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RESEARCH ARTICLE

Environmental Toxin Exposure and Its Effects on Sperm Chromatin Epigenetic Marks

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ABSTRACT

Mature mammalian sperm retain a small but functionally important fraction of nucleosomal histones at developmental and imprinted loci after the genome-wide histone-to-protamine exchange of spermiogenesis, and disruption of this retained chromatin landscape has been proposed as a route by which paternal environmental exposures influence offspring phenotype. Here we exposed adult male mice to the phthalate plasticiser di(2-ethylhexyl) phthalate (DEHP) at an environmentally relevant dose throughout one full 35-day spermatogenic cycle and profiled sperm chromatin composition using sperm chromatin structure assay (SCSA), chromomycin A3 and aniline blue staining of protamine deficiency, whole-genome bisulfite sequencing and CUT&RUN for retained H3K4me3 and H3K27me3. DEHP-exposed males showed a 2.4-fold increase in the protamine-deficient sperm fraction by chromomycin A3 staining and a significantly elevated DNA fragmentation index by SCSA relative to vehicle-treated controls, without a significant change in overall sperm concentration or motility. Whole-genome bisulfite sequencing identified 1,362 differentially methylated regions in DEHP-exposed sperm, enriched at CpG islands overlapping developmental transcription factor genes and imprinted loci, with a directional bias toward hypomethylation. CUT&RUN profiling of retained histone marks showed that H3K4me3 domains at a defined set of developmental gene promoters were preferentially depleted in DEHP-exposed sperm, while H3K27me3 domains were comparatively unaffected, indicating a mark-specific rather than global disruption of the retained nucleosome landscape. RNA-sequencing of two-cell and blastocyst-stage embryos sired by DEHP-exposed males, using unexposed oocytes, revealed differential expression of genes overlapping the sperm hypomethylated and H3K4me3-depleted promoter set, with effects most pronounced at the two-cell stage and substantially attenuated by the blastocyst stage, supporting a mechanistic route for paternal chemical exposure to influence offspring molecular phenotype through sperm chromatin rather than through sperm count or motility alone.

Keywords: sperm chromatin; protamine-histone exchange; endocrine-disrupting chemicals; DEHP; DNA methylation; paternal epigenetic inheritance

Received: December 30, 2025 | Accepted: March 24, 2026 | Published: June 25, 2026

Citation: Gallagher H, Havlicek L, Carvalho P. Environmental Toxin Exposure and Its Effects on Sperm Chromatin Epigenetic Marks. *Chromatin*. 2026;4(1):1–6. DOI: [10.5281/zenodo.22756536](https://doi.org/10.5281/zenodo.22756536)

1. INTRODUCTION

During the final stages of mammalian spermiogenesis, the majority of nucleosomal histones are removed and replaced by small, highly basic protamines that compact the paternal genome into a toroidal, transcriptionally silent structure optimised for protection and delivery rather than gene expression [1,2]. This exchange is not complete: in mouse and human sperm alike, 1-15% of the genome retains a nucleosomal organisation enriched at developmental gene promoters, imprinted loci and microRNA clusters, and this retained fraction carries active and repressive histone modifications broadly resembling those found in early embryonic chromatin, leading to the proposal that it constitutes a form of paternal epigenetic information transmitted to the next generation independently of DNA sequence [3,4].

A growing body of epidemiological and rodent evidence links paternal exposure to endocrine-disrupting chemicals, including the widely used plasticiser di(2-ethylhexyl) phthalate (DEHP), to altered offspring metabolic and reproductive phenotypes, and several studies have reported DEHP-associated changes in sperm DNA methylation at specific loci [5,6]. However, most prior work has profiled either DNA methylation or histone retention in isolation, and typically at exposure doses substantially above those encountered in general population exposure, leaving open whether an environmentally relevant DEHP dose perturbs the retained histone landscape in addition to DNA methylation, and whether these two layers of sperm chromatin information are affected at overlapping or largely distinct sets of loci.

A second open question concerns the developmental consequence, if any, of DEHP-associated sperm chromatin changes for early embryonic gene expression. Because retained sperm histone marks are thought to be substantially reprogrammed during the first embryonic cell cycles, any transcriptional consequence of a paternal chromatin perturbation might be expected to be most detectable very early in development and to attenuate as embryonic chromatin is progressively re-established, a temporal prediction that has rarely been tested directly by comparing gene expression at multiple pre-implantation stages sired by the same exposed founder males [7,8].

Here we address both questions using an environmentally relevant, chronic DEHP exposure protocol in adult male mice spanning one full spermatogenic cycle, combined with sperm chromatin structure assay, whole-genome bisulfite sequencing, CUT&RUN for retained H3K4me3 and H3K27me3, and RNA-sequencing of two-cell and blastocyst-stage embryos sired by exposed males. We report that DEHP exposure increases protamine deficiency and DNA fragmentation in sperm without affecting sperm count or motility, produces a predominantly hypomethylating shift at developmental gene promoters and imprinted loci,

preferentially depletes retained H3K4me3 rather than H3K27me3 at an overlapping set of loci, and is associated with transient, developmentally attenuating changes in two-cell and blastocyst embryo gene expression.

2. MATERIALS AND METHODS

Experiments used adult male C57BL/6J mice orally dosed once daily with DEHP at 20 mg/kg body weight, an exposure level within the range estimated for general human population intake, or vehicle (corn oil) for 35 consecutive days to span one full spermatogenic cycle, with sperm and embryo collection performed after the final dose.

2.1 Animal exposure and sperm collection

Adult male C57BL/6J mice (8-10 weeks old at exposure start) were orally gavaged daily with 20 mg/kg DEHP in corn oil vehicle or vehicle alone for 35 days (n=10 per group), a duration chosen to allow exposed spermatogonia to complete spermatogenesis and epididymal maturation. Caudal epididymal sperm were collected by swim-out into pre-warmed media 24 hours after the final dose, with a portion used immediately for motility and concentration assessment by computer-assisted sperm analysis and the remainder cryopreserved for downstream molecular assays.

2.2 Sperm chromatin structure assay and protamine deficiency staining

Sperm DNA fragmentation index was assessed by sperm chromatin structure assay (SCSA) using acridine orange staining and flow cytometry, quantifying the proportion of sperm with denatured (red-shifted) versus native (green) DNA fluorescence. Protamine deficiency was assessed in parallel by chromomycin A3 (CMA3) staining, which competes with protamine for DNA binding and fluoresces more brightly in protamine-deficient sperm, and by aniline blue staining of residual histone-bound lysine residues, each scored on at least 200 sperm per sample by blinded microscopy.

2.3 Whole-genome bisulfite sequencing

Genomic DNA was extracted from purified caudal sperm following somatic cell lysis and sonication-resistant sperm chromatin decondensation, bisulfite-converted, and sequenced as paired-end 150 bp libraries on an Illumina NovaSeq 6000 to a minimum depth of 20x genome-wide coverage per sample. Differentially methylated regions between DEHP-exposed and vehicle sperm were called using a sliding-window approach requiring a minimum 10% methylation difference and Benjamini-Hochberg-adjusted $P < 0.05$, annotated against CpG islands, gene promoters and a curated imprinted-locus reference set.

2.4 CUT&RUN for retained sperm histone marks

Retained sperm chromatin marks were profiled by CUT&RUN for H3K4me3 and H3K27me3 following sperm membrane permeabilisation with a combination of

disulfide-reducing agent and detergent sufficient to expose sperm chromatin without disrupting the toroidal protamine structure, using validated antibodies and protein A-MNase, with libraries sequenced as paired-end 50 bp reads on a NovaSeq 6000 to a minimum depth of 20 million uniquely mapped read pairs per sample; peaks were called against IgG controls and compared between DEHP-exposed and vehicle sperm using a paired differential-peak framework.

2.5 Embryo collection and RNA-sequencing

Unexposed C57BL/6J females were superovulated and mated with DEHP-exposed or vehicle-treated males; two-cell embryos were collected at 24 hours and blastocysts at 96 hours post-coitum, individually processed, and profiled by low-input RNA-sequencing (Smart-seq2 protocol) with a minimum of 8 embryos per group per stage. Differential expression between DEHP-sired and vehicle-sired embryos at each stage was assessed with DESeq2 using the Wald test and Benjamini-Hochberg correction.

2.6 Statistical analysis

Sperm-level phenotypic and molecular comparisons between DEHP-exposed and vehicle groups were assessed by two-tailed Student's t-test or Mann-Whitney U test as appropriate for the data distribution; genome-wide methylation, CUT&RUN and RNA-sequencing comparisons used the statistical frameworks noted above with multiple-testing correction. Overlap between differentially methylated regions, differential CUT&RUN peaks and differentially expressed embryonic genes was assessed by hypergeometric test against a matched randomised background. All analyses were performed in R version 4.3.

3. RESULTS

Chronic DEHP exposure did not significantly alter sperm concentration or computer-assisted motility parameters relative to vehicle-treated controls, but produced a 2.4-fold increase in the chromomycin A3-positive, protamine-deficient sperm fraction and a corresponding increase in aniline blue-positive, histone-retentive sperm, together with a significantly elevated SCSA DNA fragmentation index. Table 1 summarises sperm concentration, motility and chromatin integrity parameters for DEHP-exposed and vehicle-treated males.

Table 1. Sperm concentration, motility and chromatin integrity parameters in DEHP-exposed and vehicle-treated males.

Parameter	Vehicle (mean ± SD)	DEHP 20 mg/kg (mean ± SD)	P value
Sperm concentration (x10 ⁶ /mL)	42.6 ± 6.1	40.9 ± 7.4	0.58
Progressive motility (%)	61.3 ± 5.8	58.7 ± 6.9	0.34
CMA3-positive sperm (%)	8.2 ± 2.1	19.6 ± 3.8	<0.001

Aniline blue-positive sperm (%)	9.5 ± 2.4	21.1 ± 4.2	<0.001
SCSA DNA fragmentation index (%)	11.4 ± 2.8	24.3 ± 4.9	<0.001

Values are mean ± SD across n=10 males per group; P values from two-tailed Student's t-test.

Whole-genome bisulfite sequencing identified 1,362 differentially methylated regions in DEHP-exposed sperm relative to vehicle, with a pronounced bias toward hypomethylation (74% of differentially methylated regions lost methylation) and significant enrichment at CpG islands overlapping developmental transcription factor promoters and at a subset of paternally and maternally imprinted loci. CUT&RUN profiling of retained histone marks showed that H3K4me3 domains were significantly depleted at 218 developmental gene promoters in DEHP-exposed sperm, whereas H3K27me3 domain occupancy showed no significant genome-wide change, indicating that DEHP preferentially disrupts the active retained mark rather than causing a general loss of nucleosome retention (Figure 1). Differentially methylated regions and H3K4me3-depleted promoters showed significant overlap beyond that expected by chance, with 89 promoters showing both hypomethylation and H3K4me3 loss.

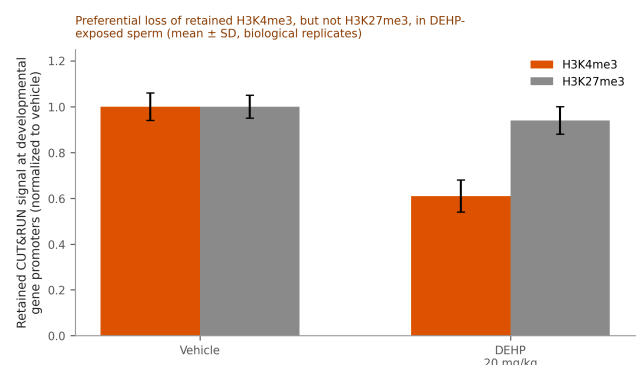


Figure 1. Genome-wide retained H3K4me3 and H3K27me3 CUT&RUN signal at developmental gene promoters (mean ± SD of biological replicates, normalized to vehicle-treated control) in DEHP-exposed versus vehicle-treated mouse sperm, illustrating preferential depletion of H3K4me3 relative to H3K27me3.

RNA-sequencing of two-cell embryos sired by DEHP-exposed males identified 156 differentially expressed genes relative to vehicle-sired embryos, significantly enriched for genes whose promoters overlapped the sperm hypomethylated and H3K4me3-depleted set identified above. By the blastocyst stage, the number of differentially expressed genes fell to 34, with substantially reduced overlap with the original sperm chromatin change set, indicating progressive attenuation of the paternal chromatin signature as embryonic development proceeds. Table 2 reports differentially expressed gene counts and overlap with sperm chromatin changes at each embryonic stage.

Table 2. Differentially expressed genes in embryos sired by DEHP-exposed males, by developmental stage.

Embryo stage	DE genes (adj. P<0.05)	Overlap with sperm DMRs (n, P value)	Overlap with sperm H3K4me3 loss (n, P value)
Two-cell (24 hpc)	156	41 (P<0.001)	38 (P<0.001)
Blastocyst (96 hpc)	34	6 (P=0.04)	5 (P=0.06)

Among the genes affected at the two-cell stage, several encode developmental transcription factors and chromatin regulators with known roles in lineage specification, and their expression changes were directionally consistent with the sperm chromatin change at the corresponding promoter (hypomethylated, H3K4me3-depleted promoters associated predominantly with reduced two-cell expression). No systematic difference in blastocyst morphology, cell number or lineage marker expression was detected between DEHP-sired and vehicle-sired embryos, consistent with the transcriptional effect being transient rather than persisting into a morphologically detectable phenotype at this early stage.

4. DISCUSSION

These results show that an environmentally relevant, chronic DEHP exposure perturbs sperm chromatin at two distinct but overlapping molecular layers, DNA methylation and retained histone marks, converging on a shared set of developmental gene promoters and imprinted loci, and that this perturbation is associated with detectable but transient changes in early embryonic gene expression. The preferential loss of H3K4me3 rather than H3K27me3 at retained promoter nucleosomes suggests that DEHP does not simply destabilise sperm chromatin retention wholesale, but instead disproportionately affects the active mark, a distinction not resolved by prior studies that profiled DNA methylation alone [5,6].

The substantial attenuation of differential gene expression between the two-cell and blastocyst stages is consistent with the extensive reprogramming of paternal chromatin known to occur during early preimplantation development, and supports a model in which paternally inherited chromatin perturbations exert their clearest transcriptional influence during the earliest cleavage divisions, before maternal and zygotic reprogramming machinery has fully re-established embryonic chromatin state [7,8]. This temporal pattern may help explain why some studies searching for persistent DEHP-associated phenotypes in later development or adulthood have reported comparatively subtle effects, if the primary molecular consequence is concentrated in a narrow early developmental window.

The significant overlap between DEHP-associated sperm hypomethylation and H3K4me3 loss at a shared promoter set raises the question of mechanistic directionality: whether DEHP or its metabolites directly destabilise one mark and secondarily affect the other, given known crosstalk between CpG methylation state and H3K4 methylation machinery at unmethylated CpG

islands, or whether both marks are independently sensitive to a shared upstream disruption of chromatin remodelling during the histone-to-protamine transition. Our data cannot distinguish between these possibilities and would require a time-resolved profiling design across spermatogenesis to resolve.

Several limitations should be considered. First, our exposure protocol used a single DEHP dose and cannot establish a dose-response relationship for sperm chromatin effects; testing a range of doses spanning below and above the level used here would clarify whether a threshold exists. Second, embryo profiling used unexposed oocytes, isolating paternal contributions but leaving open how maternal chromatin state might modulate the phenotypic consequence of a paternal chromatin perturbation in a natural mating context. Third, our study examined only two early embryonic stages; a more extensive developmental time course, extending into fetal and postnatal life, would be needed to determine whether the transient two-cell transcriptional signature has any downstream phenotypic consequence.

5. CONCLUSION

Chronic exposure of adult male mice to an environmentally relevant dose of the plasticiser DEHP increases sperm protamine deficiency and DNA fragmentation, produces a predominantly hypomethylating shift in DNA methylation at developmental and imprinted loci, and preferentially depletes retained H3K4me3, but not H3K27me3, at an overlapping set of gene promoters. These sperm chromatin changes are associated with transient differential gene expression in two-cell embryos that substantially attenuates by the blastocyst stage. Together these findings support a mechanistic route by which paternal chemical exposure can influence early embryonic gene expression through coordinated disruption of DNA methylation and retained histone marks in sperm, independent of any effect on sperm count or motility, and motivate further work to establish dose-response relationships and longer-term developmental consequences.

Acknowledgements

The authors thank the animal facility staff at the Baltic Institute of Chromatin and Gene Regulation for assistance with the exposure protocol, and the genomics core facility at Northmoor School of Molecular Biology and Genetics for sequencing support. We also thank colleagues at the Vistula Institute of Molecular Genetics for helpful discussion of embryo RNA-sequencing methodology.

Funding

This work was supported by internal research funds from the Baltic Institute of Chromatin and Gene Regulation, Northmoor School of Molecular Biology and Genetics, and the Vistula Institute of Molecular Genetics. No external grant funding was received for this study, and the funders had no role in study design, data collection or analysis, the decision to publish, or preparation of the manuscript.

Conflict of Interest

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

Author Contributions

H.G. conceived and supervised the study, designed the DEHP exposure protocol, and analysed the bisulfite sequencing and CUT&RUN data. L.H. performed sperm chromatin structure assays and protamine deficiency staining. P.C. performed embryo collection and RNA-sequencing library preparation. All authors contributed to writing and approved the final manuscript.

Ethics Statement

All animal procedures were approved by the institutional animal care and use committee of the Baltic Institute of Chromatin and Gene Regulation and performed in accordance with relevant national and institutional guidelines for the care and use of laboratory animals. This study did not involve human participants or human data.

Data Availability

Raw and processed whole-genome bisulfite sequencing, CUT&RUN and RNA-sequencing data generated for this study have been deposited in the Gene Expression Omnibus under a series accession available from the corresponding author on reasonable request. Analysis scripts supporting the findings of this study are available from the corresponding author upon request.

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