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# Benzo[*a*]pyrene-induced multigenerational changes in gene expression, behavior, and DNA methylation are primarily influenced by paternal exposure

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## Abstract

Benzo[*a*]pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH), is implicated in many developmental and behavioral adverse outcomes in offspring of exposed parents. The objective of this study was to investigate sex-dependent multigenerational effects of preconceptional effects of BaP exposure. Adult wild-type (5D) zebrafish were fed 708 μg BaP/g diet (measured) at a rate of 1% body weight twice/day (14 μg BaP/g fish/day) for 21 days. Fish were spawned using a crossover design, and parental (F0) behavior and reproductive indexes were measured. In offspring, behavioral effects were measured at 96 h post fertilization (hpf) in F1 & F2 larvae, and again when F1s were adults. Compared to controls, there was no significant effect on F0 adult behavior immediately following exposure, but locomotor activity was significantly increased in F1 adults of both sexes. Larval behavior (96 hpf, photomotor response assay) was significantly altered in both the F1 and F2 generations. To assess molecular changes associated with BaP exposure, we conducted transcriptome and DNA methylation profiling in F0 gametes (sperm and eggs) and F1 embryos (10 hpf) from all four crosses. Embryos resulting from the BaP male and control female cross had the most differentially expressed genes (DEGs) and differentially methylated regions (DMRs). Some DMRs were associated with genes encoding chromatin modifying enzymes suggesting regulation of chromatin conformation by DNA methylation. Overall, these results suggest that parental dietary BaP exposure significantly contributes to the multigenerational adverse outcomes.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kristine L Willett reports financial support was provided by University of Mississippi School of Pharmacy and by National Institutes of Health.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2023.116545.

**Keywords**

Benzo[*a*]Pyrene; Multigenerational; DNA Methylation; RNAseq; Behavior; PAH

## 1. Introduction

Susceptibility to disease can be set preconceptionally as a result of exposure to contaminants or nutritional deficits (Gómez-Roig et al., 2021; Grandjean et al., 2015; Heindel and Vandenberg, 2015). Benzo[*a*] pyrene (BaP), a carcinogenic polycyclic aromatic hydrocarbon (PAH), is a ubiquitous environmental contaminant derived from the incomplete combustion of carbon (Bukowska et al., 2022). Exposures to BaP occur from ingesting contaminated food, water, or inhalation of tobacco smoke or automotive or oven exhausts. In humans, prenatal exposure to PAHs, including BaP, are implicated in neural tube defects (Ren et al., 2011), growth deficits (Langlois et al., 2014; Siddiqui et al., 2008) and lower neurobehavioral development and higher odds of developmental delay in children as they mature (Cao et al., 2020; Perera et al., 2006, 2009, 2012).

The sex-dependence of parents' preconceptional diets and contaminant exposures on next generation adverse outcomes is recognized in human and model organisms (Perez and Lehner, 2019; Schmidt, 2018). For example, there is an association between poor paternal nutrition and higher incidence of metabolic diseases such as type 2 diabetes in offspring (Braun et al., 2017; Ferguson-Smith and Patti, 2011). Some epidemiological studies have related paternal, but not maternal, exposure to PAHs/tobacco smoke with a higher risk of childhood cancers (Boffetta et al., 2000; Cordier et al., 2004; Ji et al., 1997). Recently, the POHaD paradigm (Paternal Origins of Health and Disease) was coined to highlight the need to consider the impacts of paternal exposure on offspring (Soubry, 2018). Thus, there is growing evidence that sex-specific environmental exposures during reproductive years can alter subsequent generations and epigenetic mechanisms are implicated in this process (Ambeskovic et al., 2020; Ho et al., 2017).

We have previously demonstrated in zebrafish that BaP dietary exposure caused developmental defects in F1 and F2 offspring (Corrales et al., 2014b), and that altered DNA methylation occurred in embryos exposed directly to BaP or via their parents (Corrales et al., 2014a; Fang et al., 2013a; Fang et al., 2013b). Prior studies in medaka and rodent models found paternal BaP exposure caused more severe adverse outcomes in offspring including epigenetic modifications and reproductive dysfunction (Jorge et al., 2021; Mohamed et al., 2010; Yin et al., 2020). As mentioned above, multigenerational neurobehavioral deficits following prenatal exposure to BaP are recognized in both human offspring and model organisms (Jedrychowski et al., 2015; Knecht et al., 2017; Zhang et al., 2022) however, the molecular mechanisms associated adverse outcomes following sex-dependent preconceptional exposure to BaP have not been fully elucidated. In this study, adult zebrafish were exposed to BaP via diet for 21-days and a crossover study design was used to assess sex-dependent effects on growth and behavior on F0 and subsequent generations (F1 and F2). In addition, we quantified transcriptional and DNA methylation changes in the exposed gametes (F0) and F1 embryos.

## 2. Materials and methods

### 2.1. Fish care and handling

Wild-type 5D zebrafish (kindly provided by Dr. Robyn Tanguay at Oregon State University) were used in this study and raised under the approved Institutional Animal Care and Use Committee (IACUC) protocol (18–022). Fish were maintained in Aquatic Habitats ZF0601 Zebrafish Stand-Alone System (Aquatic Habitats, Apopka, FL) at a constant pH (7.0–7.6, 760 μS), temperature (25–28 °C), and 14:10 light-dark cycle. Fish were fed twice daily with Gemma 300 micro food (Skretting USA, UT). Sexually mature fish without any deformities or signs of disease were selected as breeders. Their eggs were collected, and larvae were raised to 4 months post fertilization (mpf) to obtain the F0 generation for the dietary exposure described below. All the experiments and exposure protocols were in accordance with IACUC approved guidelines and recommendations.

### 2.2. BaP dietary preparation and GC–MS

BaP-treated diet was prepared following a previously established protocol (Corrales et al., 2014b). To prepare the diet, 24 g of flake food were spiked with 18 mL of acetone containing BaP (0 or 1.667 μg/μL), equivalent to nominal BaP concentrations of 0 or 1250 μg/g food, respectively. The spiked flakes were immediately rotovapped to dryness and stored in amber vials at room temperature. Acetone and BaP were purchased from Fisher Scientific (Fair Lawn, NJ) and Supelco Analytical (Belfonte, PA), respectively.

BaP concentrations in the food were quantified using GC–MS following extraction with 2–3 mL methylene chloride. Approximately 10 mg of flakes were extracted at the beginning of the exposure and then weekly. A surrogate standard, BaP-d12, was added to each sample to obtain a final concentration of 0.2 μg/mL (surrogate percent recovery 140–205%). Samples were vortexed for 30 s and centrifuged for 7 min at 668 x*g*. Samples were then blown to dryness with $N_2$ and brought back up with a known volume of hexane. An internal standard, fluorene-d10, was added to obtain a final concentration of 0.2 μg/mL to determine extraction efficiency. BaP was quantitated with GC–MS using selected ion mode ($m/z$ 252). BaP was not identified in the acetone-treated control samples. Average measured BaP concentration of the treated flakes was 708 ± 26 μg BaP/g flake equivalent to 14 μg BaP/g fish/day.

### 2.3. BaP dietary exposure

Sexually mature (4 mpf) zebrafish were fed either acetone alone or BaP-treated TetraMin® Tropical Flakes (nominally 1250 μg BaP/g food equivalent to 25 μg BaP/g fish) (Exposure Timeline, Fig. 1A). Zebrafish (2 males and 2 females) in five replicate tanks per treatment group ($N$ = 5 replicate tanks for a total 20 fish/group) were allowed to acclimate for a week at 25.5–28 °C and were fed twice daily with untreated flakes and Gemma 300 micro food. During the exposure, fish were fed 1% body weight twice daily of the corresponding dose of BaP-treated flake food and once daily Gemma 300 micro food for 21 days. To determine if the dietary exposure to BaP affected somatic growth, parental fish had their weight and length recorded at beginning and end of exposure and gonad and liver weight measured at the end. Weight and length were used to calculate Fulton's condition factor, K ($K = W/TL^3$

$\times 10^5$; W = weight in g, TL = total body length in mm) which can reflect the well-being of fish (Clark et al., 2018).

At the end of day 21, a crossover breeding design was implemented to assess sex-specific contributions namely: 1) control males x control females (Cm x Cf), 2) control males x BaP females (Cm x Bf), 3) BaP males x control females (Bm x Cf), and 4) BaP males x BaP females (Bm x Bf). On days 22 and 23, eggs were collected to determine reproductive success. On day 24 (females) and day 25 (males) F0 parental behavior in an open field test (OFT) was assessed. In adult zebrafish, open field tests (OFT) in a novel environment are utilized to determine locomotor activity and thigmotaxis (wall hugging to assess anxiety) (Champagne et al., 2010). After OFT, fish were euthanized, and gonad, liver, and brain samples were collected and biobanked. Sperm or eggs from the BaP-exposed and control fish, along with 10 hpf embryos from each cross (4 crosses) were collected for RNAseq and DNA methylation analysis (graphical abstract and Fig. 1).

### 2.4. Quantification of BaP exposure induced multigenerational effects

On days 22 and 23, from each parental tank, the total number and viability of eggs were determined and a subset of 40 fertilized eggs (200 per group) were placed in 12-well plates (5 embryos/well containing 5 mL of egg water (sterilized deionized water; 60 ppm Instant Ocean; pH 7.6) with 0.05% methylene blue). Embryo survival and time to hatch was measured at 24, 48, 72, and 96 h post fertilization (hpf). Water changes in the wells were done once daily until 96 hpf.

Larval F1 zebrafish (96 hpf) from all four parental crosses (Cm x Cf, Cm x Bf, Bm x Cf, and Bm x Bf) were assessed for morphological defects and larval behaviors. At 96 hpf, a subset of 50 larvae per treatment (10 larval fish per tank, 5 tanks per treatment) were examined for developmental deformities: body shape, tail, pericardial edema, swim bladder, and yolk sac edema. Ten larvae per tank were anesthetized in buffered MS-222 and immediately placed on a microscope slide with a chamber containing 3% methylcellulose. Digital still micrographs were taken with a MicroFire camera and PictureFrame 2.3 software (Optronics®, Goleta, CA) attached to a Zeiss Stemi 2000-C stereomicroscope. Lateral images were recorded for each fish. All measurements of the larval fish were completed by blind reviewers. Deformities were evaluated and quantified in NIH ImageJ. Body length and optic vesicle areas were measured in pixels by tracing the boundaries of the space. Presence or absence of pericardial edema, yolk sac edema, body axis curvature, and swim bladder inflation were recorded. Pixel area or distance was converted to $mm^2$ or mm based on the calibrated magnification (Corrales et al., 2014b).

At 96 hpf, 10 larvae per tank (total of 50 larvae/treatment) were placed in a 96 well plate with 1 larva/well (~300 μL/well) to assess photomotor response behavior (LPR) using ZebraBox (ViewPoint, Montreal, Canada). Zebrafish LPR activity, indicative of altered locomotor and/or anxiety-like activity in response to alternating light, is known to be altered following BaP exposure (Knecht et al., 2017). Larval behavior (distance moved) was observed during a 30 min duration with light:dark cycling (0–10 min, 100% light; 10–20 min, dark, 0% light; and 20–30 min, 100% light) (Kirla et al., 2016).

At 120 hpf, larvae were transferred to 3 L tanks with 20 larvae per tank; 10 tanks per treatment group. Mortality was checked weekly until 30 days post fertilization (dpf). At 30 dpf, length and weight of 40 fish per treatment were measured, and fish were thinned to 20 fish per tank; 5 tanks per treatment group. At 90 dpf, length and weight of 30 fish per treatment were measured, and fish were thinned to 10 fish (5 male and 5 female) per tank; 4 tanks per treatment group. At 120 dpf, reproductive success of the F1 generation was assessed, F1 endpoints were collected (weight, length, fecundity, OFT), and F2 fish were processed in the same manner as the F1 larvae until 96 hpf (developmental deformities, LPR).

### 2.5. Quantification of BaP exposure induced effects in F0 generation

At the termination of the F0 exposure and after the F1 offspring were collected as described above, F0 open field behavior (OFT) was assessed in 10 fish/sex/group following similar methods to (Pandelides et al., 2020). Briefly, fish were acclimated to a darkened behavioral testing room (27–28 °C) for 10 min. Individual fish were then transferred to a system water-filled bucket (diameter 23.5 cm, depth 24.8 cm) and their exploration behavior responses were video-captured from overhead for 5 min with Noldus Ethovision 14 software. The testing area was illuminated to 9 lx. At the completion of the trial, the fish were removed from the open field arena and placed in a holding container until euthanasia. OFT was used to determine if exposure affected locomotor activity (mean velocity in mm/s), freezing time (in s, duration of freezing bouts, i.e. time periods with swimming velocities below 1 mm/s), and time spent in the periphery of the tank (as % of the total active swimming period, with swimming speeds >0.1 mm/s) as a measure for thigmotaxis (Sireeni et al., 2020; Steenbergen et al., 2011). The swim arena was divided into two regions: periphery (1 fish length from the edge, outer 50% of the arena) and center (inner 50% of the arena).

### 2.6. RNA and DNA isolation

Total RNA and genomic DNA were isolated from the same tissue using a *Quick*-DNA/RNA Miniprep Plus Kit (Zymo, Cat # D7005) following the manufacturer's instructions. Each treatment condition had 4 biological replicates. F0 sperm and egg samples (control and BaP-exposed) and the 10 hpf F1 embryos from the four crosses were sequenced. For F0 sperm, DNA was isolated, but there was not enough total RNA to sequence the transcriptome.

### 2.7. RNA sequencing analysis

Total RNA library preparation and sequencing (RNAseq) was conducted by Zymo research laboratories using approximately 250 ng of total RNA. Ribosomal RNA was removed using the method described by (Bogdanova et al., 2011). Libraries were prepared using the Zymo-Seq RiboFree Total RNA Library Prep Kit (Cat # R3000) according to the manufacturer's instructions. Libraries were sequenced on an Illumina HiSeq to a sequencing depth of >50 million read pairs (150 bp paired-end sequencing) per sample.

Raw data files were assessed for quality using FastQC (Andrews, 2010) prior to pre-processing. Trimmomatic was used for pre-processing to remove any remaining adaptor sequences and reads with low sequence quality (Phred score <20) (Bolger et al., 2014). Trimmed sequence reads were mapped and aligned to the zebrafish genome using Salmon

(Patro et al., 2017). The number of reads mapped to the annotated regions of the genome were also obtained with Salmon. Statistical analysis was conducted using DESeq2 (Love et al., 2014). Only genes with false discovery rate (FDR) of <5% were considered to be differentially expressed. Differentially expressed genes (DEGs) were annotated using BioMart (Smedley et al., 2015). The following comparisons were made: control eggs vs BaP eggs, and control M x control F 10 hpf embryos vs the three treated groups.

Differentially expressed genes (FDR <0.05) were classified based on gene ontology (molecular function) using a gProfiler package g:GOSt (Reimand et al., 2016). The up- and down-regulated datasets for each treatment were processed individually. The gene ontology (GO) terms were compared between the two (eggs) or four (10 hpf embryo) groups using the g:Cocoa, a package of gProfiler (Reimand et al., 2016). Further, REVIGO was used to remove redundant GO terms (Supek et al., 2011). Non-redundant pathways were visualized using circular gene ontology software (CirGO) (Kuznetsova et al., 2019). Only unique GO terms with a distinct set of genes were considered for further analysis. In addition, we conducted protein interaction analysis of differentially expressed genes using string data base (Szklarczyk et al., 2021).

### 2.8. Reduced representation bisulfite sequencing (RRBS)

RRBS library preparation and sequencing was done at Zymo Research Services. Genomic DNA (100 ng) was digested with 30 units of *Msp*I (NEB). Fragments were ligated to pre-annealed adapters containing 5′-methyl-cytosine instead of cytosine according to Illumina's specified guidelines. Adaptor-ligated fragments ≥50 bp in size were recovered using the DNA Clean & Concentrator™−5 (Cat#: D4003). The fragments were then bisulfite-treated using the EZ DNA Methylation-Lightning™ Kit (Cat#: D5030). Preparative-scale PCR was performed, and the resulting products were purified with DNA Clean & Concentrator™−5 (Cat#: D4003) for sequencing on an Illumina platform. Sequence reads from bisulfite-treated classic RRBS libraries were identified using standard Illumina base calling software and then raw FASTQ files were quality trimmed (removing adapter sequences and trimming reads based on Phred quality) using TrimGalore 0.6.4. FastQC 0.11.8 was used to assess the effect of trimming and overall quality distributions of the data. The following comparisons were made: control eggs vs BaP eggs, control sperm vs BaP sperm, and control M x control F 10 hpf embryos vs the three treated groups.

Bisulfite sequencing data were analyzed using the Bisulfite Analysis Toolkit (BAT; v.0.1) (Kretzmer et al., 2017), reads were aligned with *segemehl* (v0.2.0) to the zebrafish reference genome danRer10 using standard parameters of the module "BAT_calling". The resulting vcf files were filtered using the module "BAT_filter_vcf" and summarized using "BAT_summarize". The calling of differentially methylated regions (DMRs) was carried out with "BAT_DMRcalling" using *metilene* (v.0.2–8) (Jühling et al., 2016). The genomic regions enrichment of annotations tool (GREAT) was used (McLean et al., 2010) to annotate the differentially methylated regions (DMRs). The genome coordinates were converted from the latest version (GRCz11; used in methylation analysis) to older version (Zv9) using the liftOver tool in UCSC genome browser prior to analysis with GREAT.

### 2.9. Statistical analysis

All data were assessed for normality and homogeneity of variance using Shapiro-Wilk and Brown-Forsythe tests, respectively. The incidence of developmental deformities (%) was calculated and averaged per replicate tank ($n = 5$). Larval length, eye diameter, and eye area were recorded per fish and then averaged per replicate tank (n = 5). Differences between the groups were assessed either by *t*-test, $p \leq 0.05$ (F0 control vs BaP exposed), or 1-way (or 2-way when adults [sex as a second factor]) analysis of variance (ANOVA), with Student-Newman-Keuls (SNK) post hoc test (the four crosses) $p \leq 0.05$. If the data did not meet assumptions for ANOVA, an ANOVA on ranks with Dunn's posthoc test $p \leq 0.05$ was conducted. All graphs and statistical analyses were conducted using Sigmaplot 14.0 software.

## 3. Results

### 3.1. Effect of dietary BaP exposure on F0 generation

The 21-day dietary BaP exposure had no significant effect on F0 growth (length, weight, and liver somatic index, Table S1). There was also no significant effect on adult F0 fecundity ($p > 0.05$, Table S2) and open field behavior (Fig. S1). However, dietary exposure to BaP resulted in significantly lower (~62%) gonad weight and gonadal somatic index in males, but not in females ($p \leq 0.05$, Table S1).

### 3.2. Effect of F0 dietary BaP exposure on F1 generation

There was no significant effect of parental BaP exposure on the survival or incidence of developmental abnormalities in the F1 generation. There was also no effect of parental BaP exposure on adult F1 offspring gonadal somatic index, liver somatic index ($p > 0.05$, Table S1), or fecundity ($p > 0.05$, Table S2). There was no effect of BaP exposure on the length or weight in the F1 offspring of Bm x Bf cross at any time-point measured ($p > 0.05$, Fig. S2–3). However, the Bm x Cf group was significantly smaller in length (6%) at 96 hpf. At 90 dpf, this group continued to be significantly smaller in length (8%) and weight (21%) for male and female fish ($p \leq 0.05$, Fig. S2–3).

At 96 hpf, the F1 offspring of both the Bm x Cf and Bm x Bf crosses exhibited hyperactivity (28% and 42% increased total distance traveled, respectively) during the dark phase of the larval photomotor response (LPR) test ($p \leq 0.05$, Fig. 2A,B).

As adults, both male and female F1 offspring from the Bm x Bf cross were more active ($p \leq 0.05$, Fig. 2C), but thigmotaxis was unaffected by parental BaP exposure (Fig. 2D). In general, female fish moved less and spent less time in the periphery compared to males ($p \leq 0.05$, Fig. 2 C, D), and had significantly increased freezing duration (data not shown).

### 3.3. Effect of F0 dietary BaP exposure on F2 generation

There was no significant effect of parental BaP exposure on the survival or incidence of developmental abnormalities in the F2 generation for all exposed crosses. However, in the F2 generation the groups with exposed male parents were hyperactive in the light phase ($p \leq 0.05$, Fig. S4).

### 3.4. BaP-induced transcriptional changes in F0 gametes and 10 hpf F1 embryos

Transcriptional profiling of oocytes from BaP-exposed females (F0) revealed 37 differentially expressed genes (DEGs) with 18 and 19 up- and downregulated genes, respectively. For F0 sperm, there was not enough total RNA to sequence the transcriptome. For F1 generation 10 hpf embryos, the Bm x Cf cross showed differential expression of 657 genes in comparison to the control group. Among them, 245 and 412 genes were up and downregulated, respectively. In comparison, the Cm x Bf F1 embryos only had 11 DEGs (7 upregulated and 4 downregulated). When both parents are exposed to BaP (Bm x Bf), 112 genes were differentially expressed with 63 and 49 up- and downregulated genes, respectively (Fig. 3A).

There was no overlap in DEGs between the three treatment groups. Five genes were common between the paternal BaP exposure groups (Bm x Cf and Bm x Bf), and 4 genes were shared between Cm x Bf and Bm x Bf crosses (Fig. 3A). The genes common between different groups and their expression levels are shown in Fig. 3B.

GO term analysis for the two groups with the largest number of DEGs (Bm x Cf and Bm x Bf) showed enrichment of GO terms associated with protein folding, ribosome biogenesis, and cell adhesion (Fig. 3C). The entire list of DEG as well as their fold changes are provided in supplementary data (BaP_SI.xlsx).

### 3.5. DNA methylation profiling of F0 gametes and F1 embryos

DNA methylation profiling of BaP-exposed F0 showed 41 DMRs in sperm and 5 DMRs in the oocytes. In 10 hpf F1 embryos DNA methylation profiling of Bm x Cf and Cm x Bf crosses revealed 51 and 44 DMRs, respectively (Fig. 4A). When both parents were exposed, 42 DMRs were detected in F1 embryos.

Overall, more DMRs were hypomethylated relative to controls and located in the intergenic regions (50–500 kb from the transcription start site). Among the three treatment groups, only one DMR was common to all treatments. This genomic region was predicted to be associated with the *mtbp* gene (MDM2 binding protein), a key player in tumor suppression and regulation protein ubiquitination (Iwakuma and Agarwal, 2012). Ten DMRs were shared between the Bm x Cf and Bm x Bf groups, and 3 DMRs were common between Cm x Bf and Bm x Bf groups. The genes with predicted associations with DMRs that are shared between different treatment groups and tissues and their mean methylation difference are shown in Fig. 4B. The entire list and information of genes associated with DMRs are provided in the supplementary data (BaP_SI.xlsx).

GO analysis of genes associated with DMRs revealed enrichment of GO biological process terms related to retinol metabolism and histone deacetylase activity in all three treatment groups of F1 embryos. A representative GO plot of the Bm x Bf group is shown in Fig. 5. The GO plots of remaining treatments are provided in the supplementary information (Fig. S5–6).

RNAseq and RRBS raw files have been deposited in the NCBI Gene Expression Omnibus (GEO) database (GSE203631).

## 4. Discussion

This study demonstrated sex-dependent differences in BaP exposure-induced multigenerational effects. We found prenatal paternal exposure to BaP causes distinct changes in larval behavioral responses, gene expression, and DNA methylation. These observations concur with previous studies with diverse group of toxicants showing sex-specific differences in toxicant effects as well as paternal exposure-induced reproductive outcomes and persistent effects on subsequent generations (Soubry, 2018).

Significant hyperactivity was measured in the F1 offspring of the Bm x Cf and Bm x Bf crosses (which persisted into adulthood), suggesting the effect of BaP on behavior is carried through the male germline. The significant opposite effect (hypoactivity) detected in F1's from the Cm x Bf cross further highlights the complicated nature of parental exposure. The effect of BaP on hyperactivity in the Bm x Cf and Bm x Bf crosses persisted into the F2 generation, though it was in the light phase rather than dark. The multigenerational hyperactive adverse outcome was also noted following an early life stage waterborne BaP exposure in zebrafish (Knecht et al., 2017). Similarly, increased anxiety-related behaviors, but not motor activity or impaired learning/memory, were noted in adult male rats following prenatal dietary maternal PAH exposure (Crépeaux et al., 2012). Epidemiological studies also found an increased prevalence of attention deficit/hyperactivity disorder in humans prenatally exposed to air pollution and PAHs (reviewed in: (Myhre et al., 2018)).

While the exact mechanism of how BaP exposure causes neurobehavioral effects is not known, it is likely to be mediated by aryl hydrocarbon (AHR) receptor (Knecht et al., 2017) because AHR is important in various neurodevelopmental processes (Crépeaux et al., 2012; Juricek and Coumoul, 2018). Furthermore, epigenetic mechanisms such as histone modification (Zhang et al., 2022), and DNA methylation (Gao et al., 2017) are implicated in AHR agonist-induced multigenerational effects. In this study, genes associated with neurological effects that were most up- and down-regulated in F1 embryos from the Bm x Cf and Bm x Bf crosses were related to epilepsy, including *cacnb4b*, *scn1laa*, and *cux2b*. There is an association between air pollution and epilepsy (Fernandes et al., 2019), and these data support that preconceptional exposure to pollutants such as BaP may play a role in the development of neurological defects in offspring.

Paternal exposure to BaP (Bm x Cf) caused differential expression of hundreds of genes in the F1 generation embryos and many of these genes are associated with AHR function. We identified three clusters (Supplemental Fig. S7–9) based on protein interaction networks. The genes representing these clusters are associated with developmental processes such as cell proliferation and cell adhesion, key functions of AHR during development (Gialitakis et al., 2017; Zablon et al., 2021). For instance, genes encoding ephrin receptors and ephrin ligands that are pivotal for directing coordinated cell migration in development were differentially expressed (Rozbesky and Jones, 2020), and the role of AHR in ephrin-dependent cellular processes has been demonstrated (Carvajal-Gonzalez et al., 2009). It remains to be determined how these processes affect tissue organization in preconceptionally exposed embryos.

Overall, very few DEGs (37) were observed in the F0 eggs despite the 21-day dietary exposure of adult females to BaP. However, a large number of DEGs were observed in the F1 generation (10 hpf), and these effects were most influenced by paternal exposure. Many of the pathways enriched in these embryos represent processes critical during early development including protein binding/folding and rRNA processing.

The importance of germ cell methylation and the potential impacts of environmental exposures on epigenetic signaling and multigenerational outcomes is an area of intense research. DNA methylation in oocytes, while not required for oocyte development or maturation, is critical in embryonic development and genomic imprinting (Sendžikaitė and Kelsey, 2019). A recent systematic review of studies considering toxicant effects on sperm DNA methylation highlighted associations between human exposures and altered DNA methylation, but signatures were inconsistent between studies and influences on subsequent offspring health unknown (Åsenius et al., 2020). Ma and coworkers found associations between urinary hydroxyPAH concentrations and methylation status of imprinted genes (e.g. H19 and PEG3) (Ma et al., 2019). In our study, there were only five DMRs in eggs from BaP-exposed F0, but in sperm more DMRs (41) were discovered with 23 DMRs hypermethylated and 18 hypomethylated. Our analysis revealed similar numbers of DMRs in the three F1 crosses, regardless of which parent was exposed. Offspring of BaP males had the most overlap in DMR genes and all were hypomethylated. This supports the claim that zebrafish sperm may be the mediator of epigenetic consequences (Jiang et al., 2013) including BaP toxicity because the embryonic profiles were similar to that of sperm. Clasp2, grna, irx5b and pimr48 were hypomethylated in both sperm and 10 hpf offspring from BaP exposed males, whereas only grem1b was hypomethylated in eggs and F1 of exposed females. DNA hypomethylation as a result of BaP exposure also occurred in zebrafish embryos (Corrales et al., 2014a; Fang et al., 2013b; Knecht et al., 2017), in mice blood and liver (Zhao et al., 2015), and rat sperm (Zhang et al., 2019).

Other AHR agonists, like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are also associated with reproductive adverse outcomes, specifically implicating the male germline. The transgenerational effects of TCDD on male gonad histology, fertility, and transcription were attributed to hypomethylation in the male germ line (Akemann et al., 2019, 2020; Baker et al., 2016; Baker et al., 2014). Despite the significant decrease in male GSI after dietary BaP exposure, we did not find a significant effect on fecundity. It is possible that these changes to the male gonad size did not measurably affect fecundity at this early life stage (4 months) but could result in significant changes to fecundity by the time the fish are fully grown (6 months).

DNA methylation was the epigenetic modification that was the focus of this study, but histone modifications (Xia et al., 2016; Zhang et al., 2022) and microRNA dysregulation (Brevik et al., 2012) also occur following BaP exposure. Likewise, TCDD was linked to altered DMRs of genes associated with histone modification and dysregulation of histone deacetylase (HDAC) activity (Akemann et al., 2020). We found significant effects on genes associated with epigenetic processes including differential methylation near *anp32e* and significant differential expression of *mark3a* and *kdm6ba* (supplemental file BaP_SI.xlsx). It is possible that some of the differentially hypomethylated genes that we detected are

a result of HDAC activity. Furthermore, some DMRs modified by BaP exposure were associated with genes encoding chromatin modifying enzymes, suggesting regulation of chromatin conformation by DNA methylation. Dysregulation of histone deacetylase activity is associated with many types of disease (Delcuve et al., 2013) including persistent impaired cognitive function after prenatal BaP exposure (Zhang et al., 2022). Further work is needed to confirm the relationship between hypomethylation of histone modifying enzymes, their protein expression and activity, and what role HDACs play in BaP's epigenetic effects.

Our transcriptional and DNA methylation results suggest that BaP exposure altered the expression of genes associated with diverse signaling pathways including ones not related to previously established mechanisms of BaP-induced AHR activation. For example, we did not observe significant upregulation of *cyp1a* in either the exposed adult germ cells or their 10 hpf offspring. BaP is quickly metabolized by fish (Hornung et al., 2007; Zhu et al., 2008), and the transcriptional responses observed could be related to BaP metabolites (e.g., 3-hydroxybenzo(*a*)pyrene, benzo(*a*)pyrene-9,10-dihydrodiol and benzo(*a*) pyrene-7,8-dihydrodiol 9,10-epoxide). These metabolites in turn can affect several metabolic pathways (Souza et al., 2016), activate many other transcription factors (van Delft et al., 2010), and induce oxidative stress, mitogenic signaling and formation of DNA adducts (Miller and Ramos, 2001). Preconceptional exposure to BaP could therefore affect diverse physiological pathways via BaP metabolites.

In a related dietary BaP study, Alsop et al. (2007) exposed adult zebrafish (strain unspecified) to 30 or 150 μg BaP/g diet (nominal) for 260 days. They demonstrated that long-term exposure to BaP did not statistically significantly impact growth or reproduction, however, exposure to BaP caused significant depletion of retinoids, retinyl esters, and retinal (Alsop et al., 2007). We found effects on retinol metabolism by dietary exposure to BaP in the parents may be passed on to their offspring through DNA methylation because significant enrichment of pathways related to retinol metabolism were observed in the F1 generation. Alsop et al. (2007) unfortunately was not able to measure effects on the F1 generation, due to complete mortality of all F1 embryos within a few days of fertilization (including controls).

Overall, these findings highlight that preconceptional exposure to BaP causes persistent behavioral hyperactivity. While the most serious and long-term effects were dependent on both parents being exposed, the highest number of differentially expressed genes depended on male parent exposure. Sperm compared to eggs contained significantly more differentially methylated regions with hypomethylation of certain genes conserved into the F1 embryos. Molecular analyses revealed significant effects on genes associated with epigenetic processes and processes critical during early development. These findings have important implications for assessing risk to offspring following prenatal parental exposure.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data availability

RNAseq and RRBS raw files have been deposited in the NCBI Gene Expression Omnibus (GEO) database (GSE203631)

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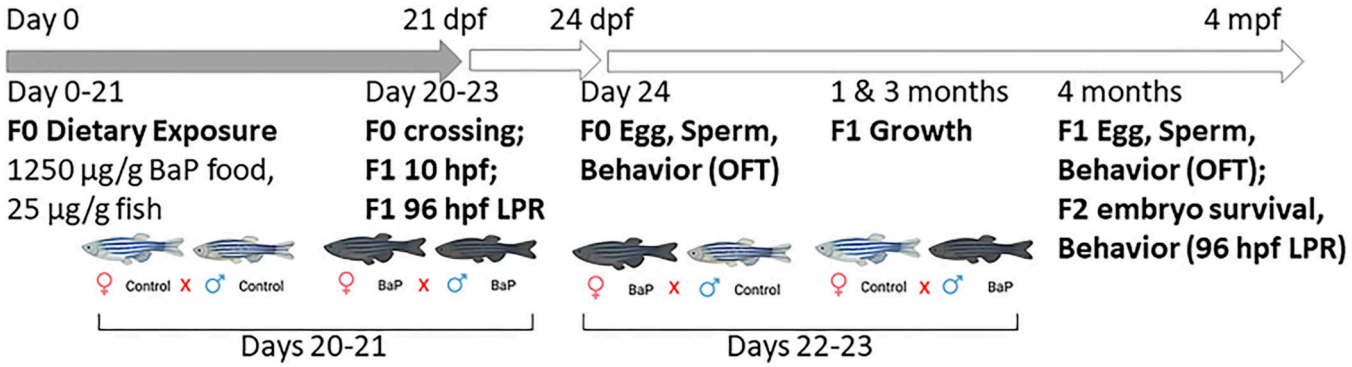



**Fig. 1.**

Exposure timeline and methodology. Adult 5D zebrafish were exposed to acetone- or BaP-treated flakes for 21 days. On days 20–23 a cross-over mating design was used to generate F1 embryos of the four crosses (Cm x Cf, Cm x Bf, Bm x Cf, and Bm x Bf). On day 24, F0 open field test (OFT) behavior was assessed prior to collection of eggs or sperm for RNASeq and RRBS. F1 embryos were raised to 10 hpf and isolated for RNASeq and RRBS. At 96 hpf, behavior of F1 larvae was assessed in a larval photomotor response assay (LPR). F1 growth was measured at 1, 3, and 4 mpf. At 4 mpf, adult F1 OFT was done, F1s mated, and F2 embryo survival and behavior (96 hpf; LPR) was measured.

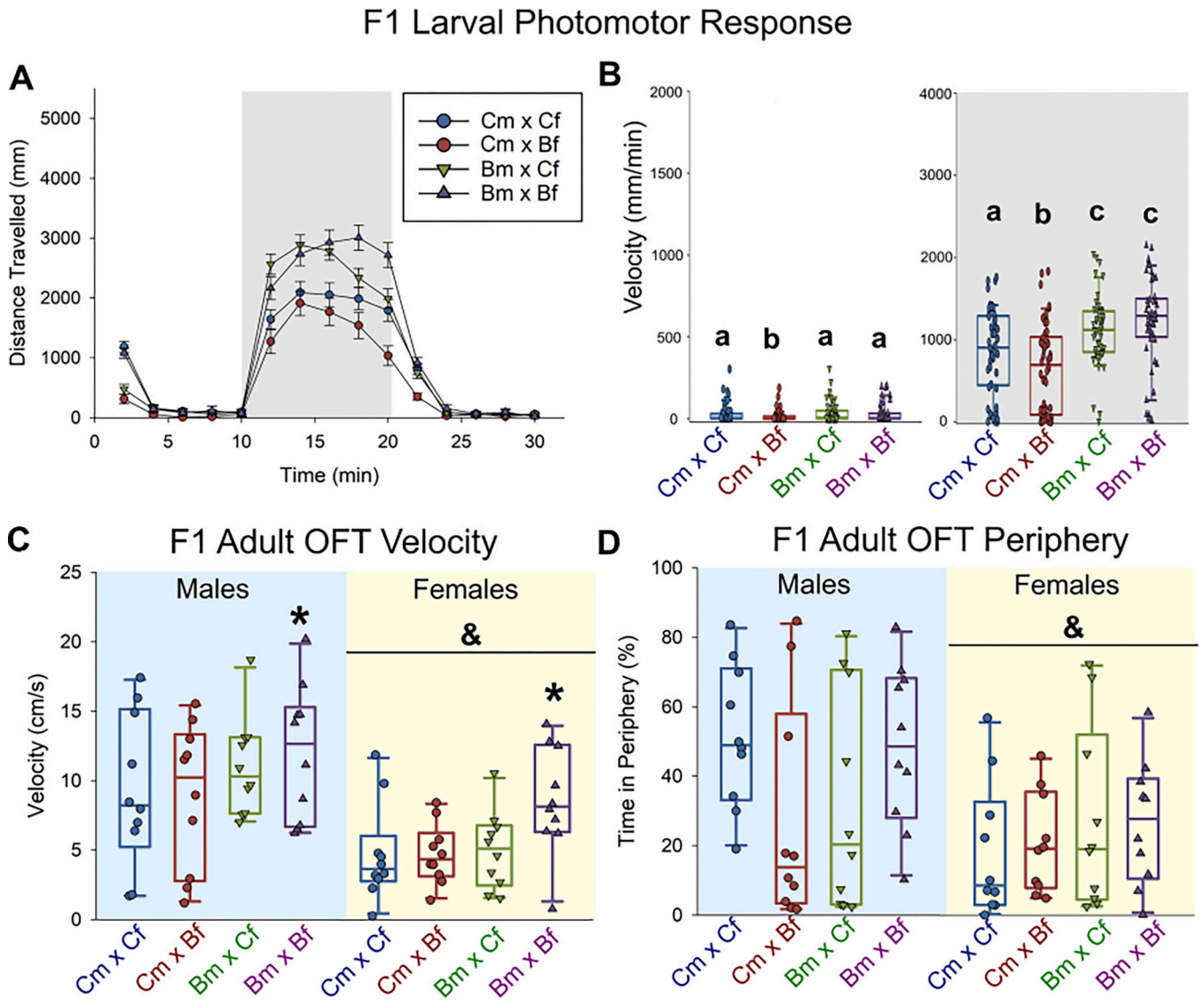
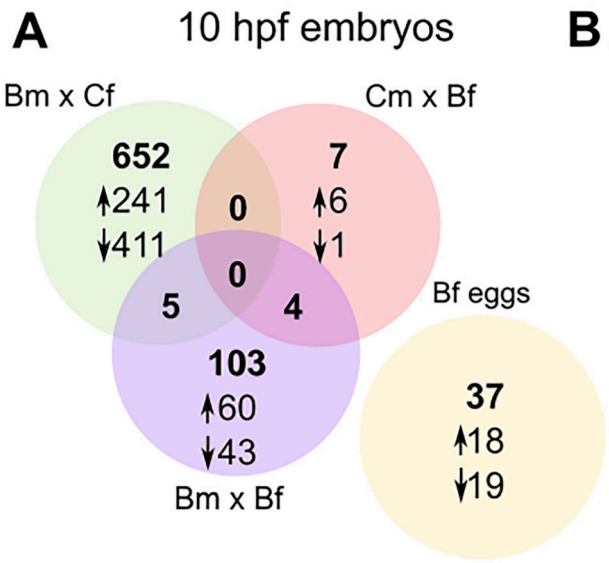



**Fig. 2.**

Impact of parental BaP exposure on offspring behavior in F1 generation (A-D). Distance traveled over 30 min larval photomotor response test (A-B, 10 min light acclimation, 10 min dark, 10 min light) of 96 hpf F1 zebrafish ($n = 50$). Within dark phase, hyperactivity was measured in Bm x Bf and Bm x Cf larval F1 zebrafish. Letters that are not in common are significantly different (ANOVA, SNK post-hoc $p \leq 0.05$). Open field behavior (OFT) of 120-day old (adult) F1 zebrafish ($n = 10$ per sex; C-D). Significantly increased velocity was detected when both parents were exposed (C). Females had significantly lower velocity and spent less time in periphery compared to males (C-D). *Indicates a significant difference from the control cross; &indicates a significant difference between males and females (two-way-ANOVA, SNK post-hoc $p \leq 0.05$).




**B**

| Gene | Bm x Bf | Cm x Bf | Bm x Cf | Bf Eggs |
|---|---|---|---|---|
| pdia3 | -0.62 | 0.54 | | |
| si:dkey-77f5.11 | 0.76 | -0.53 | | |
| golga7ba | 0.94 | -0.67 | | |
| hist1h2a11 | 0.47 | -0.37 | | |
| rab11fip1a | -1.27 | | 0.94 | |
| rnf25 | -0.89 | | 0.91 | |
| si:ch211-11k18.4 | -0.75 | | 0.54 | |
| scn1laa | -2.34 | | 1.92 | |
| cacnb4b | -6.38 | | 4.89 | |
| dspa | 0.42 | | | -0.59 |
| si:ch211-137a8.2 | | | 2.15 | 0.27 |
| CU182823.2 | | | -0.90 | 0.46 |

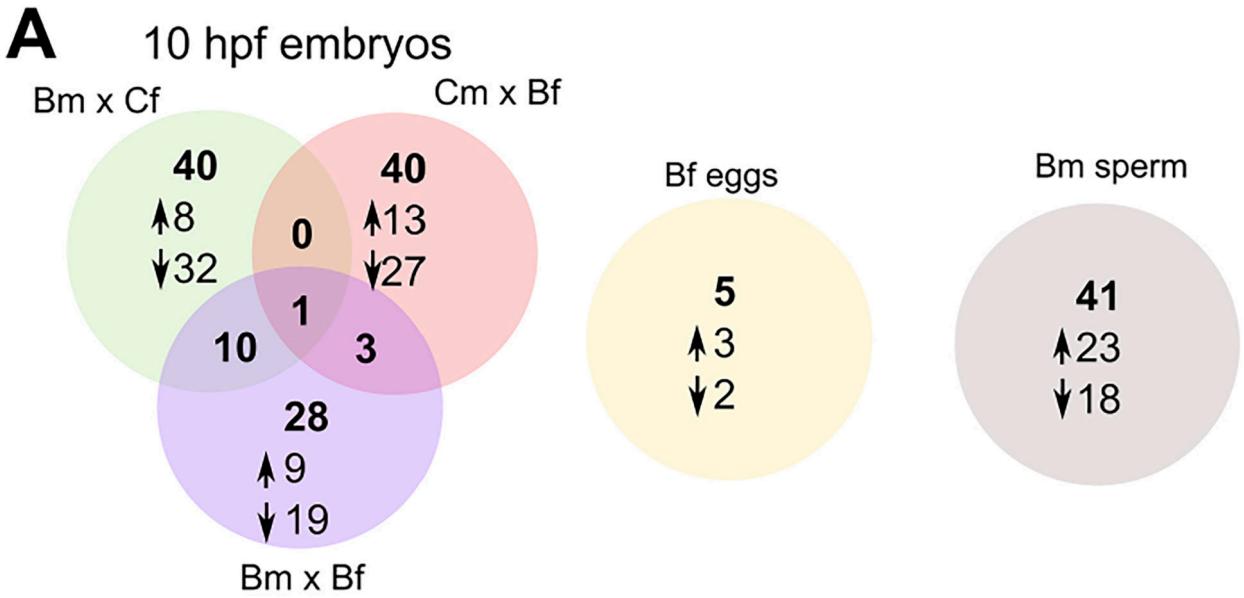
Log(2)FC: 5, 3, 1, 0, -1, -3, -5

**C**

| Group | GO | Term Name | Term ID | -Log10 (p) |
|---|---|---|---|---|
| Bm x Bf | MF | Protein folding chaperone | GO:0044183 | 2.43 |
| | MF | Misfolded protein binding | GO:0051787 | 1.83 |
| | BP | Protein refolding | GO:0042026 | 2.12 |
| | BP | Ribosome biogenesis | GO:0042254 | 2.01 |
| | BP | rRNA processing | GO:0006364 | 1.94 |
| Bm x Cf | MF | Protein binding | GO:0005515 | 1.74 |
| | BP | Cell adhesion | GO:0007155 | 3.57 |
| | BP | Anatomical structure morph | GO:0009653 | 3.37 |
| | HP | Optically empty vitreous | HP:0030663 | 1.56 |

**Fig. 3.**

Transcriptional profiles in response to BaP exposure. RNAseq was done on F0 germ cells and F1 generation 10 hpf embryos from all four parental crosses (A). Genes that overlap along with their fold change (B). GO term analysis results. GO terms enriched in the differentially expressed datasets from Bm x Cf and Bm x Bf exposures are shown (C).




B

| Gene | Bm x Bf | Cm x Bf | Bm x Cf | Bf Eggs | Bm Sperm |
|---|---|---|---|---|---|
| AL928977.1 | -22.4 | | -23.3 | | |
| CABZ01112864.1 | -37.3 | | -31 | | |
| clasp2 | -11.6 | | -15.2 | | -17.1 |
| cpne8 | -35.3 | -23.1 | | | |
| si:ch211-161m3.6 | -27.3 | | -40.3 | | |
| si:ch211-253b8.5 | -12.1 | | -15.3 | | |
| grna | -15.2 | -17.1 | | | -25.3 |
| hs2st1b | -16.2 | | -13.8 | | |
| mtbp | -17.4 | -15.5 | -13.8 | | |
| myo19 | -10.1 | | -17.3 | | |
| npy8ar | -11.8 | | -15.1 | | |
| RF00017 | -16.6 | -11.2 | | | |
| rxfp2a | -16.3 | | -12.2 | | |
| ttll5 | -17 | | -14.5 | | |
| grem1b | | -11.3 | | -19.6 | |
| irx5b | -38.5 | | | | -40.2 |
| dclk1a | -17.5 | | | | -18.5 |
| pimr48 | | | -13.1 | | -18.3 |

Scale (%): 50, 30, 15, 0, -15, -30, -50

**Fig. 4.**

RRBS analysis ($n = 4$) of eggs, sperm, and of 10 hpf embryos with significant genes hyper (up) or hypomethylated (down) (A). Differentially methylated regions (DMRs) that overlapped between the different groups, all regions that overlapped were hypomethylated relative to their respective controls (B).

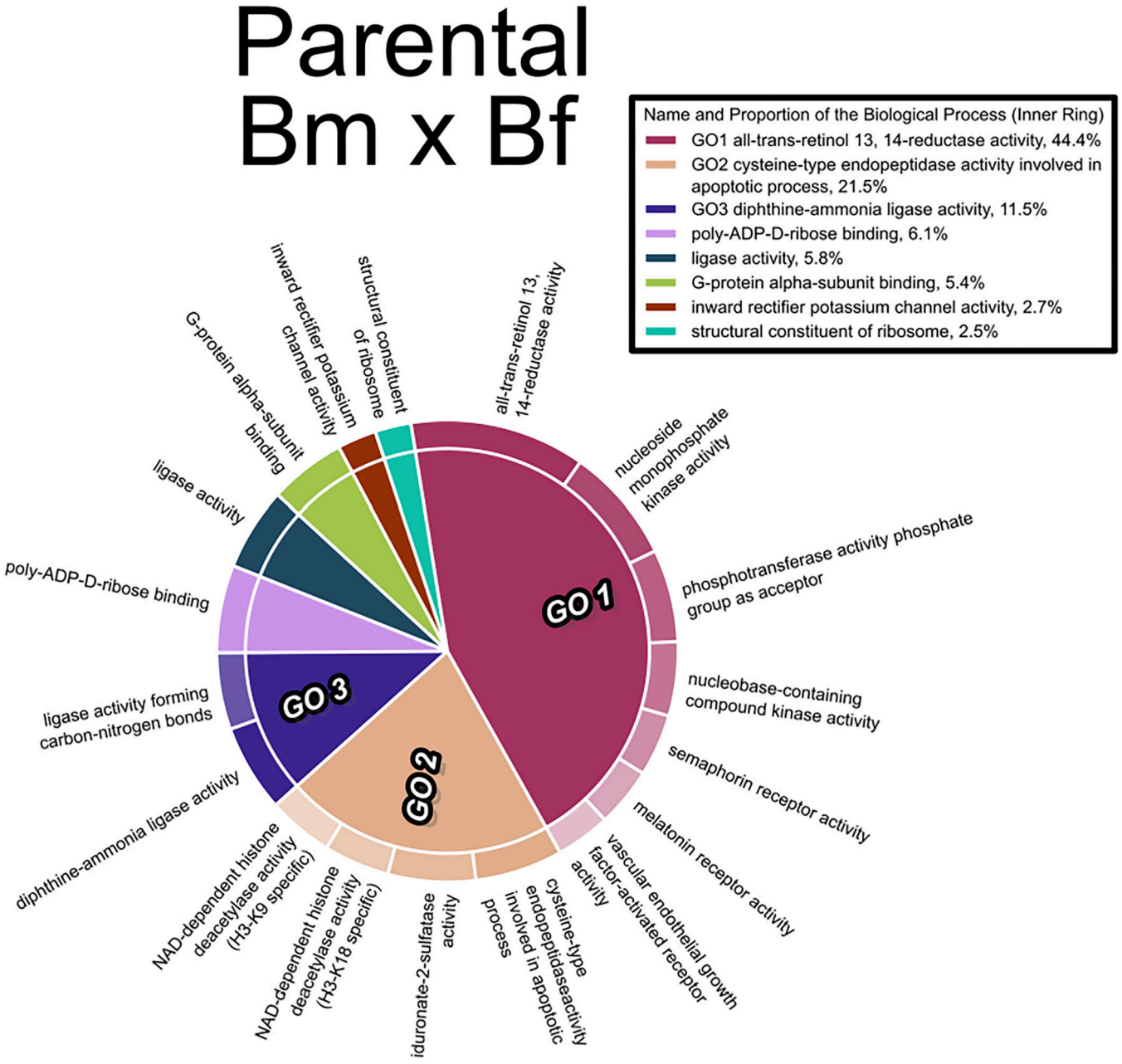



**Fig. 5.**

Gene Ontology analysis (molecular function pathways) of genes associated with DMRs in the F1 generation 10 hpf embryos from the Bm x Bf cross. The size of each GO term is determined by its statistical significance (*p*-value) and the different child terms under each parental GO term are represented by color gradient along the circumference of the plot.