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# The intergenerational toxic effects on offspring of medaka fish *Oryzias melastigma* from parental benzo[a]pyrene exposure via interference of the circadian rhythm

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ABSTRACT

Benzo[a]pyrene (BaP), a widely existed polycyclic aromatic hydrocarbon pollutant in aquatic environment, has toxic effects on marine animals and their generations, but the intergenerational immunotoxic mechanism underlying has not been clearly understood. In the study, the offspring of marine medaka (*oryzias melastigma*) which were exposed to 0.5 μg $L^{-1}$ BaP suffered from circadian rhythm oscillation disorders and severe DNA damage. Many clock-associated genes like *per1* were significantly modulated in offspring, both per1 and p53 were significantly inhibited that altered the progression of cell cycle and inhibited DNA repair, which possibly resulted in the increased mortality of offspring. The hypermethylation of the *per1* promotor and abnormal levels of $N^6$-methyladenosine ($m^6A$) suggested that the underlying mechanism was probably related to the epigenetic modification. Moreover, the offspring from paternal BaP exposure had more severe DNA damage and a higher degree of hypermethylation than those from maternal exposure. F1 larvae from BaP-exposed parents were more sensitive to BaP exposure, showing that the expression of immune and metabolism-related genes were significantly up-regulated. Taken together, the parental toxicity induced by BaP could be passed to F1 generation and the mechanism underlying was probably associated with a characteristic circadian rhythm disorder.



## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic contaminants that are ubiquitous in the environment. They are primarily derived from anthropogenic activities and accumulate in the atmosphere, soil, water and food. As a representative PAH, benzo[a]pyrene (BaP) is an important indicator used in environmental monitoring and listed as a carcinogen by the International Agency for Research on Cancer (IARC), and is also widespread in the marine environment (Hattemerfrey and Travis, 1991). Early investigations demonstrate that the maximum concentration of BaP ranges from 8.79 to 573.7 ng $L^{-1}$ in the rivers of Iran in different seasons (Karyab et al., 2014), whereas in India the content of BaP in river water has reached 8.61 μg $L^{-1}$ which is above the environmental quality standard and the overall concentration level of 17 PAHs is 157.96 ± 18.99 μg $L^{-1}$ (Singare, 2016). It is well known that PAHs cause biological disorders in aquatic organisms such as fish, which show reduced egg production, endocrine disruption, interference of embryonic and larval development, and modulation of the immune system (Corrales et al., 2014; He et al., 2011; Hoffmann and Oris, 2006; Lee et al., 2018; Reynaud and Deschaux, 2006; Wen and Pan, 2015). Notably, organisms affected by PAHs also suffer intergenerational effects and that has increasingly attached importance to investigate the intergenerational effects of pollutants on aquatic organisms (Corrales et al., 2014; Jasperse et al., 2019; Knecht et al., 2017; Seemann et al., 2017; Sun et al., 2015; Vignet et al., 2015). However, previous studies on these effects on aquatic organisms have mostly focused on development,

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reproduction, behavior, morphological and basic physiological indicators, and less research has focused on the intergenerational interference of immune status in fish offspring and the associated modulatory mechanism. It is well known that the immune system usually reacts against internal and external pathogens and is critical for the survival of the individual and the population. Alteration of the immune system caused by environmental contaminants may increase the risk of pathogen infection in fish, leading to a decline in fish populations and subsequently disturbance of the balance of the entire ecosystem.

The circadian rhythm is a transcription-translation feedback oscillator loop, with a cycle of peaks and troughs in gene expression over 24 h. The circadian clock plays an essential role in regulating basic life processes such as sleep, reproduction, metabolism, development and immunity. In particular, regarding immunity, the circadian clock circuitry not only exerts a role in regulation of innate immune function, but also in that of adaptive immune and allergic responses (Cermakian et al., 2013; Krishnaiah et al., 2017; Man et al., 2016; Patke et al., 2017). Prior studies have confirmed that environmental pollutants can directly result in circadian rhythm disorders. It has been reported that BaP exposure causes changes in the circadian clocks of mammals (Gentner and Weber, 2011), yet to our knowledge few studies have investigated the interaction between circadian rhythms and immune activity following exposure to BaP or other environmental pollutants, and even fewer studies have been undertaken on the circadian clocks of marine organisms.

In fish, previous studies have focused on the reproductive consequences and intergenerational developmental deformities resulting from environmental stimuli, with a relatively less emphasis on the immune toxicity and the underlying mechanism to the progeny (Corrales et al., 2014; Staples et al., 2011). Therefore, in this study we used the marine model fish *Oryzias melastigma* (medaka) and benzo[a]pyrene (BaP) to investigate the intergenerational toxic effects and the involved mechanism in offspring that were hatched from parents previously exposed to BaP. Understanding these effects in the case of fish exposed to BaP, and their underlying response mechanisms from parents to their offspring is of considerable scientific significance, specifically in terms of monitoring fish health and predicting the risks of pollutants to fish populations.

## 2. Materials and methods

### 2.1. Experimental design of BaP exposure

#### 2.1.1. Chemicals and reagents

BaP (Benzo[a]pyrene) was purchased from Sigma-Aldrich (HPLC, purity $\geq$ 96%, CAS:50-32-8). Dimethyl sulfoxide (DMSO, molecular biology grade, assay $\geq$ 99%) was purchased from Sangon Biotech (Shanghai, China). A range concentrations of BaP stock solution (0.75 mg $mL^{-1}$ for exposure experiment I,3 mg $mL^{-1}$, 6 μg $mL^{-1}$ and 1 μg $mL^{-1}$ for exposure experiment III) were prepared using DMSO as a co solvent and with a range dose 0.01–0.05% as mentioned below in exposure experiments. The actual concentrations of BaP in control and exposure solutions were measured using a fluorescence spectrophotometer (Cary Eclipse, Varian, USA) as shown in Supplementary Information (Table S1).

#### 2.1.2. Exposure experiment I

In the study, marine medaka at 3 months old which were reached sexual maturity were selected for this study. 120 females and 120 males were randomly divided into 6 glass aquariums, among which 3 glass aquariums were used for BaP exposure as the treatment groups and 3 for control. In each glass aquarium, 20 male and 20 female fish were assigned for mating. Each aquarium was filled with 12 L of artificial sea water at salinity 30 and fish were cultured with a photoperiod of 14 h light: 10 h dark (light on at 8 a.m. and light off at 10 p.m.) and fed with newly hatched *Artemia franciscana* twice a day. The treatment groups were exposed with a final concentration of 0.5 μg $L^{-1}$ BaP, which was reported to be an environmentally relevant concentration. Controls were treated with DMSO. The solvent DMSO were equally used with 0.01% (v/v) concentration in both control and exposed groups. During exposure time, food residue and excrement were carefully taken off and the artificial seawater were renewed per day with addition of 0.01% DMSO and 0.5 μg $L^{-1}$ BaP. Upon exposure for 14 days and 28 days, the first-generation embryos (F1 generation) were collected and incubated in dishes. It should be mentioned here that the seawater was completely renewed with clean seawater without addition of DMSO and BaP one day before embryos were spawn and collected for further culture in order to avoid their direct contact with BaP. The numbers of embryos laid in each tank (about 150 eggs per tank per day) were sufficient for embryo collection after 14 d and 28 d exposure of BaP. The collected embryos from both control and treatment groups were subsequently cultured using clean seawater without adding any chemicals. The F1 embryos were incubated until hatching to be larval fish, then the first day hatched larval fish were used for subsequent experiments, as shown in Fig. 1A. Three gonads of either F0 male or female were individually collected from each of treatment groups and controls and further kept in a −80 °C freezer for use in DNA methylation detection.

For the detection of circadian rhythm oscillations, eight zeitgeber time points (09:00, 11:00, 14:00, 17:00, 20:00, 23:00, 02:00, 05:00) were established. After 14 d and 28 d exposure, the first-generation embryos (F1 generation) were collected and incubated until hatching to be larval fish. A total of 240 larval fish (F1 larvae) were used and averaged to eight zeitgeber time points (15 larval fish per zeitgeber time point in either control or BaP exposure group). Three samples (five larval fish were mixed as one sample) were collected in parallel from the control and BaP exposure group at each zeitgeber time for statistical analysis, which were used for qPCR detection. The samples at 08:00 a.m. were excluded to avoid the effect of sudden light on. Meanwhile, the rest of F1 larval fish from parents exposed to BaP for 28 d were continued to raise until sexual maturity in a clean seawater without any exposure. After spawned, the second-generation (F2) embryos were obtained. F2 were continued to incubate until hatching, by which five F2 larval fish at first day of hatching were randomly collected as one sample and 3 triplicate samples were analyzed in subsequent experiments (Fig. 1A).

#### 2.1.3. Exposure experiment II

To understand whether BaP-exposed father alone or mother alone has any impact on their offspring, three experimental groups were arranged, that is, paternal BaP exposure group, maternal BaP exposure group and solvent control group. Each group had three aquariums as biological replicates and each aquarium included 20 male fish and 20 female fish (F0). For paternal BaP exposure groups, 20 male fish in each tank were exposed to 0.5 μg $L^{-1}$ BaP for 28 days and then mated with unexposed female medaka in clean seawater which was renewed without chemicals after 28 d exposure. Similarly, the maternal BaP exposure groups were carried out the similar exposure to 20 female fish in each tank with the same dose of BaP and then mated with unexposed male medaka. The control groups were in parallel exposed to 0.01% DMSO as solvent controls, as shown in Fig. 1B.

Three samples (five F1 larval fish at the first day of hatching were mixed as one sample) from each group were prepared for DNA methylation test and comet assay.

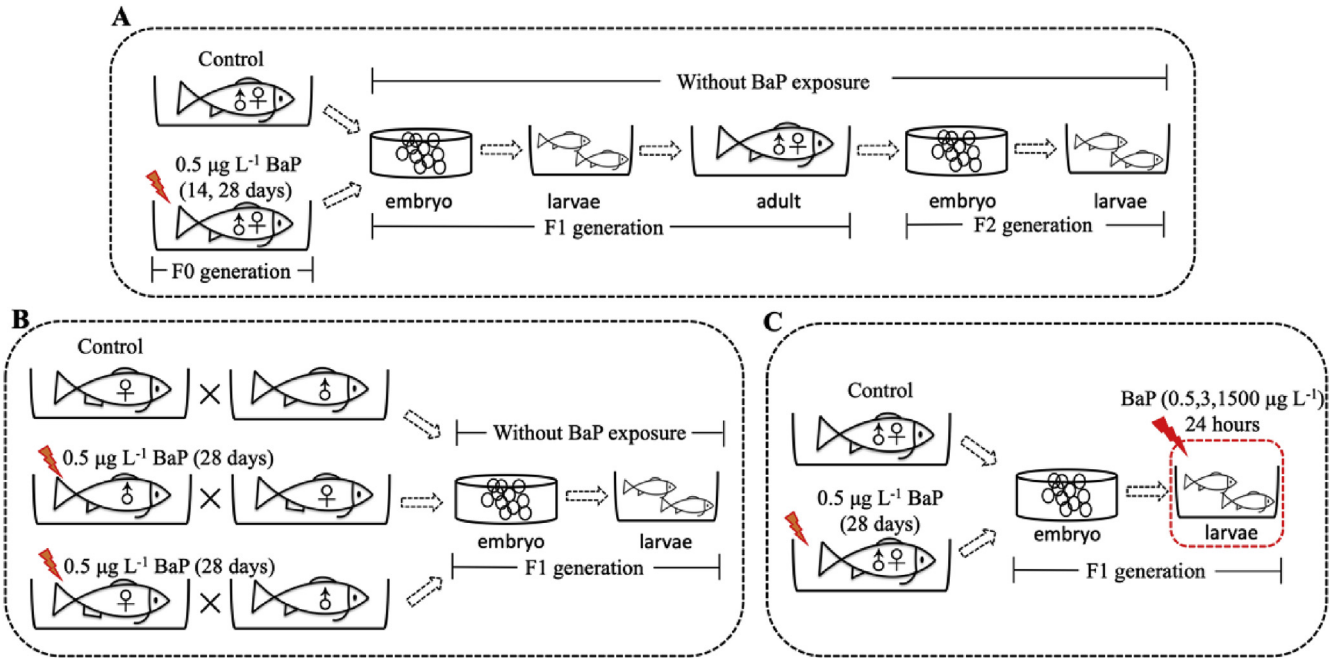


**Fig. 1.** Schematic diagrams of BaP exposure: (A) illustrating parents (F0 generation) exposed to BaP and their offspring (F1 and F2 generations) without BaP exposure; (B) separately showing paternal and maternal BaP exposure and their offspring (F1 generation) without BaP exposure; (C) showing parental (F0) BaP exposure for 28 d and the larvae (F1 generation) after being re-exposed to varying BaP concentrations.

#### 2.1.4. Exposure experiment III

This experiment aimed to investigate whether there were any different toxic effects and what subsequence would be caused while F1 fish larvae from parents (F0) with a BaP exposure history or without BaP exposure were re-exposed the same concentration of BaP. The hatchling larval fish from both BaP-exposed groups and solvent controls were separately moved to other glass crystal dishes. Each dish contained 5 fish and were exposed to a range concentrations of 0.5, 3, 1500 μg $L^{-1}$ BaP for 24 h (Fig. 1C). The doses of 0.5 μg $L^{-1}$ and 3 μg $L^{-1}$ were taken as a lower and higher concentration in the marine environment respectively as documented previously (Maskaoui et al., 2002), while the concentration of 1500 μg $L^{-1}$ was taken as a specific case which generally occurs in crude oil exploration areas where with a risk of oil spill (Anyakora et al., 2005). Each concentration of BaP was prepared using 0.05% (v/v) DMSO in each dish. Finally, three samples (five F1 larval fish were mixed as one sample) from each group were also prepared for later qPCR detection.

### 2.2. Digital gene expression profiling (DGE)

The total RNA of F1 fish larvae (exposure experiment I-14 d) from three replicates for each exposure group and control group was extracted using the Trizol Reagent (Invitrogen) following the manufacturer's instructions. The RNA concentrations were determined using a Nanodrop 2000 microvolume spectrophotometer and the quality control was performed with an Agilent 2100 bioanalyzer. The quality indicators (OD260/280, RIN) of all samples met the sequencing requirements (Table S2). The target RNAs were sent to BGI Genomics Co., Ltd, China and sequenced via BGISEQ-500 RS sequencing platforms. The differentially expressed genes between control and each exposed group were screened following the NOISeq method (Tarazona et al., 2011), using the following default criteria: fold change $\geq$ 1.5 and diverge probability $\geq$ 0.8, which has been widely used in screening the differentially expressed genes(Lin et al., 2019; Rachid Zaim et al., 2019; Wilhelmsson et al., 2019).

### 2.3. Real-time quantitative PCR (qPCR) analysis

Total RNA of F1 larval fish from experiments I and III was extracted by using the Trizol method as above and cDNA was obtained by reverse transcription using a One-Step TaKaRa Prime-script™ RT Reagent Kit (TaKaRa Biomedical Technology, Beijing, China). Specific primers were designed using NCBI/Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and listed in Table S3. Dissociation curves and standard curves were performed to ensure the specificity and amplification efficiency within 95–105%. 18s rRNA was used as reference gene and quantified to normalize the relative expression level of target genes (Bo et al., 2011). Quantitative PCR assays were performed using the Fast-Start Universal SYBR Green PCR Master Rox (Roche) and ABI 7500 System. The cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. There were three biological parallel samples in each group or at each zeitgeber time, and three technical duplicates were performed for each sample during qPCR detection. The relative expression levels of the tested genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

### 2.4. Genomic DNA extraction and bisulfite sequencing PCR (BSP)

Genomic DNA of F0 - male and F0 - female gonads and F1 fish larvae from three replicates for each control group and exposure group (exposure experiment I and II) were isolated using a TIANamp Marine Animals DNA kit (TIANGEN Biotech, Beijing, China), following the manufacturer's instructions. Then, 500 ng DNA for each sample was bisulfite-converted directly using an EZ DNA Methylation-Direct Kit (Zymo Research, USA) following standard protocols. The promoter region of the period 1 gene (*per1*) was amplified by the method of Genome Walking (Genome Walking Kit, TaKaRa). CpG islands in the promoter region and BSP-specific primers were predicted and designed using online tools: Methprimer (Li and Dahiya, 2002). The BSP-specific primer was: forward, 5′-TTTTGGTTTATAGTTTGTTGTTTATTAGTT-3'; reverse, 5′-

ATATTTTAAAAAAACAAATACCTC-3'; PCR amplification was performed with transformed genomic DNA as template and the cycling conditions were as follows: 94 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 1 min. PCR products were separated and purified and then subcloned into the pMD18-T vector (TaKaRa, Beijing, China). QUMA web-based tool (http://quma.cdb.riken.jp) was used for the bisulfite sequencing analysis of CpG methylation.

### 2.5. Detection of $N^6$-methyladenosine ($m^6A$) RNA methylation status

Total RNA of F1 larval fish from exposure experiment I and II was isolated by using Trizol method as above, and its quality was determined by 1.2% (w/v) agarose gel electrophoresis. The concentration and purity of RNA were assessed with a Nanodrop 2000 spectrophotometer. OD260/280 ratio should be > 1.9 with no DNA contamination. The EpiQuikTM $m^6A$ RNA Methylation Quantification Kit (Colorimetric) (Epigentek, USA) was used for the quantification of $m^6A$ in total RNA, following the manufacturer's instructions. 200 ng total RNA of each sample was used for detection. The detected signal was quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. Three replicates were analyzed for each experimental group.

### 2.6. Cell culture and RNA interference (RNAi)

The DIT-29 medaka liver cell line was purchased from RIKEN BioResource Center and cultured in Hyclone L-15 culture medium supplemented with 20% fetal calf serum (FCS) and 10 mM HEPES buffering agent, and the pH was adjusted to 7.2 with 1 M sodium hydroxide solution. DIT-29 cells were grown with at least three passages before experiments started. We followed the instructions of the FuGENE® 6 Transfection Reagent (Promega) for transfecting the short interfering RNAs (siRNAs) into the cell line. The siRNAs (si-p53, si-per1) were produced according to the instructions of the T7 RiboMAXTM Express RNAi System (Promega) and the sequence of each siRNA was shown in Table S3. 1 μL transfection reagent and 1 μg siRNAs were used for cell transfection. Total proteins of cells from each group were extracted after RNAi assay, and western blotting was used to detect the interference efficiency and the expression of other proteins after interference.

### 2.7. Western blotting

Proteins of the whole F1 larval fish (exposure experiment I - 28 d) were lysed and extracted by RIPA (Beyotime Biotechnology, China) and the concentration was determined by BCA assay (Pierce™ BCA Protein Assay, Thermo). Equal amounts of total proteins were separated by 8% or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skimmed milk powder, the membranes were incubated overnight with primary antibody at 4 °C, followed by incubation with an horseradish peroxidase conjugated (HRP)-conjugated secondary antibody (1: 5000) at room temperature for 1 h. Actin was used as reference protein and quantified to normalize the relative expression level of target proteins. Detection was performed using a Tanon 6100 Chemiluminescent Imaging System. The information of primary antibodies and second antibodies were shown in Table S4.

### 2.8. Comet assay (single-cell gel electrophoresis)

A single-cell suspension of F1 and F2 larval fish, whose parents were exposed to BaP for 28 days (exposure experimentI), was prepared according to the method of Kosmehl et al. (2006) with some modifications (Kosmehl et al., 2006). Briefly, the larval fish were washed three times with PBS and then scissored into 1.5 mL tubes containing a 0.25% Trypsin/EDTA solution (0.25% Trypsin, 0.02% EDTA). The samples were incubated at room temperature for 30 min, then dissociation was terminated using 10% fetal calf serum. For the comet assay, 1.5% normal-melting agarose was used for the bottom layer, and 0.75% low-melting agarose (LMP) which mixed with the single-cell suspension was used for the second layer, then spread with the coverslip. After waiting for several minutes until solidification, the coverslip was removed and the slides were placed into cold, freshly made lysing solution away from light for 1 h at 4 °C. After rinsing the remaining lysing solution, the slides were placed horizontally in the electrophoretic box filled with the electrophoresis buffer for 30 min to allow unwinding of the DNA, then electrophoresed for 30 min at 25 V in ice. The slides were then placed into neutralizing buffer for 5 min three times each, stained with DAPI for 15 min away from light, and then imaged by using confocal microscope (Zeiss Lsm 780 NLO). Three replicates were analyzed for each group, and for each replicate 50 cells were randomly selected for examination. Image analysis proceeded using CASP software (Konca et al., 2003), and Tail DNA% (Percent of DNA in the comet tail) and the OTM (Olive Tail moment:The calculation method is Tail DNA% × (Tail Mean X-Head Mean X) ([percent of DNA in the tail] × [distance between the center of gravity of DNA in the tail and that in the head in the x-direction]) were used as comet parameters to evaluate differences in mutagenic potential between experimental groups.

### 2.9. Statistical analysis

Non-observational results in the study were presented as mean ± standard deviation (SD), and all statistical analyses were conducted using IBM SPSS Statistics 21 software. A student's t-tests were performed to calculate statistical significance between exposure groups and controls, significant levels were defined at $p < 0.05$. The figures were drawn by using Graphpad Prism 8 and Microsoft Excel.

## 3. Results

### 3.1. Differentially expressed genes according to DGE

A total of 39 genes showed significantly different expression in F1 larval fish which were hatched from BaP-exposed parents (exposure experiment I) in comparison with the control group. Among them, 12 genes (4 up- and 8 down-regulated) were associated with circadian rhythms. *Per1* was the most significantly down-regulated gene, as shown in Fig. S1 and Table S5 in supporting information. To confirm the result of DGE, several genes were selected and verified their expression by qPCR. As shown in Fig. S2, the gene expression patterns detected using qPCR were almost consistent with the DGE result.

### 3.2. The effects of BaP on the circadian rhythm and immune-related genes in F1 fish larvae

In exposure experiment I, the F1 hatchling fish larvae samples were collected at 8 zeitgeber times within 24 h for detecting the rhythm of gene expression. The genes *Per1*, Cryptochrome-1 (*cry1*), and Hepatic Leukemia Factor (*HLF*) are the ones involved in maintaining the circadian clock, which were relatively highly modulated (the log2Ratio(T/C) data of *per1*, *cry1* and *HLF* were −2.08, −1.58, −1.43, respectively) among the modulated genes. Moreover, *per1* and *cry1* are known to play an important role

in the circadian rhythm signaling pathway. They are involved in the central loop of the circadian rhythm oscillator and both genes are also involved in modulating inflammation [55]. Therefore, the three genes *per1*, *cry1* and *HLF* are chosen to detect the rhythm of gene expression. They showed a 24 h oscillation pattern, with their mRNA expression levels over 24 h falling and rising over time in a "U" shape pattern. Their expression levels were highest when daytime began, and then gradually decreased over time, reaching their lowest levels at night and then gradually increased. Effects of BaP on the 24 h-pattern in mRNA expression levels of *per1*, *cry1*, *HLF*, and cellular tumor antigen *p53* are shown in Fig. 2. The mRNA levels of these genes in F1 larval fish whose parents were exposed to BaP were significantly different compared to the control group at some zeitgeber times over 24 h. With increasing duration of parental exposure, the number of zeitgeber time points which genes showed significant differentially expression in the offspring within 24 h were increased.

### 3.3. Effects of BaP on the methylation level of the per1 promoter in F1 larval fish

The partial promoter sequence was amplified and a CpG island was predicted by Methprimer (Data S1). The size of the CpG island was 203 bp with 16 CpG sites. As shown in Fig. 3A and B and Fig. S3, S4, S5 in the supporting information, the methylation level of the *per1* promoter differed in parental gonads between the control and treatment groups. The extent of *per1* promoter methylation in female gonads decreased with the duration of BaP exposure (from 53.1% to 35%), but no significant change occurred in male gonads. It is noteworthy that the degree of methylation of the *per1* promoter in male gonads was significantly higher than that in female gonads in both the control and treatment groups, and at both exposure durations. In F1 larval fish, the methylation level of the *per1* promotor in offspring from parents co-exposed to BaP was higher than in the control, and significant hypermethylation was observed in offspring from parents exposed to BaP for the longer duration (28 days), (Fig. 3B and S6). In the case of gender-specific exposure, the methylation level of the *per1* promoter in offspring from the paternal BaP treatment group was significantly higher compared to the control, and also higher than in the maternal exposure group, although the latter difference was not statistically significant (Fig. 3C and S7).

### 3.4. $N^6$-methyladenosine ($m^6A$) RNA methylation status

In the exposure experiment I, after parents were exposed to BaP for a relatively short time (14 days), there was no significant difference in $m^6A$ levels between larval fish from control and treatment groups. However, $m^6A$ levels were markedly higher than in the control when the F0 generation was exposed for the longer duration (28 days) (Fig. 4A). These results were not sex-specific: $m^6A$ in offspring exhibited a significantly higher expression in both the paternal and maternal BaP exposure experiments (exposure experiment II) (Fig. 4B).

### 3.5. Effects of BaP on protein expression levels

Results of the present study showed that after parents were exposed to BaP for 28 days, the protein per1 associated with the circadian rhythm was down-regulated in larval fish offspring. In the case of the tumor suppressor protein p53, parental BaP exposure resulted in a reduction of p53 expression in the offspring. In parallel, proteins upstream and downstream of p53 were selected for tracking of their expression levels. The phosphorylation level of p38 and p44/42 (extracellular signal−regulated kinases, ERK1/2) were significantly suppressed, both of which are belonging to the mitogen-activated protein kinases (MAPK) family. The cyclin-dependent kinase inhibitor 1 (p21), G2/mitotic-specific cyclin-B1 (cyclin B) and pho-Rb (ser807/811), which are the proteins essential for control of the cell cycle, were significantly influenced by BaP exposure. The expression of XPA (DNA-repair protein complementing XP-A cells) and XPB (General transcription and DNA repair factor IIH helicase subunit XPB), two proteins involved in nucleotide excision repair, was also inhibited in the offspring of BaP-exposed parents (Fig. 5A).

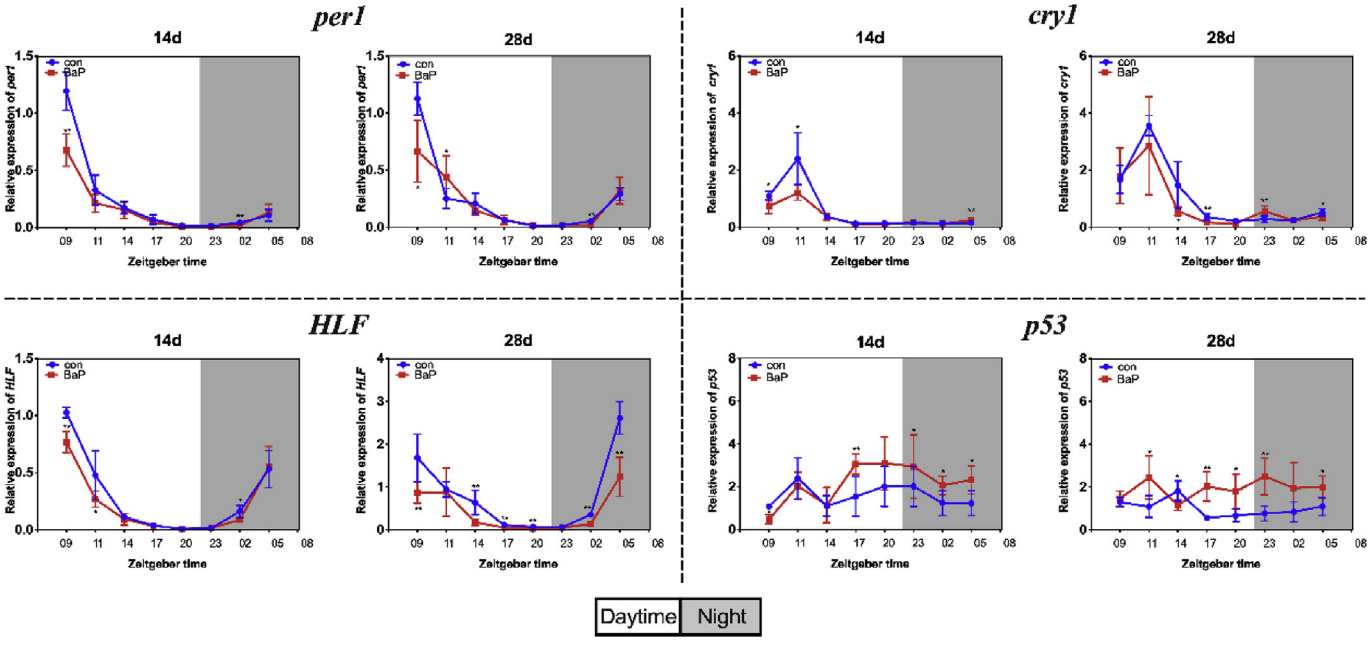


**Fig. 2.** Interference of circadian rhythm oscillations over 24 h of circadian rhythm genes (*per1*, *cry1*, *HLF*) and the immunity related gene *p53* in offspring from the BaP-exposed parental groups. Significant differences at *p < 0.05 and **p < 0.01.

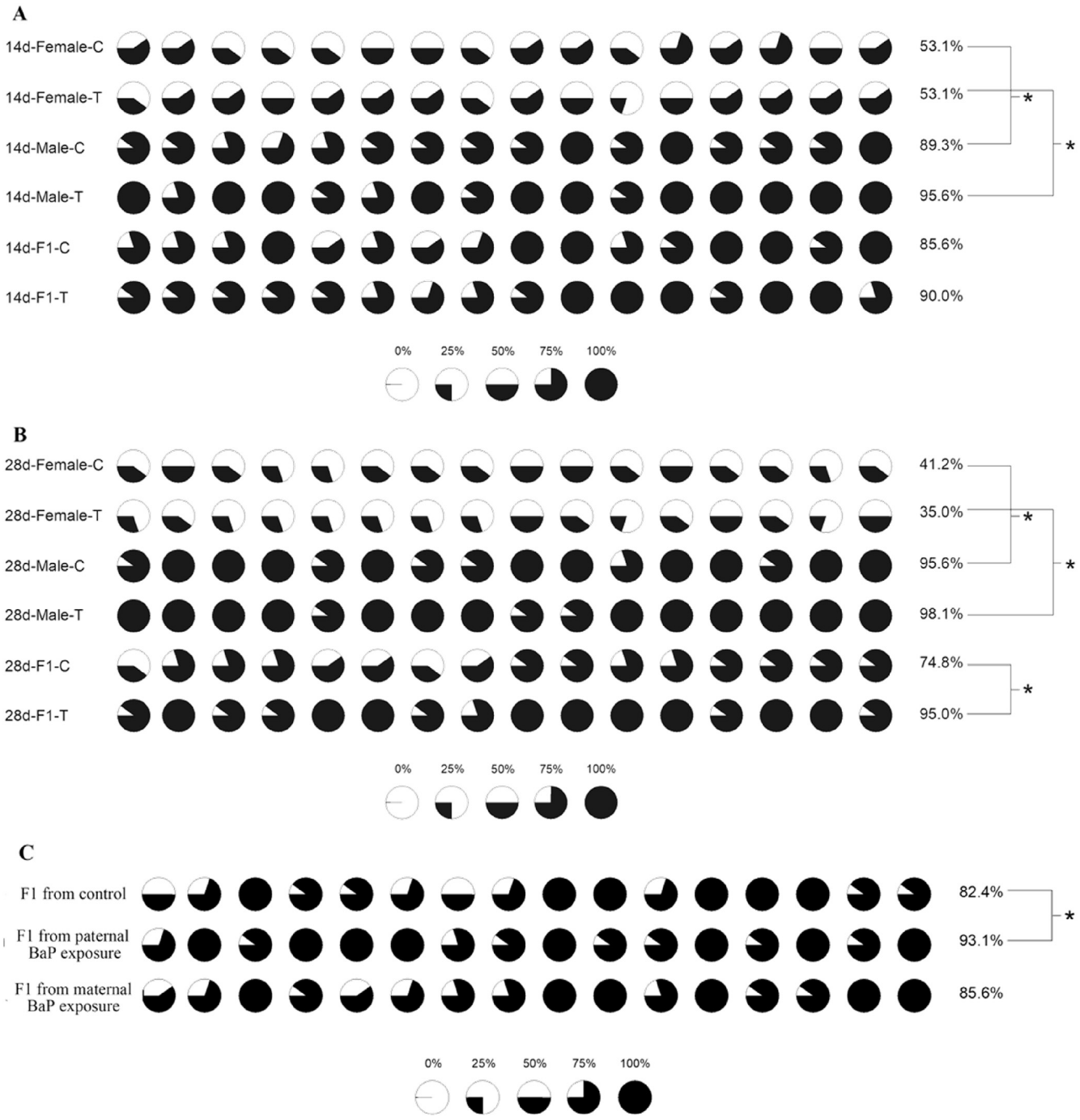


**Fig. 3.** The total methylation level of *per1* promotor in F0 gonads and F1 larval fish, shown as a percentage. (A) The total methylation level of *per1* promotor in F1 larval fish and F0 gonads after F0 generation was exposed to BaP for 14 d (exposure experiment I). (B) The total methylation level of *per1* promotor in F1 larval fish and F0 gonads after F0 generation was exposed to BaP for 28 d (exposure experiment I). (C) The total methylation of *per1* promoter in F1 larval fish with paternal and maternal BaP exposure for 28 d (exposure experiment II). "F1 from paternal BaP exposure" in the figure refers to the F1 larval fish were hatched from paternal BaP exposure group. "F1 from maternal BaP exposure" in the figure refers to the F1 larval fish were hatched from maternal BaP exposure group. Significant difference at *$p < 0.05$.

### 3.6. RNA interference (RNAi)

The relative levels of per1 and p53 proteins were examined following normalization to the level of actin. After cells were transfected with short interfering RNAs (siRNAs), per1-siRNA and p53-siRNA, significantly reduced levels of both per1 and p53 protein were determined compared to the control group. This indicated that the expression of both proteins can be effectively decreased and that siRNAs could be used in subsequent experiments. Interference results showed that the protein level of per1 was down-regulated, whereas p53 was inhibited, but there was no obvious detectable effect on p53 when there was interference with the expression of per1. Furthermore, down-regulation of cyclinB1 and DDB2, the proteins involved in the cell cycle and DNA repair, respectively, was observed (Fig. 5B).

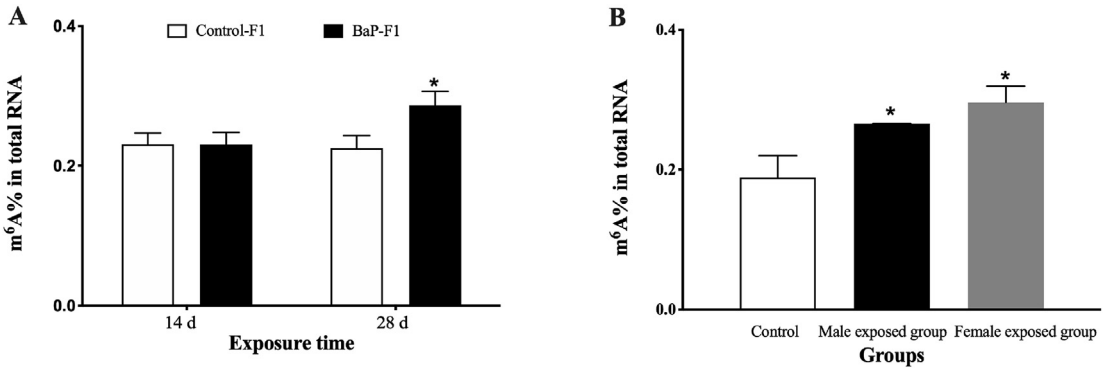


**Fig. 4.** $N^6$-methyladenosine ($m^6A$) RNA methylation status in F1 fish larvae of BaP exposed parents as well as paternal and maternal BaP exposure groups. (A) $m^6A$% in total RNA of F1 larval fish after parental BaP exposure; (B) $m^6A$% in total RNA of F1 larval fish after male and female (F0 generation) following 28 days of BaP exposure. Significant difference at *$p < 0.05$.

### 3.7. Comet assay for detection of DNA damage

Cells of larval fish whose parents were exposed to BaP for 28 days exhibited increased DNA migration resembling the shape of a comet (Fig. 6A). Also, a statistically significant increase in Tail DNA (%) and OTM were observed in this treatment compared with the control (Fig. 6C). It is important to note that in the gender-specific BaP exposure test, the offspring of BaP-exposed fathers revealed serious DNA damage with a visible comet tail, but maternal BaP exposure did not cause obvious DNA damage to offspring (Fig. 6B). Statistical analysis demonstrated that the numerical values of Tail DNA (%) and OTM in the offspring of BaP-exposed fathers were markedly higher than in the other two groups, but there was no such difference between the control and maternal BaP exposure groups (Fig. 6D). In second-generation larvae, the degree of DNA damage in offspring from treatment groups was also found to be more severe than in offspring from the control (Fig. 6E and F), but there was lesser damage than in the F1 larvae, as shown in Fig. 6G.

### 3.8. qPCR for genes expression after F1 fish larvae exposed to BaP

In the exposure experiment III, the changes of gene expression after F1 fish larvae from parents (F0) with a BaP exposure history or without BaP exposure were re-exposed the same concentration of BaP were shown in Fig. 7. When the F1 larvae were separately exposed to 0.5 and 3 μg $L^{-1}$ BaP, the transcriptional levels of *cyp1a1* (Cytochrome P450 1A1) and *cyp27a2* (Cytochrome P450 27A2) were significantly higher than those in control. In addition, the expression pattern of *myd88* (Myeloid differentiation primary response protein MyD88), *tlr3* (Toll-like receptor 3) and the apoptosis-related gene *bcl2* (Apoptosis regulator Bcl-2) was consistent after exposure, that is, the expression was significantly up-regulated at 0.5 and 1500 μg $L^{-1}$ group, but not at 3 μg $L^{-1}$ group. The expression level of *caspase9* was significantly higher than that of the control after exposure to three concentrations of BaP, but the expression of antimicrobial peptide-*hepcidin1* was not significantly altered.

## 4. Discussion

The present study investigated the intergenerational effects on offspring transferred from parents chronically exposed to an environmentally relevant concentration of BaP. In the preliminary experiment, it was observed that the offspring embryos suffered

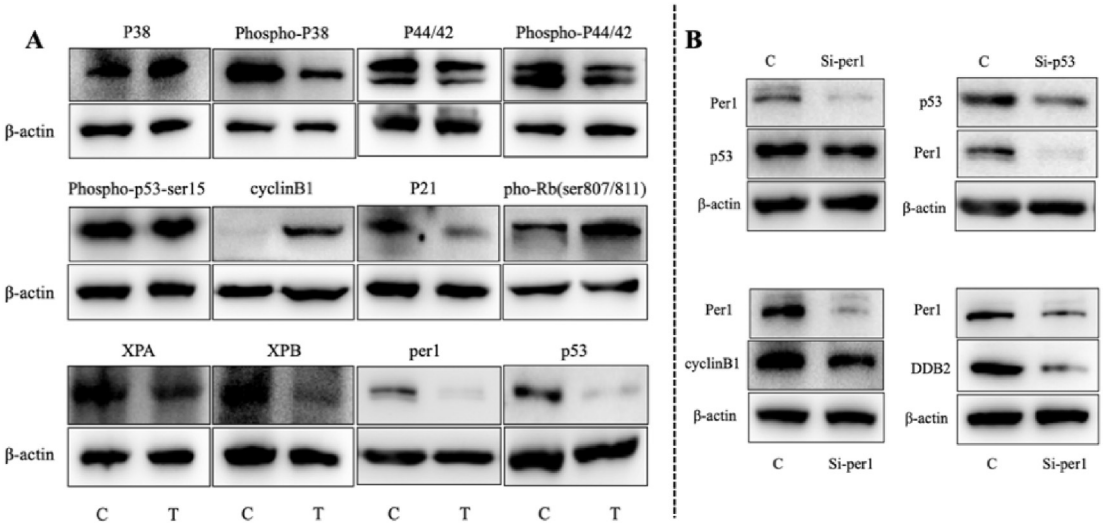


**Fig. 5.** (A) Proteins associated with either immunity or cell cycle or DNA repair showed inhibition in offspring of parental BaP exposure. C refers to the parental control group; T refers to the parental BaP exposure group. (B) The protein expression levels of the RNAi experiment using DIT-29 cells. C refers to the control group; Si-per1 or Si-p53 refers to the group in which interference in per1 and p53 was detected.

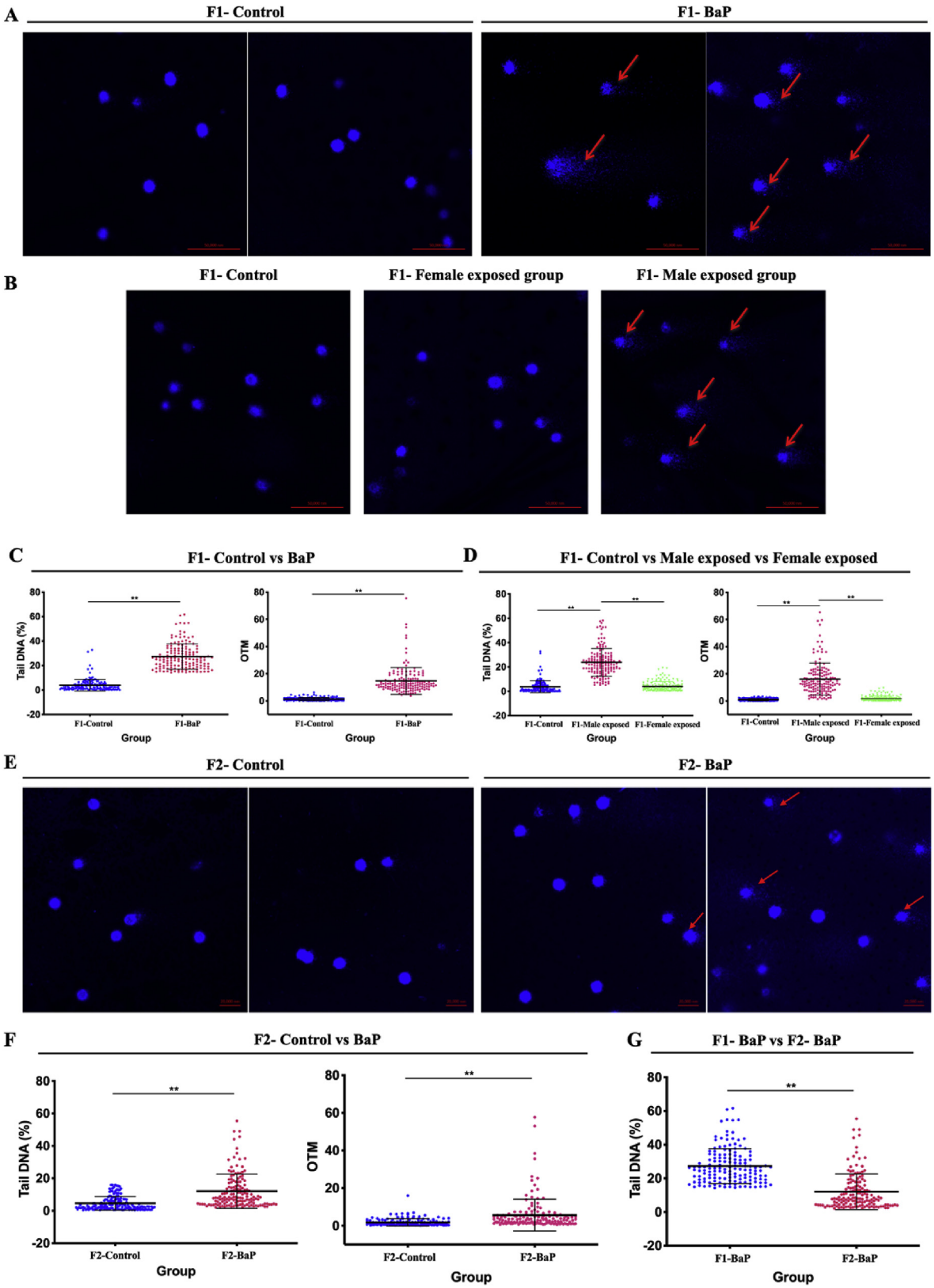


**Fig. 6.** DNA damage in the offspring of BaP-exposed parents and fathers. Three replicates were analyzed for each group, and for each replicate 50 cells were randomly selected for examination. Only several clear and representative images of comets in each group were selected and shown herein. (A, C) In F1 larval fish from the parental control and BaP exposure groups. (B, D) In F1 larval fish from the paternal vs. maternal BaP exposure groups. (E–F) In F2 larval fish from the parental control and BaP exposure groups. (G) Comparison of the degree of damage between F1 and F2 generation fish larvae from BaP-exposed parents. The red arrow marks the DNA damaged "comet tail". Each dot represents one cell. Significant difference at $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

arrested development and even death after parental BaP exposure for 14 days and 28 days, and the offspring hatched from BaP-treated parents had a higher mortality rate from the embryonic stage to hatching than the control (method and result were shown in supporting information-Fig S8), suggesting that the toxic effect might be passed to the offspring from their parents with a long-term BaP exposure. Thus, a very interesting question was raised why the offspring suffered severe damage without direct exposure, which was worth investigating.

To better understand the potential toxic effects and underlying mechanism that occurred in offspring from BaP-exposed parents, molecular analysis via DGE sequencing was undertaken using the F1 larval fish to obtain more bioinformatics of modulated genes. DGE analysis showed that some genes associated with immune functions and DNA damage were significantly modulated. More interestingly, 12 modulated genes associated with the circadian rhythm were screened that led to us speculate that the alteration of the circadian rhythm in the progeny might be related to the toxic effects on offspring from parents exposed to BaP. Therefore, we wondered whether these genes present rhythmic fluctuations in the offspring of parental long-term BaP exposure. In the study, it was found after qPCR detection using the offspring samples that the expression patterns of circadian rhythm associated genes such as *per1*, *cry1*, *HLF* as well as cellular tumor antigen *p53* were all altered within a 24 h oscillation pattern, and their expression significantly differed at certain zeitgeber times over 24 h in the offspring of BaP-treated parents relative to control. Therefore, the above results demonstrated that an intergenerational toxic effect possibly occurred in offspring from BaP-exposed parents that modulated their circadian rhythm and *p53* expression.

The circadian rhythm is associated with various biological functions, including but not limited to growth, reproduction, metabolism and immunity, and is currently a popular research topic in multidisciplinary studies. Many factors can cause disturbance of the circadian rhythm, such as inflammation and bacterial infection. Alteration of the circadian rhythm can lead to serious consequences such as metabolic disorders and tumorigenesis (Bellet et al., 2013; Haspel et al., 2014; Papagiannakopoulos et al., 2016; Skene et al., 2018). Moreover, the circadian rhythm can be altered by environmental pollutants, which are reported to directly modulate the diurnal patterns of physiological functions in mammals. For example, 7-day intranasal BaP exposure alters the circadian pattern of blood pressure in rats, probably due to inflammation occurring in the lungs (Gentner and Weber, 2011). Endocrine disruptors, such as cadmium (Cd), have been shown to affect the circadian rhythm in mammals, by disturbing the 24 h pattern of transcriptional expression of Cu/Zn, Mn, superoxide dismutase (SOD), catalase and other genes in rat adenohypophysis (Jimenez-Ortega et al., 2012). Based on the results from previous and present studies, it is possible that BaP had an intergenerational impact on the biorhythm stability of descendants by way of causing circadian rhythm disturbance in vivo.

Additionally, it was found in this study that the transcriptional expression of *per1* was the most significantly down-regulated in offspring, and that there was a pronounced difference in the *per1* promoter methylation level between offspring derived from BaP parental treatment groups and the control. Furthermore, the degree of *per1* promotor methylation in the gonads of male fish exposed to BaP was higher (by 42.5% and 63.1%) than in the female gonads, and males and females not exposed to BaP differed in the extent of initial methylation. As already established, DNA methylation is one kind of epigenetic modification and occurs almost exclusively in the context of CpG dinucleotides, also associated with gene silencing (Portela and Esteller, 2010). Therefore, based on the above results we speculate that the occurrence of hypermethylation may be one potential mechanism explaining the remarkable regulation of *per1* in offspring from BaP-exposed parents, from which it can be inferred that BaP exposure may change the epigenetic connection of parents and offspring. Environmental factors and toxicants, such as metals, air pollution, benzene, bisphenol A (BPA), and dioxin, are reported to be linked to aberrant changes in epigenetic pathways *in vitro*, in human and other animal studies (Baccarelli and Bollati, 2009). Many studies have shown that environmental chemicals and exogenous elements can alter epigenetic marks, which are potentially transmitted to offspring leading to increased risk of disease. For instance, previous studies have shown that maternal BaP exposure could elicit an increase in promoter hypermethylation of interferon gamma (IFN$\gamma$), but lower global methylation in umbilical cord white blood cells (Herbstman et al., 2012; Tang et al., 2012). As reported, male mice received BaP for 12 weeks, the methylation levels of imprinting genes were altered and a similar alteration was observed in the F1-2 generations which revealed a potential transgenerational effect (Zhang et al., 2019), Similarly, our study also confirmed the intergenerational toxic effect of BaP by detecting methylation levels. In addition, different from a previous study that environmental factors could induce abnormal methylation modification of circadian genes in placental tissue but the effect on offspring has not been clarified (Nawrot et al., 2018). In the study, there was a discrepancy (not significant) between the 14 d-F1-control and 28 d-F1-control was observed and the reason might be related to the samples collection from the fish at 14 days or 28 days which were raised in different tanks.

In addition to DNA changes detected in descendants from BaP-exposed parents in this study, the presence of $m^6A$ also significantly increased in these offspring relative to the control. Modification of $m^6A$ is reported to play an important role in cell differentiation and development, regulate DNA damage responses and the circadian clock, and modulate hepatic lipid metabolism and other major biological processes (Deng et al., 2018; Xiang et al., 2017; Zhong et al., 2018). The $m^6A$ data presented here supports the suggestion that epigenetic modification may be involved in transferring toxic effects from parents exposed to BaP to their offspring.

The expression of p53 was significantly inhibited in F1 larval fish after parental long-term exposure (28 days) by western blotting, suggested that parental BaP exposure is time-dependent on the regulation of p53 in offspring. It is known that a wide variety of regulators govern the activity of p53, which, in turn, controls many distinct biological processes, such as immune regulation, stress response, inflammation, apoptosis, metabolism, ROS control, etc (Kastenhuber and Lowe, 2017; Munoz-Fontela et al., 2016; Vousden and Prives, 2009). p53 is crucial as a reversible DNA damage-induced cell cycle checkpoint, and activates downstream genes to retard the G1 or G2/M phase in order to facilitate DNA repair that supports genomic stability prior to further cell division. Once the DNA damage occurs, the downstream p53 signaling pathway is activated and the apoptosis process is initiated to eliminate the damaged cells. However, results from our study showed that the phosphorylation expression of p44/42 and p38 were inhibited in offspring, i.e., that cell proliferation and differentiation and the signal transduction of the stress response were blocked. Given that both p44/42 (ERK) and p38 belong to the MAPK family and are involved in signal transduction from the cell surface to the nucleus, the downstream p53 signaling pathway could not be activated and the apoptosis reaction was also inactivated. An additional finding of this study was that the critical proteins XPA and XPB, which are involved in nucleotide excision repair, were also markedly inhibited along with the p53 signal pathway, and thus the DNA damage could not be immediately repaired as is usual. Therefore, we speculated

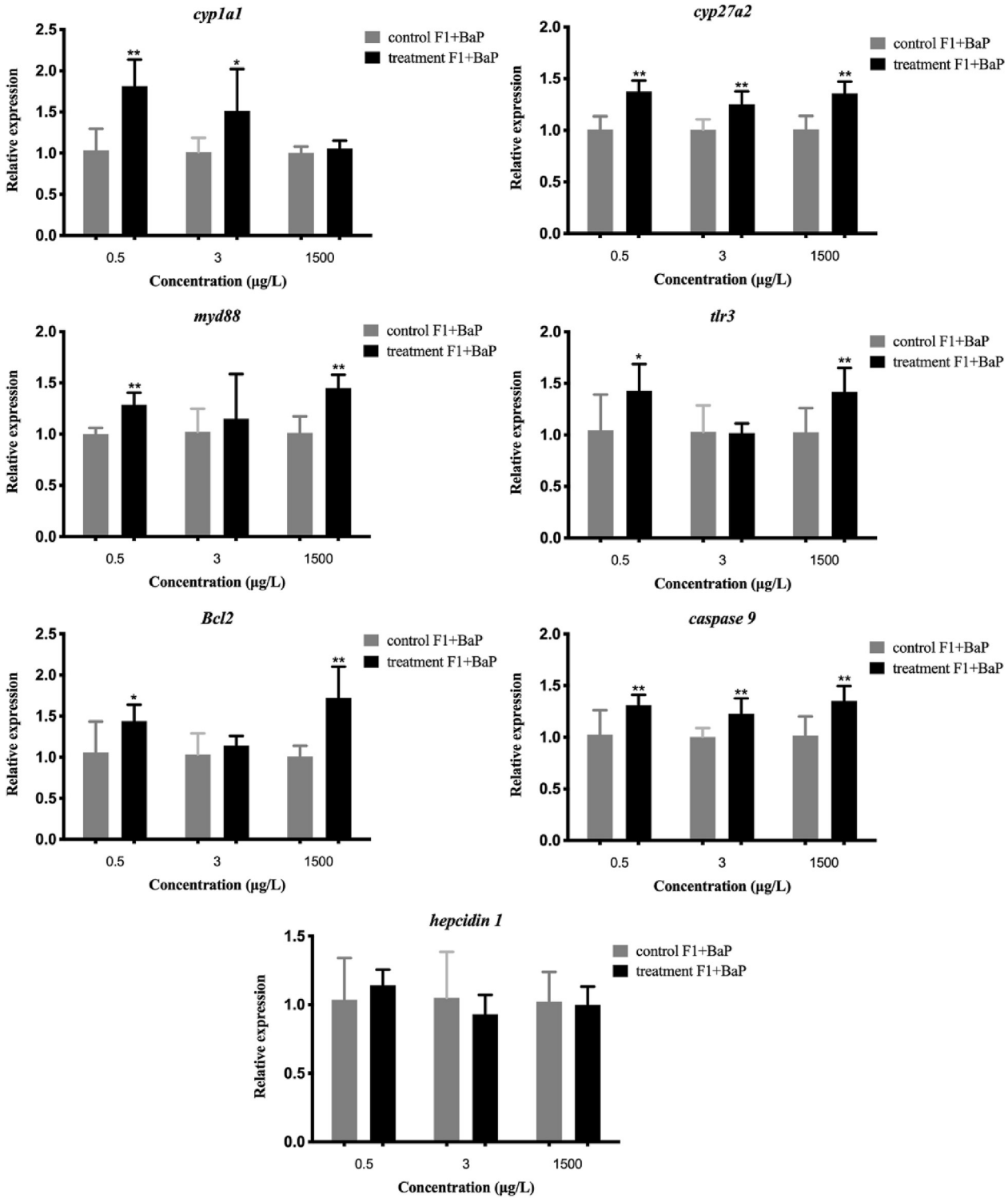


**Fig. 7.** The changes of gene expression after F1 larvae from control and exposure groups were re-exposed to the same concentration gradient of BaP, (i.e., 0.5, 3, 1500 μg $L^{-1}$) for 24 h. Significant difference at * $p < 0.05$, and **$p < 0.01$.

that the accumulation of DNA damage might be one of the reasons for the increased mortality of offspring.

As reported, the circadian rhythm, DNA damage and repair, cell cycle and p53 signaling pathway are closely related to the immune system although each of them has individual functions (Balomenos and Martinez-A, 2000; Chatzinikolaou et al., 2014; Curtis et al., 2014; Ermolaeva et al., 2013; Geiger et al., 2015; Kastenhuber and Lowe, 2017; Munoz-Fontela et al., 2016; Poliezhaieva and Ermolaeva, 2017; Vousden and Prives, 2009; Zebell and Dong, 2015). Therefore, we believe that parents with BaP exposure will affect the immunity of their offspring, which was further demonstrated in the results of experiment III (Fig. 7) that the offspring whose parents were exposed to BaP were more sensitive to exogenous pollutants and the immune related genes were markedly

modulated. However, it remains uncertain whether there is a relationship between the alteration of the circadian rhythm and immunomodulation, i.e., whether the circadian rhythm varies with the change of immune responses in fish or vice versa. The role of the circadian rhythm in vivo has been extensively studied in mammals, and a mutual interaction between the circadian rhythm and immunity is also observed in some previous studies. Circadian oscillators have also been identified in other peripheral tissues (liver, heart, kidney etc.) and cells (fibroblasts, macrophages, T cells, etc.) where many important biological processes occur (Keller et al., 2009; Scheiermann et al., 2013; Schibler et al., 2003). Almost every immune response (innate and adaptive) oscillates in a circadian manner, and that the circadian clock acts as a gatekeeper reducing immunity-related costs and improving organismal health and resistance (Man et al., 2016). In the study, both *per1* and *p53* were modulated in offspring following parental BaP exposure, suggesting that *per1* and *p53* might be coordinated in causing immunomodulation in descendants. The communication between the circadian rhythm and the immune response exists in fish might be interfered after BaP exposure and an intergenerational immunomodulation involving the circadian clock might occur in the offspring of BaP-exposed parents.

In this study, the degree of methylation of the *per1* promotor in BaP-exposed male fish gonads was greater than in the control and also greater than in the gonads of BaP-exposed females, meanwhile, it is noteworthy that paternal BaP exposure had a more appreciable impact on offspring than maternal exposure, the severity of DNA damage increased in the offspring of BaP-exposed fathers, whereas the effect on offspring from maternal BaP exposure was considerably lower and comparable to that in the control group. Trevor et al. also found the same that male offspring appeared more susceptible to BaP as compared to females in mice by detecting the DNA methylation in lung tissues of offspring (Fish and Benninghoff, 2017), which presents a similar result as we observed in fish. Numerous studies have indicated that males and females differ in their immune responses to external stimuli (Aldridge et al., 2018; Klein and Flanagan, 2016; Ye et al., 2018). However, fewer studies have examined the differences in the effects of paternal and maternal BaP exposure on their offspring. DNA damage can be transferred from the impaired germ cells of the parents to offspring, most likely through the damaged sperm. There is evidence that male germ cells lose the ability to repair DNA after meiosis in mammals. Thus, DNA damage carried by paternal sperm may cause damage in fertilized eggs and developing embryos, which leading to genomic instability and may persist for generations (Adiga et al., 2010; Harrouk et al., 2000). Sperm are highly sensitive to genotoxic substances and also particularly susceptible to oxidative stress. Meanwhile, it is generally accepted that males are much more vulnerable than females with respect to some features of immune capacity. We thus hypothesize that the toxic effects on male fish when exposed to BaP might persist and be inherited by the next generation through sperm, as evidenced by one report that the sperm DNA methylome is inherited by zebrafish early embryos (Jiang et al., 2013). This conjecture is also supported by other studies in which males and females were found to have different immunological responses to foreign and self-antigens (Klein and Flanagan, 2016). Moreover, sex-specific immunomodulatory action is also observed in fish when both sexes are exposed to environmental estrogen (Ye et al., 2018). Therefore, it is reasonable to assume that the gender of fish and possibly other marine animals is one of the critical factors to consider in the assessment of environmental risk and the impact of exogenous stimuli. The toxic effects and even the underlying mechanisms caused by the same stimulus on different sexes are diverse. In particular, there may be differential impacts or damage on offspring or even multiple generations from different-sex parents.

Following these studies, it remains uncertain whether F1 generation larvae from parents (F0) with a BaP exposure history or without BaP exposure had a similar or different response when both were exposed to the same concentration of BaP (Fig. 7). The result indicated that the offspring with BaP-exposed parents were more sensitive to the additional BaP exposure than the offspring from control groups. Furthermore, the toxic effects on the F2 generation from parents (F0) exposed to BaP were relatively lower than on the F1, with relatively milder DNA damage to the first generation. This suggests that the toxic effects caused by parental BaP exposure had a trans-generational effect. It remains to be determined whether the negative effect will persist for a longer time in later generations.

## 5. Conclusion

In conclusion, the present study demonstrated that marine medaka exposed to environmentally relevant concentrations of BaP induced remarkable disturbance in their circadian rhythm oscillation patterns, and that the toxic effects can be transferred to the next generation. The observed circadian oscillation disturbance may be one of the causes of immunomodulation in offspring from exposed parents. Based on this study, the alteration of the circadian rhythm can be considered a potential biomarker for the toxic effects of organismal exogenous stimulations. In addition, since the offspring from BaP exposed parents suffered from DNA damage and the expression of per1, p53 and its downstream proteins were inhibited, we speculated that there might be a link between circadian rhythm and immunity in marine fish. Methylation of *per1* and abnormal levels of $m^6A$ suggested that the toxic effect in offspring following parental BaP exposure was probably due to epigenetic modification. Finally, the magnitude of toxic effects in offspring was highly dependent on gender, and the underlying mechanisms differed somewhat between males and females.

## Author contributions

Kejian Wang designed the research work, supervised and revised the manuscript; Xiaohan Yin performed the experiments, co-designed and wrote the paper; Yong Liu and Rabia Zeb participated in part of the fish maintenance and BaP exposure trials; Huiyun Chen assisted in the analysis of results of the comet assay using confocal microscopy; Kejian Wang and Fangyi Chen supervised and revised the research work. All authors discussed the results and commented on the manuscript. Funding for this research was generated by grants awarded to K.J.W.

## Declaration of competing interest

The authors declare no competing financial interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115437.

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