanthrene impacts the cardiac development in F1 zebrafish larvae and potential reasons

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## Abstract

To explore the impact of embryonic exposure to phenanthrene (Phe), a typical tricyclic polycyclic aromatic hydrocarbon, on cardiac development in next generation, fertilized zebrafish embryos were exposed to 0.05, 0.5, 5 and 50 nM Phe for 96 h, and then transferred to clear water and raised to adulthood. The cardiac development in F1 larvae generated by adult females or males mated with unexposed zebrafish was assessed. Malformation and dysfunction of the heart, such as increased heart rate, arrhythmia, enlarged heart and abnormal contraction, were shown in both paternal and maternal F1 larvae. A greater impact on the distance between the sinus venosus and bulbus arteriosus was exhibited in maternal F1 larvae, while paternal F1 larvae displayed a more severe impact on heart rate and arrhythmia. The transcription of genes related to cardiac development was disturbed in F1 larvae. DNA methylation levels in the promoter of some genes were associated with their transcription. The expression of acetylated histone H3K9Ac and H3K14Ac in maternal F1 larvae was no significantly changed, but was significantly downregulated in paternal F1 larvae, which might be associated with the downregulated transcription of *tbx5*. These results indicate that exposure to Phe during embryogenesis adversely affects cardiac development in F1 generation, and the effects and toxic mechanisms showed sex-linked hereditary differences, highlighting the risk of Phe exposure in early life to heart health in next generation.

**Keywords** PAHs · Embryonic exposure · Cardiac development · Epigenetic modifications · Transgenerational effects

## Introduction

As a class of ubiquitous environmental pollutant, polycyclic aromatic hydrocarbons (PAHs) are commonly detected in the aquatic environment and human bodies (Song et al. 2013). Most of the PAHs detected in the environment are low molecular weight PAHs ( $\leq 3$  rings). Phenanthrene (Phe), a tricyclic PAH, is involved in the list of 16 priority PAHs by the US Environment Protection Agency (USEPA). Among the 16 PAHs detected in drinking water of China, Phe is generally the most plentiful individual PAH (Zhang et al. 2019). In the Yangtze River Delta, China, the concentration

of Phe in drinking water was the highest among detected PAHs (maximum value 145 ng/L) (Liu et al. 2021). The levels of Phe ranged from 0.019  $\mu\text{g/L}$  to 2.05  $\mu\text{g/L}$  in the Bohai Region, China (Zhang et al. 2017). Phe levels in river water samples in India reached 1.54–8.35  $\mu\text{g/L}$  (Duttagupta et al. 2020). Studies have reported that Phe possesses reproductive and developmental toxicities to fish (Zhang et al. 2013; Chen et al. 2021). Phe exposure impacts skeletal, cardiac and ocular development in fish embryos (Brette et al. 2017; Incardona et al. 2004; Huang et al. 2013; Zhang et al. 2013). It has been reported that crude oil including Phe altered cardiac rhythm and force via disrupting electrical activity and  $\text{Ca}^{2+}$  influx of myocytes (Brette et al. 2014; 2017). It is demonstrated that neurotoxic effects of exposure to benzo(a)pyrene (BaP) could be transmitted to subsequent generations (Knecht et al. 2017). However, whether cardiac defects caused by embryonic exposure to Phe can be transmitted to next generation is still unclear.

Exposure to environmental chemicals in early life could cause adverse effects in adulthood and even be persisted into subsequent generation through epigenetic regulation

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(Chen et al. 2021). Many chemicals can induce epigenetic modifications including DNA methylation and histone acetylation, then alter the transcriptional levels of relative genes (Kamstra et al. 2015). Some studies have showed that epigenetic changes mediate the effects of PAHs. Exposure of embryos to BaP decreased the levels of whole-genome DNA methylation, and led to genome-wide transcriptional alterations in zebrafish (Fang et al. 2013). Exposure of embryonic zebrafish to BaP reduced the levels of global DNA methylation and caused neurobehavioral and physiological defects in F1 and F2 generation (Knecht et al. 2017). Prenatal exposure to a mixture of PAHs increased anxiety-like behaviors, impaired neurocognitive function accompanied with elevated methylation in the promoter of the brain-derived neurotrophic factor 1 in mice (Miller et al. 2016). During DNA replication, altered DNA methylation status could be stably inherited and mediates continuous toxicological damages to the following generations (Skinner et al. 2012). Studies in humans have revealed that maternal exposure to PAHs alters gene methylation levels in offspring (Herbstman et al. 2012). Epidemiological reports suggest that epigenetic changes caused by PAH exposure are related to a greater risk of disease in offspring (Corrales et al. 2014). However, little attention is paid to investigate the association between epigenetic modification of related genes and developmental defects caused by Phe.

Zebrafish is a wonderful model for developmental research, its organogenesis is easy to be observed due to the embryonic transparency. Notably, despite only 70% of genes in zebrafish are shared with humans, about 99% of embryonic indispensable genes are homologous in human embryonic development (Kamstra et al. 2015). Transgenerational studies in zebrafish are increasing, since histone acetylation and promotor-specific DNA methylation of development-related genes can be accurately analyzed. Following a complex choreography, epigenetic modifications from gametes are largely erased and reprogrammed during early embryos (Bollati and Baccarelli 2010). Studies have demonstrated that the toxic effects of environmental chemicals are passed on to offspring through the male germline, whereas, the contribution of female germline is poorly understood (Zama and Uzumcu 2010). During early embryogenesis, zebrafish inherit DNA methylome pattern from sperm rather than oocytes (Jiang et al. 2013). However, the difference of transgenerational effects of chemicals between maternal and paternal inheritance is little known.

In the current study, Phe, a representative of low-ring number PAHs, was applied to study the transgenerational effects of embryonic exposure to PAHs on cardiac development in F1 larvae. The effects resulting from maternal and paternal inheritance were compared, and possible mechanism was explored via transgenerational changes in epigenetic modifications.

## Materials and methods

### Production of zebrafish embryos

All of the experiments conducted were in accordance with the guidelines of Animal Ethics Committee of Xiamen University. Wild-type TU zebrafish were used to produce embryos, they were raised in embryo culture medium (3.5 g/L NaCl, 0.05 g/L NaHCO<sub>3</sub>, 0.05 g/L CaCl<sub>2</sub>, 0.05 g/L KCl). Fertilized eggs within 0.5–1.0 hpf (hour post-fertilization) were used for Phe exposure.

### Exposure protocols and zebrafish husbandry

Phe (purity > 98%, Sigma–Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO, analytical grade) to get a series of stock solutions (0.001, 0.01, 0.1 and 1 mg/mL), and exposure medium was prepared by adding the stock solution into the embryo culture solution. Approximately 100 fertilized embryos (0.5–1.0 hpf) were cultured in 30 mL Phe exposure medium at 0, 0.05, 0.5, 5 and 50 nmol/L in a glass dish. Solvent control was set by adding an identical volume of DMSO [0.09% (v/v)]. Both control and exposure media were changed twice a day. Six replicates were designed for each treatment. After embryonic exposure for 96 h, the larvae were transferred to zebrafish culture solution (3.5 g/L NaCl, 0.05 g/L NaHCO<sub>3</sub>, 0.05 g/L CaCl<sub>2</sub>, 0.05 g/L KCl) for one month and then raised to adulthood (120 d) in a continuous flow system (ESEN-AW-DU5SS, ESEN EnvironScience, China), the conditions of zebrafish raised were: temperature (28 ± 1 °C), pH (7.2–7.4), light cycle (14 h light:10 h dark), and dissolved oxygen (7–8 mg/L). The fish were fed with brine shrimp twice daily. The rate of successful growth (to adult) for each treatment group is exhibited in Table S1.

### Obtain of F1 embryos

Six F0 adult (4 months old) female and male zebrafish embryonic exposed to Phe were randomly chosen from each treatment and mated with unexposed males and females to produce their F1 embryos. The maternal and paternal F1 embryos were hatched separately. The 100 fertilized eggs were collected from each mating pair and raised for 72 hpf, the development of embryos was observed using a Nikon TE300 microscope. The gross malformation was assessed following the methods of Westerfield (2000): malformation rate (%) = the number of malformed embryos/the number of survival embryos × 100%.

### Analysis for cardiac morphology and function

F1 larvae at 72 hpf were immobilized in 3% methyl cellulose, and 20-s videos were recorded under a positive

microscope (Nikon, Tokyo, Japan). Heart rate (HR) was firstly measured, and interbeat variability was assayed employing the software of NIS-Elements Imaging (Nikon, Tokyo, Japan) to evaluate cardiac arrhythmia. The frame number between cardiac contraction initiations was recorded. In the 20-s videos of each larva, three different time points at the beginning, middle and end were randomly selected to obtain standard deviations (SDs) (Incardona et al. 2009), a regular rhythm would possess the same number of frames between beats, therefore a lower SD represents a regular rhythm.

The end-diastolic volume (EDV) and end-systolic volume (ESV) were measured according to the method of Chen et al. (2008), and the volume of stroke volume (SV) was calculated as:  $SV = EDV - ESV$ , and cardiac output (CO) as:  $CO = SV \times HR$ . Three larvae per replicate were randomly examined to obtain a mean value, six means for each treatment were obtained ( $n = 6$ ). To assess the distance between sinus venosus and bulbus arteriosus, 8 larvae from each replicate were measured to acquire a mean value, the mean values from 6 replicates were used for statistical analysis.

### Quantitative real-time PCR (RT-qPCR)

Thirty F1 larvae collected from each replicated at 72 hpf were pooled a subsample, six subsamples were applied for total RNA extraction. The method of TRIzol (Invitrogen, Carlsbad, CA) was employed for RNA extraction, and RT-qPCR was performed based on the method of Huang et al. (2014). cDNA was synthesized using a commercial kit (Accurate Biology, China). Primers are listed in Table S2. The relative mRNA expression was determined applying the method of  $2^{-\Delta\Delta C_t}$  with  $\beta$ -actin as the internal reference.

### Bisulfite sequencing

F0 larvae and the F1 larvae produced from the adult zebrafish were randomly selected for analysis of the gene methylation. Thirty larvae were pooled a subsample, four subsamples were assessed in each treatment. The genes specifically associated with the cardiac development were selected for methylation assessment. The CpG islands in the promoter region upstream of the gene were determined applying UCSC (<http://genome.ucsc.edu/>). We employed the Methylation Primer software to design the bisulfite specific primers (Table S3). The detailed procedure for bisulfite sequencing was described in the supporting materials. The number of CpG sites is divided by total number of methylated CpG to obtain the percentage of CpG methylation (Fang et al. 2013).

### Western blotting analysis

Fifty F1 larvae from each adult fish were pooled a subsample. Total protein from subsample ( $n = 4$ ) was extracted with RIPA lysis buffer (Solarbio, Beijing, China). After total protein concentrations were determined, the proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes, which were blocked with 5% nonfat milk at room temperature for 1 h. Then membranes were incubated with primary antibodies at 4 °C overnight. Primary antibodies used were as follows: acetylation of histones H3 Lys-9 (H3K9) (1:1000, ABclonal, China) and H3K14 (1:1000, ABclonal, China). Respective secondary antibody (1:10,000) was used at room temperature for 1 h, and the protein band was detected with ECL (Advansta, USA) and analyzed with the software of Quantity One (Bio-Rad, USA).

### Quantification of Phe in the exposure solutions, F0 larvae and adult gonads

Phe in exposure media freshly made up with the stock solutions was determined after solid-phase extraction (SPE). Phe was firstly extracted using Supelclean™ LC-18 SPE Tube (500 mg, 6 mL, Sigma–Aldrich, USA) according to the method provided by the manufacturer. After added with d10-Phe as a surrogate standard, exposure solution (20 mL) was passed through the SPE tube. Phe was eluted from the SPE tube and concentrated with nitrogen flow. Finally, the volume was adjusted to 500  $\mu$ L with n-hexane. The concentrations of Phe were determined by GC–MS/MS instrument (Agilent, USA). The recoveries of Phe were  $90.6\% \pm 4.7\%$  ( $n = 3$ ). The detection limit of Phe was 6 ng/L.

Phe accumulation in F0 larvae and the adult gonads after 96 h embryonic exposure was determined based on the previous method of Chen et al. (2021) using a GC–MS/MS instrument (Agilent, USA).

### Data processing

Statistical analysis was executed applying SPSS software 25.0 (SPSS Inc., Chicago, IL, USA). All data of this study were firstly checked normality by Kolmogorov–Smirnov test and homogeneity by Levene's test. Data are showed as the mean  $\pm$  standard deviation (SD). One-way analysis of variance (ANOVA) was performed followed by the Dunnett's test to assess significant differences ( $P < 0.05$ ) between treatment groups and solvent control group.

To analyze differences between paternal and maternal inheritance in the effects, the sex and concentration were used as variables, two-way ANOVA was performed with prism7 (GraphPad Software, Inc., USA) followed by Bonferroni's post hoc test. The correlation between mRNA

transcription and levels of promoter methylation or acetylated histone was assessed by Pearson's test using GraphPad Prism 7.

We calculated a change rate of index from the Phe treatment vs solvent control, which was applied to compare the impact degree of paternal and maternal heredity on the cardiac development in F1 larvae,

## Results

### Phe accumulation in the F0 larvae and adult gonads

The actual concentrations of Phe in the exposure solutions and the F0 larvae exposed to Phe for 96 h are shown in Table S4. In the ovary and testis of adult zebrafish, Phe accumulation in all treatments was lower than the detection limit (3.6 ng/g tissue).

### The morphological malformations and solvent effects

The occurrence rate of morphological abnormalities including pericardial edema and yolk sac edema was significantly elevated in F1 larvae as F0 embryos were exposed to the higher concentrations of Phe (Table S5).

Among the indicators measured in this study, there was no statistical difference between the solvent and the blank control. Hence, the solvent control group was used as the reference to calculate the fold changes of each analytical index.

### Effects on cardiac function

In maternal F1 larvae, significantly elevated heart rate (1.09- and 1.13-fold) was displayed in the 5 and 50 nmol/L treatments (Fig. 1A), and the irregularity of rhythm was significantly enhanced (1.69-fold) in the 50 nmol/L treatment (Fig. 1B).

In paternal F1 larvae, a significant elevation in heart rate (1.06-, 1.08- and 1.15-fold) was displayed in the 0.5, 5 and 50 nmol/L treatments (Fig. 1A), and the irregularity of rhythm was significantly enhanced (2.19- and 2.51-fold) in the 5 and 50 nmol/L treatments (Fig. 1B).

No significant changes in EDV, ESV or SV, CO were exhibited in either maternal or paternal F1 larvae (Fig. 1C, D, E, F). Paternal F1 larvae exhibited a greater impact on heartbeats ( $P < 0.0001$ ), arrhythmia ( $P < 0.0001$ ) and SV ( $P < 0.0001$ ) than maternal F1 larvae (Fig. 1A, B, E; Table S6).

### Effect on the heart morphology

In the solvent treated fish, the F1 larvae presented a natural S-shaped atria and ventricles with no visible pericardial edema. However, the hearts of F1 larvae from the Phe treatments were dose-dependently elongated and showed string-like formation (Fig. 2A).

Compared with the solvent group, the sinus venosus—bulbus arteriosus (SV-BA) distance showed a significant increase both in maternal F1 larvae (1.05-, 1.07-, 1.1- and 1.13-fold) and paternal F1 larvae (1.05-, 1.05- and 1.06-fold) in all Phe treatments (Fig. 2B). Compared to paternal F1 larvae, a greater impact ( $P < 0.0001$ ) on the distance between sinus venosus and bulbus arteriosus (Fig. 2B; Table S6) was exhibited in maternal F1 larvae.

### Transcriptional levels of genes related to cardiac development in F1 larvae

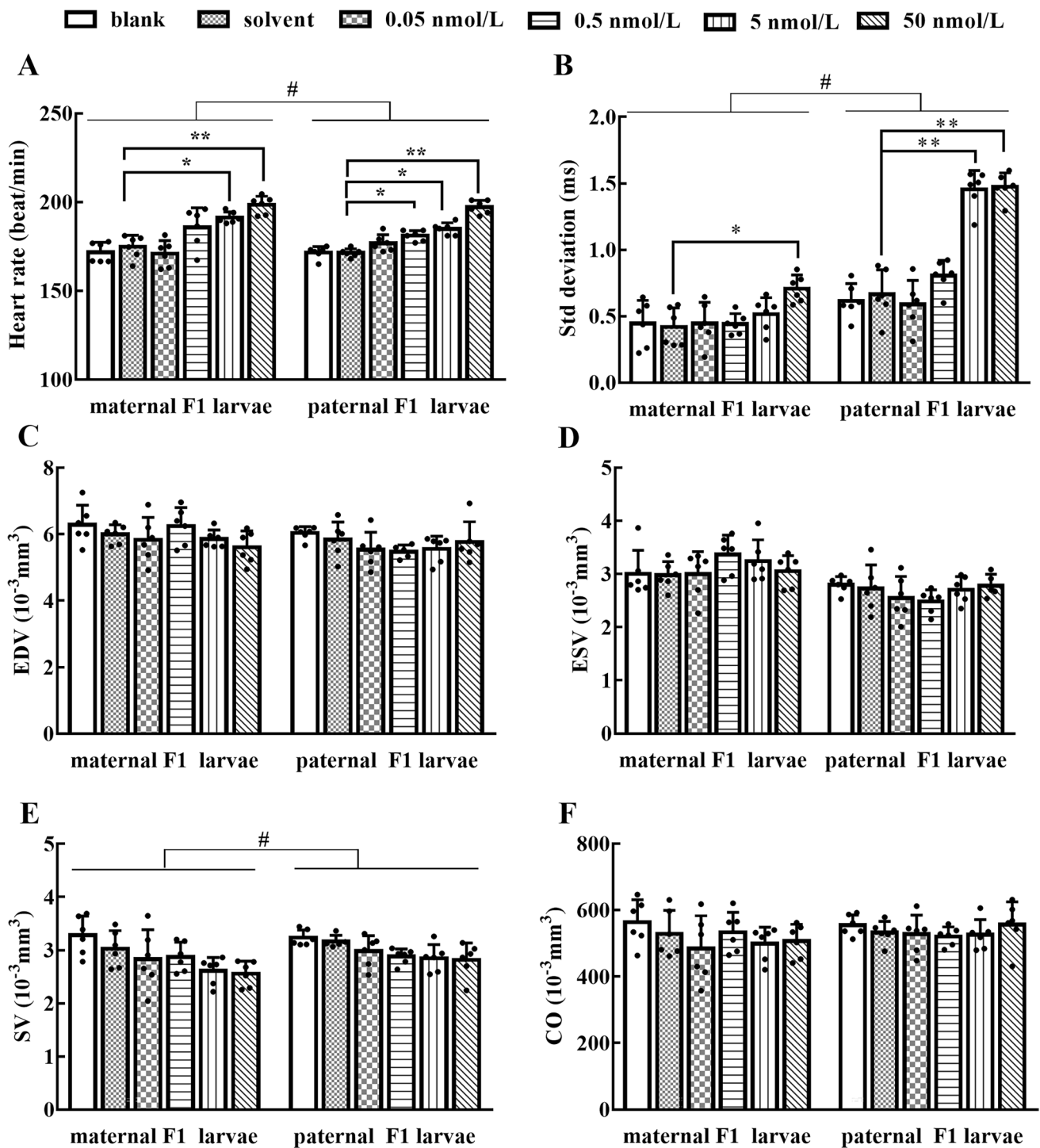
Maternal F1 larvae showed a significant increase in mRNA levels of *tbx5* (1.43- and 1.42-fold), *nkx2.5* (1.39- and 1.37-fold) in the 5 and 50 nmol/L groups. Transcription of *cdh2* (1.44- and 1.39-fold) was significantly upregulated in the 0.5 and 50 nmol/L groups. The mRNA level of *cyp26a1* was significantly upregulated (1.64-fold) in the 5 nmol/L groups. However, mRNA levels of *myh6* (by 39.2%) in the 5 nmol/L treatment and *gata4* (by 33.5%), *myl7* (47.9%) in the 50 nmol/L group were significantly reduced; significantly downregulated transcription of *tnt2* (36.9% and 33.8%) in the 5 and 50 nmol/L treatments was observed (Fig. 3A).

Paternal F1 larvae exhibited a significant downregulation in the transcription of *tbx5* (32.4%), *gata4* (30.8%) and *cdh2* (32.7%) in the 50 nmol/L group. Significantly reduced mRNA levels of *tnt2* (35.5% and 32.6%) were exhibited in the 5 and 50 nmol/L groups. However, the transcriptional level of *cyp26a1* (1.24-fold) was significantly upregulated in the 50 nmol/L group. *Myh6* transcription displayed no significant alteration in all Phe groups (Fig. 3B).

### The levels of promotor methylation in the genes

Reduced methylation levels of *tbx5* were shown in the Phe-treated F0 larvae and F1 larvae from adult fish of both sexes (Fig. 4A, B). The DNA methylation status of *tbx5* in these F1 larvae was coincident with that of F0 larvae (Fig. 4A, B, C). Methylation levels of *gata4* and *cdh2* were detected in two sequences, showing different alterations (Fig. 4). After embryonic exposure to 50 nmol/L Phe, maternal F1 larvae showed significantly downregulated methylation level in *cdh2-1*, while paternal F1 larvae exhibited an upregulated methylation level in *cdh2-2* and showed a significant difference in the 50 nmol/L group when T test was used (Fig. 4B, C). In paternal F1 larvae,



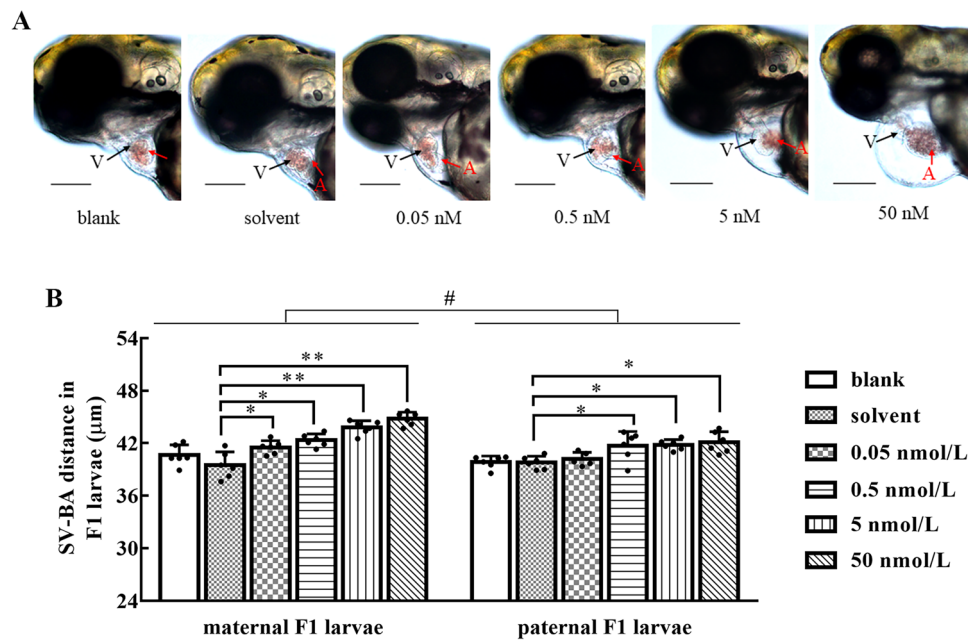


**Fig. 1** Embryonic exposure to phenanthrene (Phe) for 96 h affected cardiac function in F1 larvae. (A) Heart rate; (B) Cardiac arrhythmia; (C) EDV: Volume of the ventricle at end-diastole; (D) ESV: Volume of the ventricle at end-systole; (E) SV: Stroke volume; (F) CO: Cardiac output. Values are represented as the mean ± SD (3 larvae per

replicate, six replicates. n=6). One-way ANOVA was performed to analyze the statistical difference of the F1 larvae from the same sex followed by Dunnett’s test (\*  $P < 0.05$ , \*\*  $P < 0.01$  vs solvent control). Two-way ANOVA was used to analyze sex-dependent differences followed by Bonferroni’s post hoc and indicated with # ( $P < 0.05$ )

the methylation level of *gata4-1* was significantly reduced in the 0.05 nmol/L group, but was significantly increased in the 50 nmol/L group with T test (Fig. 4C).

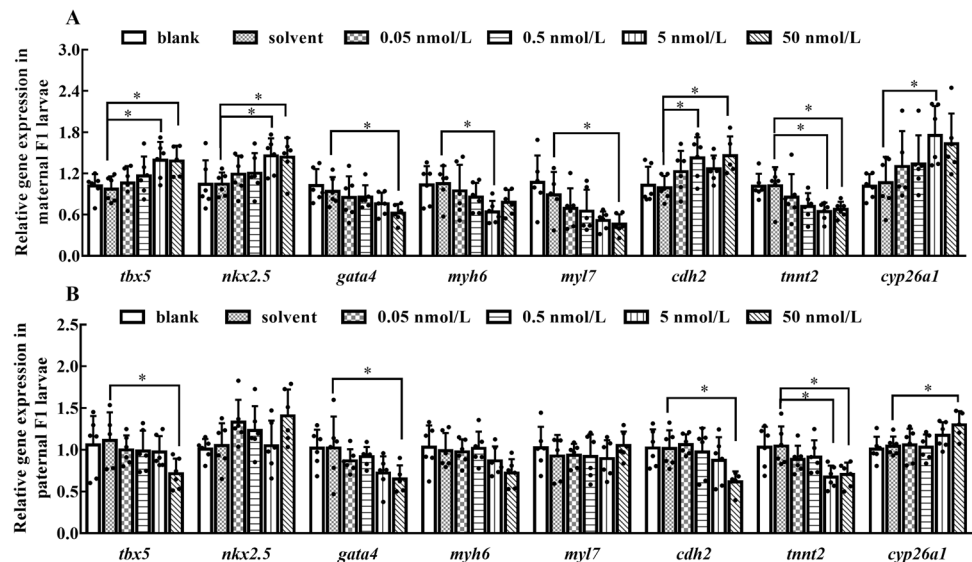
Pearson’s correlation analysis indicated that the methylation levels of *tbx5* and *cdh2-1* were well matched with their transcription levels in maternal F1 larvae (Fig. 6A, B). In



**Fig. 2** Embryonic exposure to phenanthrene (Phe) for 96 h affected cardiac morphology in F1 larvae. (A) Representative images of heart morphology with increasing concentrations of Phe. (V: ventricle; A: atrium). Scale bar=100 µm. (B) The distance between sinus venosus and bulbus arteriosus of F1 larvae. Values are represented as the mean ± SD (8 larvae per replicate, six replicates. n=6). One-way

ANOVA was performed to analyze the statistical difference of the F1 larvae from the same sex followed by Dunnett's test (\*  $P < 0.05$ , \*\*  $P < 0.01$  vs solvent control). Two-way ANOVA was used to analyze sex-dependent differences followed by Bonferroni's post hoc and indicated with # ( $P < 0.05$ )

**Fig. 3** Embryonic exposure to phenanthrene (Phe) for 96 h disturbed the transcription of the genes associated with cardiac development in F1 larvae. (A) maternal F1 larvae. (B) paternal F1 larvae. Values are represented as the mean ± SD (n=6). One-way ANOVA was performed to analyze the statistical difference of the F1 larvae from the same sex followed by Dunnett's test (\*  $P < 0.05$ , \*\*  $P < 0.01$  vs solvent control)



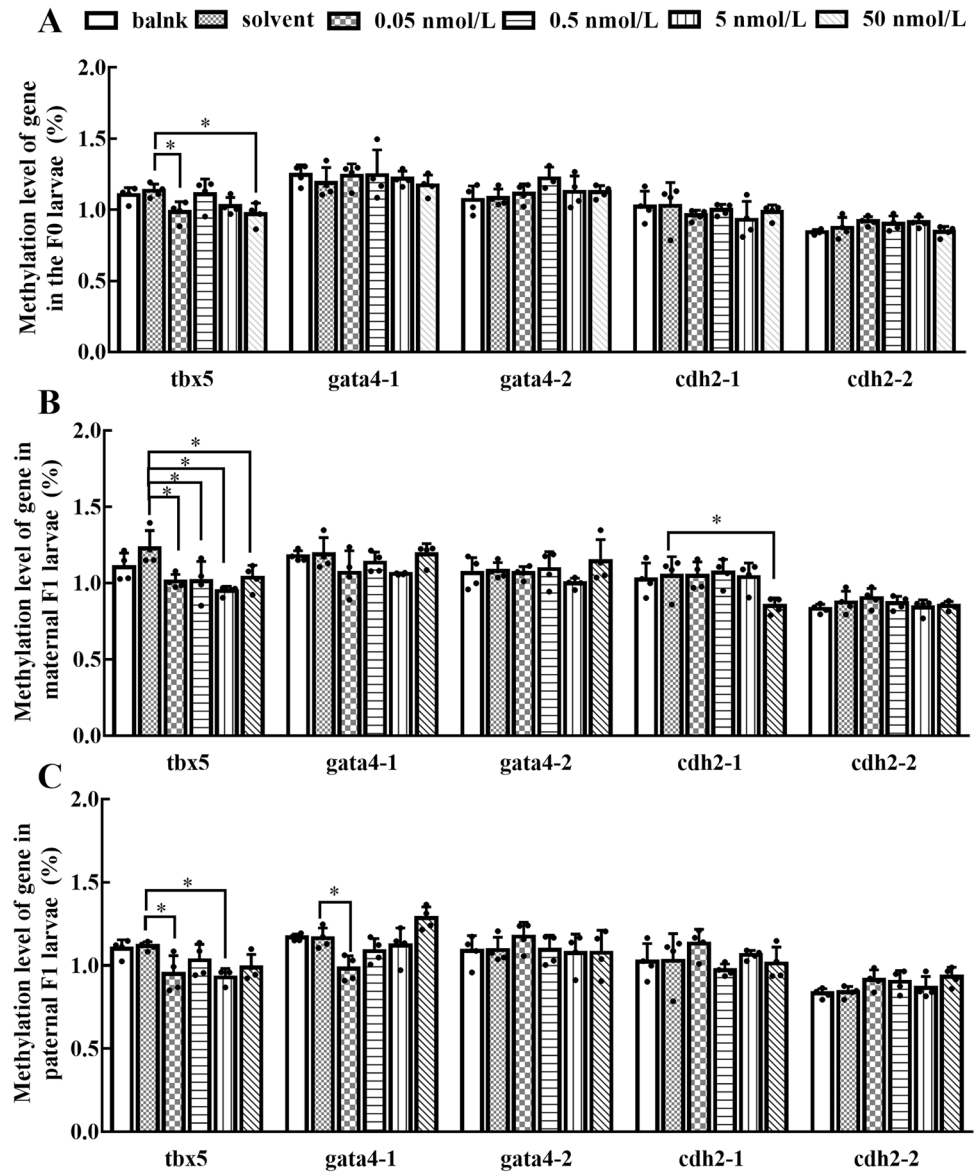
paternal F1 larvae, the methylation levels of *gata4-1* and *cdh2-2* were matched with their mRNA levels (Fig. 6C, D).

### The expression of acetylated histone in F1 larvae

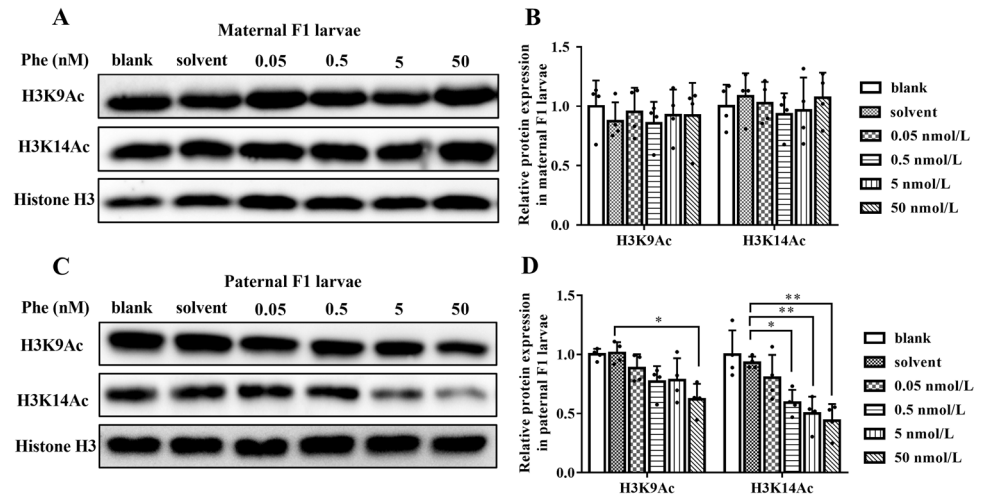
Since altered methylation levels of *tbx5* in paternal F1 larvae were not matched with its transcription, we analyzed

the histone acetylation levels. The results showed that there were no significant changes in the expression of the proteins examined in maternal F1 larvae (Fig. 5A, B), while the expression of acetylated H3K9 (28.8%) and H3K14 (36.3%, 46.1% and 52.6%) was significantly downregulated in a dose-dependent manner in paternal F1 larvae (Fig. 5C, D), Pearson's test showed a significant correlation between

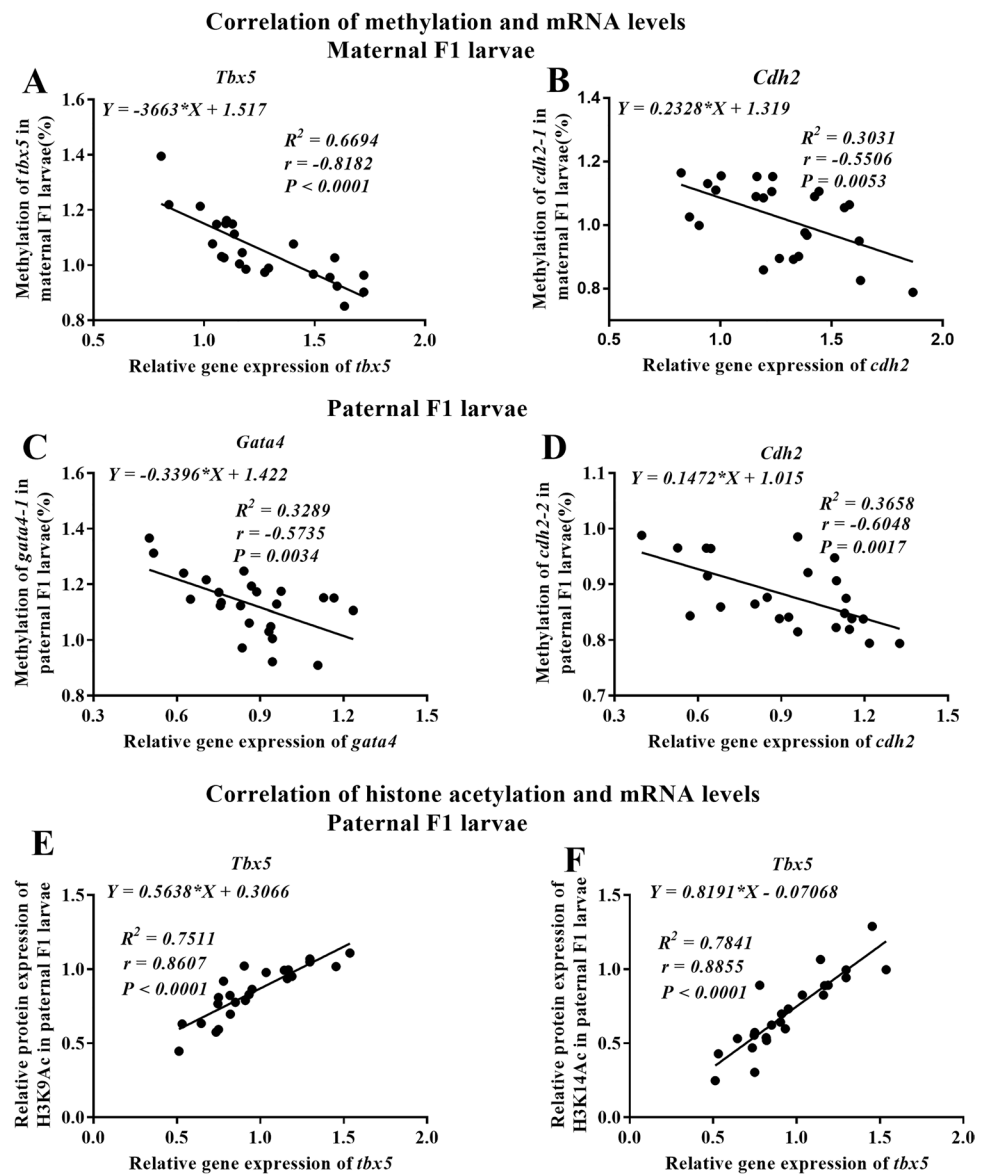
**Fig. 4** Embryonic exposure to phenanthrene (Phe) for 96 h altered the levels of DNA methylation in F0 and F1 larvae. (A) F0 larvae; (B) maternal F1 larvae; (C) paternal F1 larvae. Values are represented as the mean  $\pm$  SD (n=4). One-way ANOVA was performed to analyze the statistical difference of the F1 larvae from the same sex followed by Dunnett's test (\*  $P < 0.05$  vs solvent control)



**Fig. 5** Embryonic exposure to phenanthrene (Phe) for 96 h regulated the protein expression of H3K9Ac and H3K14Ac in F1 larvae. Values are represented as the mean  $\pm$  SD (n=4). One-way ANOVA was performed to analyze the statistical difference of the F1 larvae from the same sex followed by Dunnett's test (\*  $P < 0.05$  vs solvent control)



**Fig. 6** The Pearson correlation coefficient between the transcription of mRNA and epigenetic modifications in F1 larvae as F0 embryos were exposed to phenanthrene (Phe) for 96 h. Correlation of the promoter methylation and the mRNA levels of *tbx5* in maternal F1 larvae (A), *cdh2-1* in maternal F1 larvae (B), *gata4* in paternal F1 larvae (C) and *cdh2* in paternal F1 larvae (D). Correlation of the protein expression of H3K9 (E) and H3K14 (F) and the mRNA levels of *tbx5* in paternal F1 larvae



the expression of acetylated histone and the transcription of *tbx5* (Fig. 6E, F).

## Discussion

The present study found that embryonic exposure to Phe induced cardiac defects including tachycardia, arrhythmia and malformation in F1 larvae. The phenotypes of these adverse effects were consistent with those caused by acute exposure of embryos to Phe in our previous study (Zhang et al. 2013), suggesting that impact on the same genes related to cardiac development might be inherited to F1 larvae via epigenetic modifications such as histone acetylation and DNA methylation. Notably, the impaired effect on SV-BA distance in maternal F1 larvae was

greater than in paternal F1 larvae, while impact on heart rate and arrhythmia was more grievous in paternal F1 larvae than in maternal F1 larvae. The reason may be that transgenerational effects of exposure to environmental pollutants have sex-dependent characteristic. Exposure to Tris (1,3-dichloro-2-propyl) phosphate in early life impacted the development of neuro system in F1 larvae through inherited disorder of thyroid hormone levels in maternal zebrafish but not in paternal zebrafish (Li et al. 2021). Paternal exposure to environmental concentrations of Bisphenol AF (10 µg/L) for 28 days was toxic to offspring and affected the development of F1 larvae in a gender-dependent manner (Zhang et al. 2022). Phe exposure during early life differentially impacted the heart rate in maternal and paternal F1 larvae, suggesting that the risk of Phe to fishes should be adequately evaluated.



Understanding toxicological mechanisms is crucial for risk assessment of contaminants at the molecular level. During the early development of zebrafish, cardiac-specific genes, involving *gata4*, *nkx2.5*, *tbx5*, *myh6* and *myl7*, play an essential role in the differentiation and specialization of the heart. As the main cardiac transcription factors, *Gata4*, *Tbx5* and *Nkx2.5* can regulate the development of cardiac morphological structure and the cardiac conduction system (Takeuchi and Bruneau 2009). In medaka (*Oryzias melastigma*), the reduced expression of *gata4* disrupted cardiac morphogenesis after Phe exposure (Zheng et al. 2020). Exposure of zebrafish embryos to 2-bromomethyl naphthalene led to cardiac malformations by upregulating *nkx2.5* expression (Park et al. 2022). In our study, the cardiac malformation in F1 larvae might be caused by the decreased expression of *gata4* and increased expression of *nkx2.5*. In the differentiation of contracting cardiomyocytes, *tbx5* plays an important role (Takeuchi and Bruneau 2009), whose over-expression or inhibition can result in looping failure (Pi-Roig et al. 2014). The greater transcriptional alterations in *nkx2.5* and *tbx5* might be a reason for the more serious impairment on the SV-BA distance in maternal F1 larvae than in paternal F1 larvae. Retinoic acid deficiency is identified as a factor leading to pericardium edema in zebrafish embryos, and the reduction of retinoic acid is always accompanied by upregulation of *cyp26a1* (encoding retinoic acid metabolism enzyme) (Sun et al. 2015). The present study exhibited that embryonic exposure to 50 nmol/L Phe induced obvious pericardium edema in paternal and maternal F1 larvae, which might be related with the significantly increased transcription of *cyp26a1*. In zebrafish, *Myl7* (myosin light polypeptide 7), *Myh6* (atrial myosin heavy chain polypeptide 6) and *Tnnt2* (cardiac troponin T2) are important for cardiac contractile function and myocardial differentiation (Maves et al. 2009). Knockdown of *cmcl2* (also known as *myl7*) resulted in reduced contractility and ventricular chamber volume (Chen et al. 2008). *Cadherin2* (coded by *cdh2*), a cell-adhesion molecule, is essential for cardiomyocyte differentiation in zebrafish (Bagatto et al. 2006). In this study, the altered transcription of the aforementioned genes was associated with the developmental defects of the F1 heart.

The cardiac development is especially susceptible to environmental pollutant exposure, and sustained heart damage is also noticed in fish exposed to PAHs (Huang et al. 2014). Parental contaminant exposure can produce more susceptible offspring embryos who tend to developmental abnormalities (Yu et al. 2011). Paternal exposure to bisphenol A (BPA) for 14 days resulted in severe cardiac malformation accompanied by atrioventricular separation and extended ventricle with cardiomyocytes forming an irregular and interrupted wall in F2 zebrafish progeny (Lombó et al. 2015). Long-term maternal exposure to bisphenol S (BPS) or short-term (21 days) exposure

to BPA increased the rate of malformation, and heart rate was significantly increased in F1 zebrafish embryos (Fan et al. 2018). The bioaccumulation and transfer of toxic substances in the germ cells may be a reason for the difference between paternal- and maternal-mediated subsequent generational effects (Fan et al. 2018; Zhang et al. 2022). However, in the present study, the accumulation of Phe in the gonads of F0 adult zebrafish could not be detected after embryonic exposure, indicating that the effects on unexposed F1 generation was not due to the transfer of Phe into the parental gonads, and the mechanism may be related to epigenetic modification.

Growing evidence suggests that the transgenerational transmission of developmental defects caused by environmental factors is related to epigenetic modifications, including DNA methylation and histone acetylation, which can change epigenetic information in developing individuals and ultimately affect the phenotype of offspring (Ci and Liu 2015). Hypermethylation of promoter regions of gene usually results in decreased gene expression, and vice versa (Moore et al. 2013). Our results showed that the methylation of *cdh2* was decreased in maternal F1 larvae and displayed an increased trend in paternal F1 larvae, these changes were matched with their transcriptional alterations, respectively. The difference of methylation in F1 larvae might be due to the parental influence on offspring development in a sex-dependent manner, which has been indicated in a study on exposure to Bisphenol AF in zebrafish (Zhang et al. 2022).

Reduced methylation level of *tbx5* might be responsible for elevated *tbx5* transcription in maternal F1 larvae. However, the decreased methylation of *tbx5* cannot explain the downregulation of *tbx5* mRNA levels in paternal F1 larvae. It has been reported that histone deacetylation will play an essential role in inhibiting the expression of genes, when the level of CpG sites cannot cause a long suppression (Curradi et al. 2002). The H3K9 and H3K14 protein are the histone H3 subunits that are the primary targets of modifications most closely related to transcriptional regulation (Cunliffe 2016), their reduced protein levels may result in downregulated transcription of gene, since gene silencing is related to reduced histone deacetylation (Cheung et al. 2000). During early development of zebrafish, transcriptional activity of *tbx5* was potentiated by acetylation, and knockout/knockdown of *kat2a* or *kat2b* (encoding acetyltransferases) caused cardiac defects (Ghosh et al. 2018). In the present study, Pearson's analysis suggested that downregulated expression of H3K9Ac and H3K14Ac protein might be associated with the reduced mRNA levels of *tbx5* in paternal F1 larvae. Simultaneously, this result indicated a difference between maternal and paternal epigenetic alteration caused by embryonic exposure to Phe, since the expression of H3K9Ac and H3K14Ac protein exhibited no significant alteration in maternal F1 larvae.

During zebrafish embryonic development, the patterns of DNA methylation and histone acetylation are highly dynamic, especially during epigenetic reprogramming. The pattern of paternal DNA methylation is stably inherited after fertilization, whereas the maternal DNA is hypomethylated and reprogrammed into a sperm-like pattern (Jiang et al. 2013). However, a study manifested that exposure to methoxychlor could stimulate epigenetic transgenerational inheritance via the female germline (Manikkam et al. 2014). Exposure of parental zebrafish to tetrachlorodibenzo-p-dioxin (TCDD) altered DNA methylation patterns not only in the F0 generation but also in the offspring (Olsvik et al. 2014). The current study found that the DNA methylation patterns of *tbx5* in paternal and maternal F1 larvae were coincidence with that in F0 larvae. These results supported that parental exposure to chemicals could produce imprinted-like DNA sequences, even in the absence of further exposures, and epigenetic alterations could be transmitted to subsequent generations (Skinner et al. 2012).

## Conclusion

The current results confirmed that exposure to Phe during embryonic stages led to the defects of cardiac development in both paternal and maternal F1 progeny. The underlying mechanism might be associated with epigenetic modifications including DNA methylation and histone acetylation resulting from embryonic exposure. The effects and toxic reasons showed sex-linked hereditary differences. In humans, congenital heart disease is one of the most common congenital anomalies, accounting for one-third of all major birth defects (Fahed et al. 2013), its etiology could be related to maternal exposure to environmental pollutants and gene-environment interactions (Fahed et al. 2013; Lage et al. 2012). This study highlights a necessity for further assessing its ecological and health risks, since Phe is ubiquitously detected in the environment, especially in food and drinking water.

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**Data availability** All data and materials generated or analyzed during this study are included in this published article.

## Declarations

**Ethics approval** This study was approved by the Animal Ethics Committee of Xiamen University.

**Consent to participate** Not applicable.

**Consent to publish** Consent was given by all the authors.

**Conflict of interest** The authors declare no competing interests.

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