A Father's Long-Lasting Legacy : The multigenerational burden of in utero exposure to Arctic pollutants on the sperm epigenome and folic acid supplementation as a shield across generations

Thèse

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Résumé

Les polluants organiques persistants (POPs) sont très préoccupants dans les écosystèmes arctiques car ils sont résistants à la dégradation, semi-volatils et lipophiles. En raison des courants océaniques et atmosphériques naturels, l'Arctique est constamment contaminé par des POPs. Une fois dans l'Arctique, les POPs affectent non seulement la faune (par exemple les ours polaires), mais aussi la population autochtone, en particulier en raison de leur dépendance à leur régime alimentaire traditionnel. De multiples disparités de santé sont liées aux POPs, il n'est donc pas surprenant qu'il existe de grandes différences de santé entre les Canadiens autochtones et non autochtones. À l'échelle moléculaire, les POPs ont été associés à des changements épigénétiques suite à une exposition *in utero* et adulte. L'hérédité épigénétique paternelle a été décrite dans des cohortes humaines et des modèles animaux allant du ver à la souris. L'exposition aux contaminants environnementaux au cours des moments clés du développement a été corrélée avec des changements physiologiques chez la progéniture future qui s'étendent souvent sur plusieurs générations. Malgré ces observations, les mécanismes moléculaires sous-jacents de l'hérédité non génétique (épigénétique) restent inconnus.

Ici, nous émettons l'hypothèse que l'exposition *in utero* à un mélange de POPs représentatif de l'environnement induit des épimutations dans la lignée germinale paternelle qui sont ensuite transmises à travers la lignée paternelle compromettant ainsi le développement embryonnaire précoce sur plusieurs générations (non) exposées. Nous émettons en outre l'hypothèse qu'une intervention nutritionnelle, utilisant une supplémentation en acide folique alimentaire (vitamine B9), atténue et / ou réduit les épimutations spermatiques induites par les POPs et les phénotypes embryonnaires précoces générationnels associés.

Pour tester ces hypothèses, les rates Sprague-Dawley fondatrices (F0) ont été gavées avec un mélange de POPs représentatif de l’environnement (500 µg de BPCs plus les autres POPs / kg de poids corporel) ou de l'huile de maïs et ont reçu un régime d'acide folique 1X ou 3X représentant l'apport des aliments enrichis ± prise d’une multivitamine. Les traitements ont été administrés cinq semaines avant la reproduction et jusqu'à la mise bas. Seules les femelles fondatrices F0 ont été directement exposées au mélange de POPs et / ou au régime d'acide folique 3X. Les descendants F1-F4 suivants ont reçu un régime d'acide folique 1X. À 90

jours, les mâles F1 ont été reproduits avec des femelles non traitées pour obtenir les portées F2. Similairement, les descendants des générations F3 et F4 ont été générés. Pour chaque génération, les spermatozoïdes des mâles (âgés de 150 jours) ont été prélevés et des embryons au stade deux cellules ont été collectés des femelles.

Cette thèse démontre que de multiples mécanismes épigénétiques spermatiques, à savoir les miARN, la méthylation de l'ADN et la marque d'histone H3K4me3, sont significativement affectés par l'exposition *in utero* aux POPs et sont partiellement sauvés par la supplémentation en acide folique de manière inter- (F1, F2) et parfois transgénérationnelle (F3, F4). Bien que la lignée paternelle F3 n'ait pas montré de paramètres spermatiques altérés, elle a produit les pires résultats de grossesse par rapport à toutes les autres générations. Ceci est particulièrement intéressant car l'expression des gènes des embryons à deux cellules F4 a révélé le plus grand nombre de gènes exprimés différentiellement en raison de tous les traitements. Ces altérations précoces du développement pourraient être le fondement de résultats phénotypiques défavorables ultérieurs.

En plus de ce qui précède, dans un chapitre complémentaire, nous démontrons que l'exposition aux POPs environnementaux est associée à l'expression du gène du tissu adipeux de la mère ours polaire sauvage et de ses oursons. Ceci peut être lié à un dysfonctionnement métabolique, mettant ainsi en évidence les différences physiologiques de réponse entre les adultes et leurs petits.

Les résultats de cette thèse servent de preuves pour des concepts essentiels qui ont des implications majeures pour les populations humaines et fauniques.

// Abstract

Paternal epigenetic inheritance has been described in human cohorts and animal models ranging from worm to mouse. Exposure to environmental contaminants during key developmental time points has been correlated with physiological changes in future offspring that oftentimes last over multiple generations. Despite these observations, the underlying molecular mechanisms of nongenetic (epigenetic) inheritance remain unknown. Persistent organic pollutants (POPs) are of great concern in Arctic ecosystems as they are resistant to degradation, semi-volatile and lipophilic. Due to naturally-occurring ocean and atmospheric currents, the Arctic is persistently contaminated with POPs. Once in the Arctic, POPs affect wildlife (e.g. polar bears) and also the Indigenous human population particularly through their reliance on the traditional diet. Multiple adverse health outcomes are related to POPs, and an increasing body of evidence showed big health discrepancies between Indigenous and non-Indigenous Canadians. On a molecular scale, POPs cause epigenetic changes following *in utero* and adult exposure.

Here we hypothesize that *in utero* exposure to an environmentally-relevant Arctic POPs mixture induces epimutations in the paternal germline that are subsequently transmitted through the paternal lineage thereby compromising early-embryonic development in multiple (un)exposed generations. We further hypothesize that a nutritional intervention, using dietary folic acid (Vitamin B9) supplementation, mitigates and/or reduces the POPs induced sperm epimutations and associated generational early-embryonic phenotypes. Folic acid's potential protective role against environmental pollutants, such as BPA, DDT and air pollutants, has been demonstrated multiple times. Folic acid functions as a methyl donor in the methyl cycle, a cycle that is of high importance during prenatal development as rapid cell division and epigenetic reprogramming occur.

To test these hypotheses, founder Sprague-Dawley rat dams (F0) were gavaged with an environmentally-relevant POPs mixture (500 µg PCBs plus remaining POPs/kg body weight) or corn oil and were fed a 1X or 3X folic acid diet representing intake from fortified foods ± additional supplementation. Treatments were administered five weeks before reproduction and until parturition. Only F0 founder females were directly exposed to the POPs mixture

and/or 3X folic acid diet. Subsequent F1-F4 offspring received a 1X folic acid diet. At 90 days of age, F1 males were bred with untreated females to obtain F2 offspring. Likewise, F3 and F4 generation offspring were generated. For each generation, sperm were collected from males (150 days of age) and two cell embryos from females.

This thesis demonstrates that multiple epigenetic mechanisms in sperm, specifically non-coding micro RNAs (miRNAs), DNA methylation and histone mark H3K4me3, are significantly affected by *in utero* POPs exposure and are partially restored by folic acid supplementation in an inter- (F1,F2), sometimes, transgenerational (F3, F4) manner. Although the F3 paternal lineage did not show altered sperm parameters, it did display the worst pregnancy outcomes compared to all other generations. This is particularly interesting as F4 two-cell embryo gene expression revealed the highest number of differentially expressed genes due to all treatments. These early developmental alterations could be the foundation for later adverse phenotypic outcomes.

In a complementary chapter, we hypothesized that exposure to POPs differentially alters genome-wide gene transcription in the adipose tissue from mother polar bears and their cubs, highlighting molecular differences in response between adults and young. Results confirm that environmental POPs exposure is associated to wild polar bear mother and cub adipose tissue gene expression, which may be linked to metabolic dysfunction; thereby highlighting physiological differences in response between adults and their young.

The outcomes of this thesis serve as essential proof-of concept that has major implications for human and wildlife populations.

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List of abbreviations

5,10-Methylene THF	5,10-Methylenetetrahydrofolate
5caC	5-Carboxylcytosine
5fC	5-Formylcytosine
5hmC	5-Hydroxymethylcytosine
5hmU	5-Hydroxymethyluracil
5mC	5-Methyl cytosine
5-methylTHF	5-Methyltetrahydrofolate
AID	Activity-induced cytidine deaminase/apolipoprotein B mRNA editing complex
ALH	Mean amplitude of lateral head displacement
AP	Apyrimidinic
BCF	Frequency of head displacement
BER	Base excision repair
BFrs	Brominated flame retardants
BPA	Bisphenol A
b-TSH	Blood thyroid-stimulating hormone
BW	Body weight
C	Cytosine
CASA	Computer-assisted sperm analysis
CGIs	CpG islands
ChIP-Seq	Chromatin Immunoprecipitation Sequencing
CTCF	CCCTC-binding factor
CTRL	Control group
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DE	Differentially expressed
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DMRs	Differentially methylated regions
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
DOHaD	Developmental Origins of Health and Disease
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
EXP-5	Exportin-5
FA	Folic Acid
FCM	Flow cytometric

FDR	False discovery rate
FSH	Follicle stimulating hormone
G	Guanine
GD	Gestational day
GnRH	Gonadotropin-releasing hormone
GO	Gene-ontology analysis
H	Histidines
H3K4	Lysine 4 histone H3
H3K9	Lysine 9 histone H3
HAT	Histone acetyltransferase
HCH	α/β/γ-Hexachlorobenzene
HDAC	Histone deacetylase
HDL	High-density lipoprotein
IAP	Intracisternal A particle
ICRs	Imprinting control regions
ICSI	Intracytoplasmic Sperm Injection
Igf2	Insulin-like growth factor 2
IVF	*in vitro* fertilisation
K	Lysines
LH	Luteinizing hormone
LIN	Linearity coefficient
LINE-1	Long interspersed nucleotide elements
MARs	Matrix attachment regions
me	Methylation
miRNAs	Micro RNAs
Msp1	Methylation-insensitive restriction enzyme
MTHFR	Methylenetetrahydrofolate reductase
NTDs	Neural tube defects
OCPs	Organochlorine pesticides
PARP1	Poly(-ADP-ribose) polymerase 1
PBDE	Polybrominated diphenyl ethers
PCA	Principal component analysis
PCBs	Polychlorinated biphenyls
PFCAs	Perfluoroalkyl carboxylates
PGCs	P*rimordial germ cells*
piRNAs	Piwi-interacting RNAs
PND	Postnatal day
POHaD	Paternal Origins of Health and Disease
POPs	Persistent Organic Pollutants
PPARG	Proliferator-activated receptor gamma
pre-miRNA	Precursor miRNA

pri-miRNA	Primary miRNA
PRMT5	Protein arginine methyltransferase 5
PTMs	Post-translational modifications
Q	Quartile
R	Arginines
RBC	Red blood cell
RISC	RNA induced silencing complex
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RRBS	Reduced representation bisulfite sequencing
SAM	S-Adenosyl methionine
SAM	S-adenyl methionine
SCSA	Sperm chromatin structure assay approach
SHMT	Serine hydroxymethyltransferase
sncRNAs	Small non-coding RNAs
sRNAs	Small non-protein-coding regulatory RNAs
SSCs	Spermatogonial stem cells
STR	Straightness coefficient
TDG	DNA glycosylase
TET	Ten-eleven translocation
THF	Tetrahydrofolate
Thy	Thymine
TPs	Transition proteins
tsRNAs	tRNA-derived small RNAs
TUNEL	Terminal deoxunucleotidyl transferase-driven dUTP Nick End Labelling
UNECE	United Nations Economic Commission for Europe
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Linear velocity
WGBS	Whole genome bisulfite sequencing
WHO	World Health Organization

To our future generations

Acknowledgements

Thank you, Janice, for being such a phenomenal director, for supporting my development and challenging me throughout my graduate career. You always gave me the opportunity to explore "outside the box" scientific avenues including science policy/communication and wildlife biology. To set up an additional passion project involving polar bears and visiting Toronto Zoo to present the results were definitely some of the main highlights. I am grateful for your consistent effort to provide advice, resources, and great scientific connections that helped make this fantastic project happen. You are an inspiring director and I hope we will keep in touch in the future. Also, please feel free to visit Vienna, at any time!

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Caroline and Georges, my beauparents, and Alexis my rock and my home. I could not have done this without you and your belief in me.

Foreword

The present thesis is part of a larger research study called "Molecular foundations of intergenerational transmission of the paternal environment" funded by the Canadian Institute of Health Research (CIHR) which involves a multidisciplinary collaboration between various teams and knowledge users. The research proposal was originally designed by Janice L. Bailey (Laval University), Sarah Kimmins (McGill University), Jacquetta Trasler (McGill University Health Centre), Amanda MacFarlane (Health Canada) and Hélène Jacques (Laval University).

Below, the contribution of my co-authors and I are discussed in further detail. In addition, Chapters 1 and 3 have been successfully published, whereas Chapters 2 and 4 will soon be ready for submission.

Chapter 1 (published):

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Throughout all three studies (Chapter 1-3), I worked closely alongside three team members including, Maryse Lessard, Phanie L. Charest and Pauline Navarro to establish and maintain the experimental animal model. As a team we all played vital roles during the organization and execution of organ, semen and two-cell embryo collection days. I participated in setting up the final study design, performed data acquisition, analysis, interpretation, validation and drafted the complete manuscript. Our research professional, Mathieu Dalvai, managed the experimental animal model and supported me in my laboratory experiments. Bioinformatic pipeline analyses of the miRNA sequencing data were performed by Charles Joly-Beauparlant under supervision of Arnaud Droit. Marie-Odilde Benoit-Biancamano from Montreal University, supported us throughout necropsy and organ collection during the early stages of the project. Janice Bailey thoroughly revised the final manuscript after which our co-authors made revisions as well.

Chapter 2 (manuscript in preparation for publication):

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Besides animal colony maintenance and sample collection in collaboration with Maryse Lessard, Phanie L. Charest and Pauline Navarro, I received training to acquire ChIP-Seq and RRBS techniques in respectively Sarah Kimmins and Serge McGraw labs. After, I successfully set up both techniques in our own lab at Laval University. I was responsible for setting up the final study design, experimental optimization, performed data acquisition, analysis, interpretation, and drafted the complete manuscript. Mathieu Dalvai provided support in the preparation of the experimental set-up. Bioinformatic pipeline and data analyses of the RRBS data were performed by Charles Joly-Beauparlant under supervision of Arnaud Droit. ChIP-seq bioinformatic analyses were carried out by Ariane Lismer under supervision of Sarah Kimmins. Janice Bailey and Mathieu Dalvai afforded guidance in final data interpretation.

Chapter 3 (published):

Lessard, M.[1*], Herst, P.M.[1*], Charest, P.L.[1], Navarro, P.[2], Joly-Beauparlant, C.[3], Droit, A.[3], Kimmins, S.[4], Trasler, J.M.[5], Benoit-Biancamano, M.O.[6], MacFarlane, A.J.[7], Dalvai, M.[1] and Bailey, J.L.[1] **(2019)** Prenatal Exposure to Environmentally-Relevant Contaminants Perturbs Male Reproductive Parameters Across Multiple Generations that are Partially Protected by Folic Acid Supplementation. *Scientific Reports*, 9(1), 13829. Submitted the 7th of June 2019. Accepted the 23rd of August 2019.

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This paper is a result of a close collaboration between me and Maryse Lessard during which we combined her phenotypic results with my two-cell embryo transcriptome data as they complemented each other very well. As for the two-cell embryo project, I participated in the

embryo collection, set up the final study design, performed all experimental procedures, final analysis, interpretation and drafted part of the manuscript. Under supervision of Arnaud Droit, Charles Joly-Beauparlant performed the bioinformatic pipeline analyses on the two-cell RNA sequencing data. Mathieu Dalvai supported during the creation of the final study design. Janice Bailey thoroughly revised the final manuscript after which our co-authors made revisions as well.

Chapter 4 (in review with Environmental Science and Technology):

Herst, P.M.[1], Routti, H.[3], Joly-Beauparlant, C.[2], Bodein, A.[2], Aars, J.[3], Droit, A.[2], Dalvai, M.[1], Gagné, D.[4] and Bailey, J.L.[1] **(2020)** Exposure to persistent organic pollutants is related to adipose tissue gene expression in mother polar bears and their cubs from Svalbard, Norway.

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In contrast to the other three studies, this distinct but pertinent study was carried out in collaboration with Heli Routti from the Norwegian Polar Institute in Tromsø, Norway. Polar bear samples were collected by Heli Routti and Jon Aars in the Spring of 2011, 2012 and 2013. With the support of Janice Bailey, Mathieu Dalvai and Heli Routti, I took part in the construction of the study design and importation of the samples. I conducted the experimental procedures, final analyses, interpretation and drafted the complete manuscript. Charles Joly-Beauparlant together with Antoin Bodein performed the bioinformatic pipeline analyses under supervision of Arnaud Droit. Dominic Gagné provided support during the validation experiments. Heli Routti and Janice Bailey revised the manuscript, followed by the other co-authors.

Introduction

1. Environmental Pollution

Being the biggest environmental cause of disease and premature death worldwide, pollution is a serious and increasing threat to global health. According to the World Health Organization (WHO), almost 8.9 million people die annually due to pollution-related diseases, of which 8.4 million (94%) occur in underdeveloped/poor countries (Suk *et al.*, 2016). This is particularly shocking, knowing that the total number of deaths caused by AIDS, tuberculosis and malaria combined is three times less (Suk *et al.*, 2016). Also, these numbers will only rise as pollution is becoming worse in many parts of the world (Landrigan *et al.*, 2015; Landrigan *et al.*, 2018).

1.1 The Industrial Revolution

The industrial revolution transformed our agrarian society and economy to one dominated by industry and machine manufacturing. Technological advancement and the use of new energy sources, led to the improvement of food production, infrastructure, conservation, use of resources and forms of transportation. The industrial revolution was the foundation for today's arguable wealthier and better living – as it still has an enormous negative impact on the environment.

Pollution caused by industrial emissions, and exhaust has immensely increased in the past 500 years especially in low- and middle-income countries. An estimate of 140,000 new pollutants have been created and widely dispersed in the environment since 1950 (Landrigan *et al.*, 2011). Examples of such pollutants include mercury, dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs) and chlorofluorocarbons, many of which are still present in the environment due to their long half-life and capacity to bioaccumulate. Far too few, especially contemporary classes of chemicals, have undergone **pre-market safety and toxicity evaluation,** causing a gap in information about the possible contribution to the global burden of disease (Landrigan *et al.*, 2018).

Today's pollution is a substantial problem, that not only endangers the health of billions of people, but also the Earth's ecosystem by worsening climate change, biodiversity loss, ocean acidification, desertification, and loss of the water supply (Landrigan *et al.*, 2018). Within the present study, we will focus on persistent organic pollutants.

1.2 Persistent Organic Pollutants (POPs)

POPs are anthropogenic organochlorine compounds that persist in the environment as they withstand photolytic, biological and chemical degradation, remaining intact for years. From a chemical structural perspective, POPs contain chlorine (Cl-) atoms bound covalently to a carbon structure (Figure 1) (Shukla *et al.*, 2014). These synthetic compounds were used in industrial processes, agricultural applications and arose as unintentional by-products from industrial processes.

1.2.1 *Several members of the POPs category*

Possibly the most well-known POPs member is *polychlorinated biphenyl (PCB)* (Figure 1). PCBs are a broad group of compounds that include 209 aromatic chlorinated hydrocarbons (Hopf *et al.*, 2014). They were extensively used as industrial lubricants and coolant fluids, particularly inside parts of consumer electronics, due to their high stability and resistance to oxidation (Silberhorn *et al.*, 1990). PCBs were first manufactured commercially in the 1920s and subsequently sold under the name "Arochlor".

Dichlorodiphenyltrichloroethane (DDT) was originally synthesized by graduate student Orthmar Zeidler while generating novel substances as part of his dissertation research at the University of Strasbourg in 1874 (Mulliken *et al.*, 2005). It was only until 1939 that DDT became widely used as synthetic insecticide – particularly during World War II to protect militants from mosquito-transmitted malaria but also typhus and other vector born diseases (Figure 1). After the war, DDT was used as an agricultural insecticide in the United States and other countries until the mid 1970s (Ritter *et al.*, 1995). Cotton was the largest agricultural use of DDT, accounting for more than 80% of USA's use. In the human liver, DDT is slowly metabolized into its metabolites including dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD) which are known to be more persistent than DDT (Ritter *et al.*, 1995).

Another insecticide, introduced around the end of World War II, is *chlordane*, which was used on a wide variety of agricultural crops including vegetables, maize, oilseeds, potatoes, sugarcane, sugar beets, fruits, nuts, cotton and jute (Figure 1). Chlordane is a mixture of chlorinated hydrocarbons including heptachlor, nonachlor and related compounds (Ritter *et al.*, 1995).

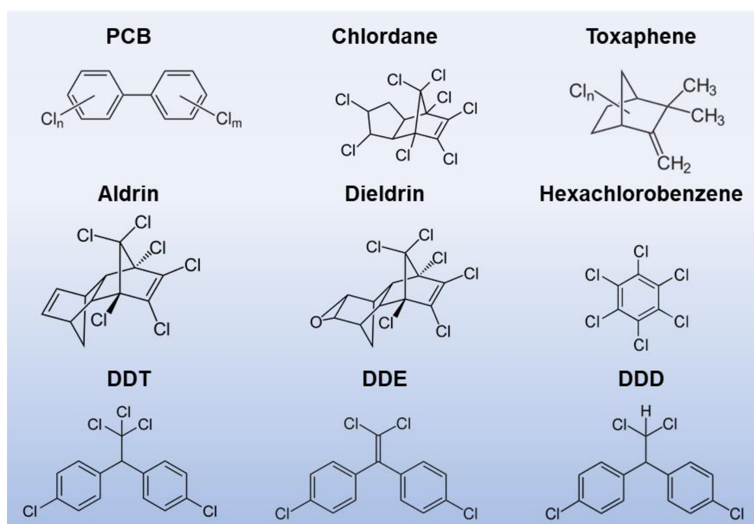

Figure 1. Chemical structures of multiple persistent organic pollutants. © 2019 by P. Herst

Toxaphene, an insecticide mixture of chlorinated camphenes, was primarily used on cotton, cereal grain fruits, nuts and vegetables but also on livestock for tick and mite control (Figure 1) (Ritter *et al.*, 1995). Along with the other insecticides, *α/β/γ-hexachlorobenzene (HCH),* a chlorinated hydrocarbon fungicide, was introduced in the late 1940s. Due to its anti-fungal properties, HCH was mainly utilized for seed treatment, especially on wheat. HCH was also a by product of the production of industrial chemicals, e.g. lindane (Ritter *et al.*, 1995).

Aldrin and *dieldrin* were widely used shortly after World War II (Figure 1). Aldrin was used as an insecticide in soil to protect crops and wood structures as it strongly binds to soil particles and resists leaching into groundwater (Ritter *et al.*, 1995). Aldrin is readily metabolized to dieldrin in both animals and plants, hence, little aldrin residues are detected in animals (Berntssen *et al.*, 2012).

1.2.2 *Distribution of POPs into the environment*

Once released, POPs partition into air, water, soil, snow/ice and other environmental media according to their physical-chemical properties (Ma *et al.*, 2011). Their semi-volatile character and high environmental half-lives result in long-range atmospheric transport and global planetary distribution, especially towards colder regions where they deposit (Figure 2). This phenomenon is also known as "grasshopping", which occurs through successive steps of volatilization and deposition (Gouin *et al.*, 2004) (Figure 2).

Due to their lipophilic character, POPs bioaccumulate in fatty tissues of living organisms, move up the food chain and biomagnify (El-Shahawi *et al.*, 2010; Riget *et al.*, 2019) (Figure 2). This results in POPs being omnipresent in food products especially those that contain animal fat like fatty fish, meat, and milk products. It therefore may come as no surprise, that chronic POPs exposure mostly occurs through dietary consumption of POPs-contaminated foods. Inhalation, *in utero* exposure via the mother and breast feeding, however, are also routes of exposure (Mitro *et al.*, 2015; Porpora *et al.*, 2013). Indigenous populations, living in the Arctic, are particularly exposed as they traditionally rely on "country foods" as a source of nutrition that contributes to their socio-economic wellbeing (Laird *et al.*, 2013).

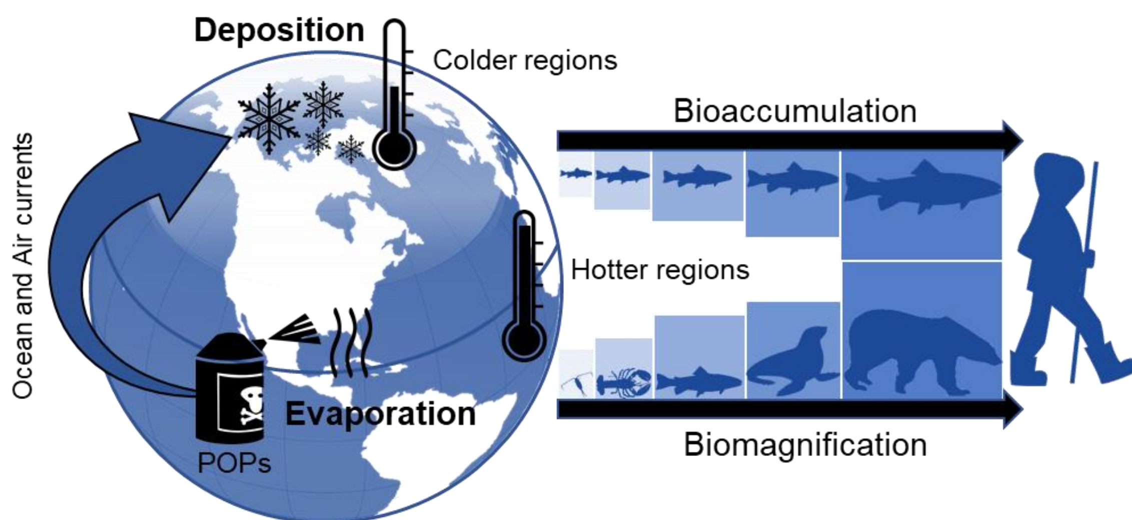

Figure 2. Global distribution of POPs via grasshopper effect followed by bioaccumulation and biomagnification. © 2019 by P. Herst.

Previous biomonitoring studies showed that indeed Inuit populations living in the Arctic face elevated exposures of POPs. Levels of PCBs in breast milk were up to five times higher than levels found in non-indigenous of Southern Quebec (Dewailly *et al.*, 1989). Another study, monitoring women from an Arctic community, demonstrated that the PCB blood levels exceeded Health Canada's 'tolerable' guideline for 39% in women of reproductive age (Kinloch *et al.*, 1992). Shortly after, the presence of multiple POPs in maternal and umbilical cord blood plasma of Inuit and non-indigenous women and their newborns from the Northwest Territories and Nunavut was confirmed (Walker, 2003).

Besides the Arctic, elevated levels of POPs have also been reported in other human populations worldwide, including the Great Lakes regions, Mexico, Northern Europe, throughout Africa and Asia .

1.2.3 *Increasing environmental POP concentrations due to warmer climate*

The physical-chemical properties of POPs vary directly or indirectly with temperature, wind speed, precipitation, and solar radiation. Consequently, climate change is likely to influence their environmental fate and behaviour by altering physical, chemical, and biological drivers of partitioning between the atmosphere, water, soil/sediment, and biota (Macdonald *et al.*, 2002; Noyes *et al.*, 2009). In fact, POPs, deposited in water and ice, are forecasted to revolatilize into the atmosphere leading to increased contaminant exposure (Alava *et al.*, 2018; Nizzetto *et al.*, 2010).

The Artic is particularly vulnerable, as it is warming more than twice the global average (Serreze *et al.*, 2011). Increased concentrations of POPs have also been observed in polar bears (*Ursus maritimus*) and sea birds due to changes in diet composition, and alterations in pollutant exposure pathways driven by climate change (Dietz, Desforges, Eulaers, *et al.*, 2018; Routti *et al.*, 2019).

1.2.4 *Health deficits linked to POPs exposure*

According to the World Health Organization, low levels of POPs can lead to increased cancer risk, endocrine disruption, neurobehavioral impairments, altered immune system, genotoxicity, reproductive disorders and birth defects (Organization, 2020). Although the

mechanisms have not been fully elucidated, associations between POPs and metabolic diseases, including diabetes type 2 and obesity have been previously made as well (Arisawa *et al.*, 2005; Magliano *et al.*, 2014). Some POPs can alter endocrine and reproductive systems by mimicking or antagonizing endogenous hormones and interfering with the synthesis, transport and metabolism of endogenous hormones (Mnif *et al.*, 2011). DDT-exposed men living in an endemic malaria region in South Africa, showed inverse associations between serum DDE levels and sperm quality including sperm chromatin integrity (Aneck-Hahn *et al.*, 2007; de Jager *et al.*, 2009). Studies of exposure to PCBs in adult men observed links between PCB and decreased sperm chromatin integrity as well as affected levels of free testosterone (Vested *et al.*, 2014). Fetuses, infants and children are particularly vulnerable to the disruptive effects of POPs exposure because of their rapid growth and organ development resulting in possible long-term effects. Low maternal plasma POP levels, during early pregnancy, were associated with reduced fetal growth in a recent cohort including women from multiple ethnic groups (Ouidir *et al.*, 2019). Exposure to POPs in pregnant Indigenous women is correlated to a shorter pregnancy duration, which is associated with reduced infant birth weight, length and head circumference (Lopez-Espinosa *et al.*, 2011). Perhaps most concerning, environmental contaminants have been associated with epigenetic changes following *in utero* and adult exposure, and these changes may be transmitted to subsequent generations through the germline, thereby increasing the risk of developing disease later on in life (Baccarelli *et al.*, 2009; Desaulniers *et al.*, 2009; Manikkam *et al.*, 2012; Nilsson *et al.*, 2018).

1.2.5 *The Stockholm Convention - regulating POPs worldwide*

To protect human health and the environment from the adverse affects of specific POPs, several countries put legal regulations into force against the utilisation of certain POPs in the 1970s. The first regional agreement, called the Aarhus Protocol on POPs, was adopted by the United Nations Economic Commission for Europe (UNECE) Convention on Long-Range Transboundary Air Pollution in 1998 and was entered into force in 2003 (Byrne, 2015; Teran *et al.*, 2012). A *global agreement* called The Stockholm Convention was implemented by the United Nations Environment Programme and adopted in 2001. It was put into force in 2004. The treaty requires parties to take measures to (1) eliminate the production and use, (2)

restrict the production and use, and/or (3) reduce the unintentional releases of the chemicals as described by the treaty.

The Stockholm convention initially targeted 12 POPs, referred to as the ‘dirty dozen,’ which include: aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzene, mirex, toxaphene, PCB, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans. As the Stockholm Convention is a “living document”, new chemicals have been added to the list over time and some are scheduled for either elimination, restriction or reduction of unintentional production release (Convention, 2019).

2.1.5.1 Exemptions to the Stockholm Convention, the DDT controversy

While DDT exposure in industrialized areas has dramatically dropped, it remains high in developing countries where it is used as malaria vector control. Malaria continues to be one of the most serious public health problems in lower and middle-income countries. In 2016, there were an estimated 216 million confirmed clinical cases worldwide, causing almost half a million deaths, which took mostly place in sub-Saharan Africa (WHO, 2017). To reduce overall and infant mortality, the World Health Organization permits indoor house spraying as it is effective, locally safe and affordable – alternatives are not yet available. Indoor house spraying might reduce ecologic effects, however, it does expose humans to high amounts of DDT that may cause adverse health and, in particular, reproductive outcomes (Beard *et al.*, 2006).

2. The epigenetic landscape of sperm

"*The branch of biology that studies the causal interactions between genes and their products which bring the phenotype into being*" – Conrad Waddington, embryologist and developmental biologist, United Kingdom, 1942.

The epigenome refers to the information that lies "above" the DNA sequence and regulates gene expression through mechanisms like (1) DNA methylation, (2) histone/chromatin modifications, and (3) coding and non-coding RNA. These epigenetic modifications are mitotically and/or meiotically heritable. In contrast to the genome, the epigenome is cell specific, dynamic, responsive to environmental stressors and its modifications occur without intrinsic changes in the primary DNA sequence. Persistent organic pollutants can have a profound effect on the sperm epigenome and have been associated with paternal epigenetic inheritance (Consales *et al.*, 2016; Manikkam *et al.*, 2012; Rusiecki *et al.*, 2008; Skinner *et al.*, 2018; Skinner, Manikkam*, et al.*, 2013; H. Wu *et al.*, 2015). To grasp this concept, we have to establish a thorough base of the sperm epigenome. Compared to somatic cells, the sperm epigenome is unique and highly specialized due to its structure, function, and mostly to its diverse requirements for successful fertilization.

2.1 Sperm chromatin structure

A mature spermatozoon consists of (1) a head, which contains a haploid nucleus and a sac of enzymes, the acrosome, that enables the sperm to penetrate the egg's outer coat, (2) a middle piece, and (3) a tail, the flagellum, which propels the sperm towards the egg (Figure 3) (Alberts et al., 2002). In contrast to somatic cells, sperm contain almost no cytoplasm, after spermiogenesis, and are left with only certain modified organelles, such as mitochondria located in the mid-piece, that are necessary for sperm function. The primary levels of sperm chromatin structure can be divided into three categories: (1) coiled DNA into "doughnut loops" or toroids by protamines, (2) 1-15% histone-bound chromatin, and (3) DNA attached to nuclear matrix at MARs (matrix attachment regions) at every 50 kb intervals throughout the genome (Figure 3) (Ward, 2010).

During the postmeiotic phase of spermatogenesis, the majority of histones is replaced by nuclear proteins called protamines, thereby establishing a highly condensed genomic structure. Protamines are small, arginine-rich proteins that bind DNA with high affinity and wrap it in a supercoiled structure (Balhorn, 2007). Interestingly, not all histones are replaced by protamines, as 1-2% of DNA remains bound to histones in murine spermatozoa (Balhorn *et al.*, 1977; Brykczynska *et al.*, 2010); and up to 15% in human spermatozoa (Gatewood *et al.*, 1990; Tanphaichitr *et al.*, 1978). This extensive repackaging not only reduces the nuclear volume but also protects the paternal genome when moving through the female reproductive tract towards the oocyte.

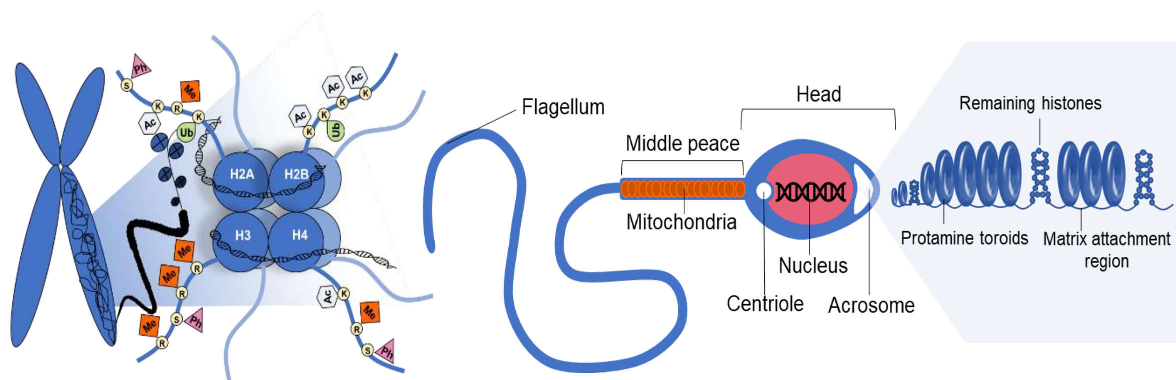

Figure 3. Comparison between the chromatin structure of somatic cells (left) and mature sperm (right). In somatic cells, DNA (black) is coiled roughly twice around an octamer of histones (blue) including H2A, H2B, H3 and H4. Histone tails protruding from the nucleosomal core can contain various post-translational modifications (PTMs). PTMs can alter the chromatin structure (e.g. opening, closing the structure, or recruiting histone modifiers) and thus play direct regulatory roles in gene expression. Well-known examples of PMTs are histone acetylation, phosphorylation, methylation and ubiquitination. Closed chromatin is referred to as heterochromatin and open chromatin as euchromatin. In sperm, most histones are replaced by protamines resulting in a highly compact chromatin structure. Adapted from Ward (2010). © 2019 by P. Herst

The entire sperm chromatin is organized into loop domains that are attached every 20-120 kb in length to the proteinaceous nuclear matrix at MARs (Figure 3) (Ward, 2010). Previous studies have demonstrated that the sperm nuclear matrix plays a role in DNA replication (Shaman *et al.*, 2007), DNA degradation (Shaman *et al.*, 2006) and transcription (Martins *et al.*, 2004). Furthermore, the sperm nuclear matrix appears to play a functional role during embryogenesis since structurally disrupted nuclear matrices do not support embryonic

development after intracytoplasmic sperm injection in contrast to those with intact matrices (Ward *et al.*, 1999). Sperm nuclear matrix instability has also been observed in infertile cryptochidic patients (Ankem *et al.*, 2002; Barone *et al.*, 2000).

2.2 DNA methylation

In sperm, DNA methylation plays an important role in various biological processes including silencing transposable elements, DNA compaction, paternal genomic imprinting, and X-chromosome inactivation (Larson *et al.*, 2016; D. Miller *et al.*, 2010; Stewart *et al.*, 2016; Zamudio *et al.*, 2015). Abnormal DNA methylation is associated with impaired male fertility, affected embryo quality and increased susceptibility to disorders in the offspring (Aston *et al.*, 2015; Jenkins *et al.*, 2014). Sperm DNA methylation alterations may also persist into the early embryo and influence the transcriptome and epigenome in somatic tissues (Ben Maamar *et al.*, 2019; Wei *et al.*, 2014).

Compared to somatic cells, DNA methylation in sperm in highly distinct and arises particularly from genome-wide reprogramming, during which DNA methylation is globally erased and re-established, in the developing embryo and primordial germ cells but also spermatogenesis (Carrell *et al.*, 2010; Eckhardt *et al.*, 2006). From a mechanistic perspective, DNA methylation involves the transfer of a methyl group ($-CH_3$) from S-adenyl methionine (SAM) to the C5 position of cytosine to form 5-methyl cytosine (5mC) (Figure 4). In mammalian genomes, DNA methylation marks are often found in regions where cytosine (C) is followed by guanine (G) nucleotide (5' prime to 3' prime) called CpG dinucleotides. The p indicates the phosphate group between C and G. Regions with a high frequency of clustered CpG dinucleotides are called CpG islands (CGIs). DNA hypomethylation (low levels of 5mC) is a gene activation mark, whereas DNA hypermethylation is a silencing mark as it interferes with the gene transcription machinery (Gannon *et al.*, 2014). Around 90% of all CpGs are methylated in sperm in contrast to oocytes with 40% (Erkek *et al.*, 2013; Kobayashi *et al.*, 2012). Overall DNA methylation is greatly reduced in sperm. As shown by a previous rodent study, 6.8% of CpG cites remained stable across sperm and embryonic developmental stages, of which the majority was hyper-methylated (L. Wang *et al.*, 2014). Highly conserved hypo-methylated regions were mainly observed around gene promotors (Y. Li *et al.*, 2018).

During embryonic development and spermatogenesis (until the pachytene phase of meiosis) d*e novo* DNA methylation is facilitated by DNA methyltransferases (DNMTs) DNMT3a and DNMT3b (Oakes *et al.*, 2007; Okano *et al.*, 1999). DNMT3l, a DNMT without catalytic activity, is especially active in gonocytes in fetal testes of mice starting 15.5-18.5 days post coitum (Bourc'his *et al.*, 2001; La Salle *et al.*, 2004). DNMT1 maintains the methylation pattern by copying it from the parental DNA strand onto the newly synthesized daughter strand during DNA replication. DNMT1 is highly expressed in primordial germ cells (PGCs), spermatogonia and throughout multiple stages of spermiogenesis up till the pachytene stage (Jue *et al.*, 1995).

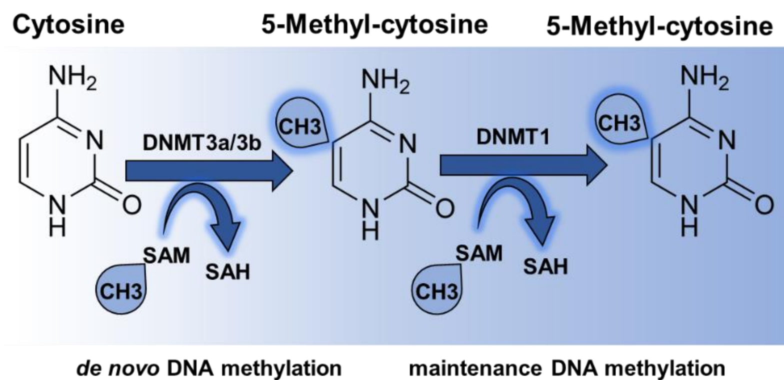

Figure 4. Schematic overview of DNA methylation. De novo DNA methylation is carried out by the DNA methyl transferase 3a/3b (DNMT3), opposed to the maintenance of DNA methylation that is performed by DNMT1. Adapted from (McGowan *et al.*, 2010). © 2019 by P. Herst.

Although DNA methylation is relatively stable, active and passive loss of DNA methylation have been observed previously. DNA demethylation is a crucial process that takes place during spermatogenesis, and genome-wide epigenetic reprogramming in embryonic development (Oakes *et al.*, 2007).

Active DNA demethylation requires enzymatic reactions to convert 5mC back to its naked cytosine form (Figure 5) (Mayer *et al.*, 2000; Oswald *et al.*, 2000). DNA methylation marks are removed through a series of oxidation and/or deamination reactions that result in a

product recognized by base excision repair (BER) pathway. Then, BER replaces the modified base with a naked cytosine (Moore *et al.*, 2013). More precisely, the oxidation pathway commences with the conversion of 5mC to 5-hydroxymethylcytosine (5hmC) by a family of dioxygenases including ten-eleven translocation (TET) enzymes. Subsequently, 5hmC is hydroxylated to generate 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) which are recognized by thymine DNA glycosylase (TDG). TDG removes the glycosidic bond resulting in an apyrimidinic (AP) site. The deamination pathway involves the deamination of 5hmC to 5-hydroxymethyluracil (5hmU) by 'activity-induced cytidine deaminase/apolipoprotein B mRNA editing complex' (AID) deaminase. 5hmU is then transformed to an AP site. 5mC can also directly be converted to thymine (Thy) causing a T:G mismatch. Resulting AP sites and T:G mismatches can be repaired by the base excision repair (BER) mechanisms leading to the incorporation of a naked cytosine (Figure 5) (Bayraktar *et al.*, 2018).

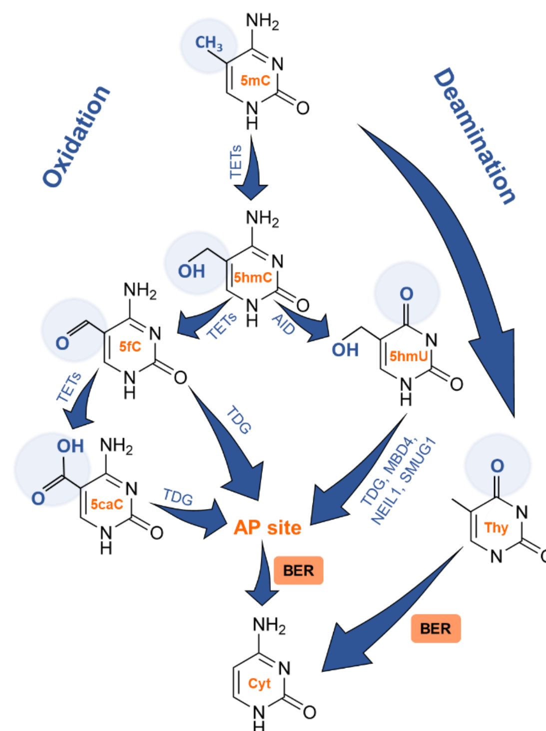

Figure 5. Active DNA demethylation pathways. Oxidation: 5mC bases can be oxidized by TET enzymes to 5hmC followed by 5fC and 5cAC. Next, TDG transforms 5caC into an AP site. Deamination: alternatively, 5mC bases deaminated by AID/APOBEC to form 5hmU which is further processed into an AP site as well. AP sites resulted from both pathways are then repaired by BER. Alternatively, 5mC is directly converted into Thy, and T:G mismatches are repaired by BER. See text for abbreviations. Adapted from (Bayraktar *et al.*, 2018). © 2019 by P. Herst

Passive DNA demethylation takes place in dividing cells when DNMT1 is inhibited or absent during successive rounds of DNA replication (Seisenberger *et al.*, 2013). As a result, methyl groups are lost by 'dilution', and the overall methylation level is reduced upon each cell division (Figure 5).

2.3 Histone modifications

As mentioned earlier, a small percentage of histones is retained by the sperm genome in mammals (Ben Maamar *et al.*, 2020). Initially, researchers hypothesized that these histones were a result of an inefficient replacement process. It is becoming clearer, however, that these histones contribute to the paternal information transferred to the oocyte and the developing embryo (Brykczynska *et al.*, 2010; Carone *et al.*, 2014; Hammoud *et al.*, 2009).

Studies have shown that histone-bound chromatin is highly enriched at regulatory regions and, particularly, at genes that regulate embryonic development e.g. HOX genes (Arpanahi *et al.*, 2009; Hammoud *et al.*, 2009). They also found histones to be enriched at imprinted gene clusters. These results imply that histones in sperm facilitate the regulation of transcription in the early embryo (Casas *et al.*, 2014; Gatewood *et al.*, 1987).

Histone marks H3K4me2/3 and H3K27me3 are of interest, as they play a key role in normal development and maintenance of transcription patterns. Unlike acetylation and phosphorylation, histone methylation does not affect the histone's charge. Histone methylation occurs on all basic residues: lysines (K), arginines (R), and histidines (H). Lysines can be mono-, di-, or tri-methylated; arginines can be (a)symmetrically di-methylated (Bannister *et al.*, 2011); and albeit rare, histidines can be monomethylated (Al-Hadid *et al.*, 2014). Histone K and R methylation play important roles in regulating transcription, maintaining genomic integrity, and contributing to epigenetic memory (S. S. Ng *et al.*, 2009). H3K4me2/3 is enriched at certain developmental promotors and H3K4me3 localizes to HOX gene clusters, noncoding RNAs and, paternally expressed imprinted loci (Brunner *et al.*, 2014). H3K4me3 is enriched at promotors of genes that are highly expressed during spermatogenesis (Brykczynska *et al.*, 2010; Hammoud *et al.*, 2009). Overexpressing

H3K4 demethylase KDM1A during spermatogenesis impairs embryonic gene expression, fetal development and survivability over multiple generations in mice (Siklenka *et al.*, 2015). H3K27me3, on the other hand, is enriched at repetitive regions and developmental promotors that are repressed in the early embryo (Brunner *et al.*, 2014; Carone *et al.*, 2014; Hammoud *et al.*, 2011). In infertile men, retention of both H3K4me and H3K27me was decreased at developmental transcription factors and several imprinted genes (Hammoud *et al.*, 2011). These results highlight the epigenetic differences, particularly at imprinted and developmental loci, observed in infertile compared to fertile men.

These findings support that histone modifications and their positioning are not non-functional remnants and are indeed essential for normal sperm function and embryonic development.

2.4 Small non-coding RNAs

Although transcriptionally inactive, mature sperm transmit a complex array of RNAs (coding and non-coding) to the embryo along with DNA and chromatin (Grunewald *et al.*, 2005). Early on, it was thought that these RNAs were a result of incomplete expulsion of cytoplasmic elements during nuclear condensation. Recent studies, however, have shown that some of these RNAs may affect the developing embryo and/or its subsequent generations (Q. Chen *et al.*, 2015; U. Sharma *et al.*, 2016). In somatic cells, they play an essential role in chromatin remodeling but also transcriptional and post-transcriptional regulation.

The majority of RNA molecules in sperm are fragments of longer transcripts, including ribosomal RNA as well as testes- and spermatogenesis-specific mRNAs (Casas *et al.*, 2014). Besides longer transcripts, sperm also contain many small non-coding RNAs (sncRNAs) which are <200 nt. Examples of sncRNAs are micro RNAs (miRNAs), Piwi-interacting RNAs (piRNAs) and tRNA-derived small RNAs (tsRNAs) which will be described below.

2.4.1 miRNA

miRNAs are short-single-stranded RNAs of circa 20-24 nucleotides that negatively regulate expression of target genes at the post-transcriptional level by binding to 3’-untranslated regions of target mRNAs.

miRNAs are differentially expressed throughout the different stages of male germline development and maturation and are indispensable during post-fertilization stages including sex differentiation, germline establishment, preimplantation development, and implantation of embryos (Reza *et al.*, 2019). Although, sperm make an almost insignificant contribution to the total miRNA content of the zygote compared to the oocyte, inhibition of sperm-delivered miRNAs leads to developmental delays in the zygote (Amanai *et al.*, 2006; Hammoud *et al.*, 2014). In addition, traumatic stress in early life of males alters the sperm miRNA profile, behavioral and metabolic responses in the offspring (Gapp *et al.*, 2014).

On a molecular level, each mature miRNA is partially complementary to multiple mRNA targets and directs the RNA-induced silencing complex (RISC) to identify the target mRNAs for inactivation. In mammals, genes from miRNAs are transcribed to a primary miRNA (pri-miRNA) after which it is processed to a precursor miRNA (pre-miRNA) by class 2 RNase III enzyme. This pre-miRNA is then transported to the cytoplasm with the help of exportin-5 (EXP-5). There, they are further processed to become mature miRNAs by Dicer and RISC (He *et al.*, 2004). miRNAs guide RISC to interact with mRNAs and determine post-transcriptional repression.

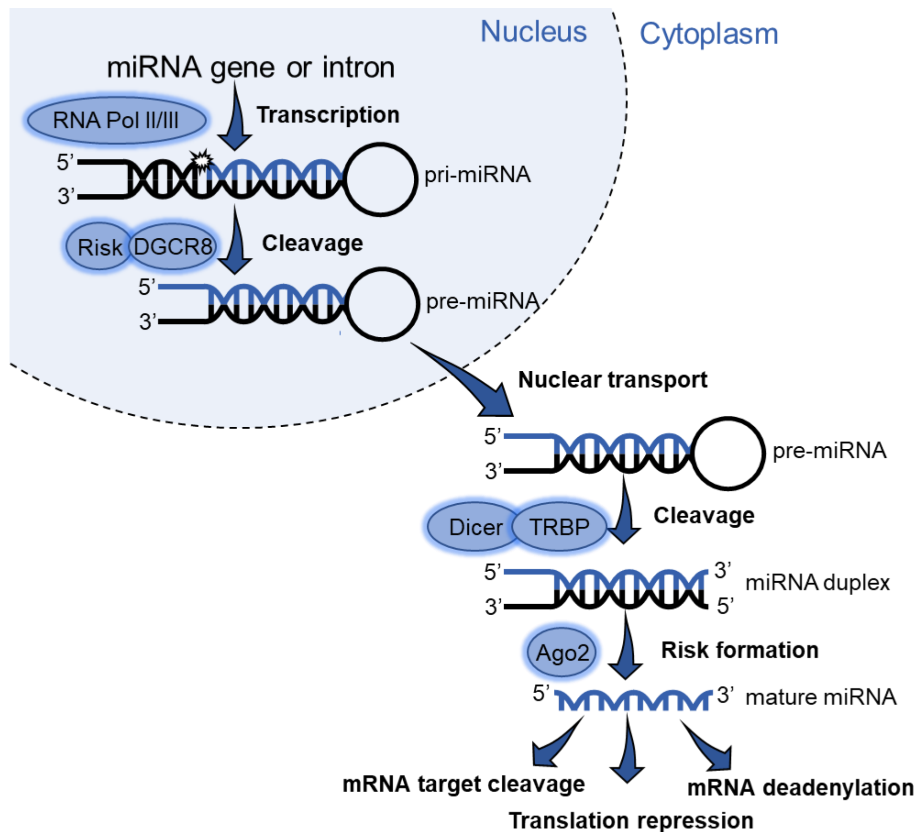

Figure 6. miRNA cleavage pathway. Adapted from (Winter *et al.*, 2009). © 2019 by P. Herst

2.4.2 piRNA

PIWI-interacting RNAs (piRNAs) protect the genome from invasive transposable elements in the germline by using their antisense orientation, of the transposon transcripts, and induce silencing by hybridizing them (Ishizu *et al.*, 2012). Mutations in PIWI protein function or piRNA biogenesis result in germ cell death, defects in gametogenesis and ultimately sterility (Clark *et al.*, 2014). Although piRNAs have been suggested to be germline-specific, recent studies have shown that piRNAs also play important roles in non-gonadal cells such as neurons in mammals (E. J. Lee *et al.*, 2011).

piRNAs arise from intergenic repetitive elements in the genome called piRNA clusters. The biogenesis of piRNAs is independent from Dicer and thus requires other nucleases (Iwasaki *et al.*, 2015). piRNAs can be classified into two major groups, dependent on their biogenesis, including primary and secondary piRNAs (Iwasaki *et al.*, 2015). Primary piRNAs are

generated by still not well understood mechanisms, whereas secondary piRNAs arise from an amplification process called the ping-pong cycle (Toth *et al.*, 2016). Within the ping-pong cycle, mature sense primary piRNAs guide PIWI proteins to the complementary sequences on antisense transcripts from the same piRNA cluster. Then, PIWI proteins use their slicer activity to cleave the target antisense transcript to generate a new 5' end, which is bound by another PIWI protein. Subsequently, the 3' end is trimmed to the length of mature piRNA, leading to a mature antisense secondary piRNA. These secondary piRNAs are able to target sense transcripts transcribed from the piRNA cluster (Weick *et al.*, 2014).

2.4.3 tsRNA

tsRNAs were first discovered through sperm RNA-seq analyses in mouse sperm (Peng *et al.*, 2012). Compared to miRNAs, tsRNAs (~29-34 nt long) are more abundant and make up the majority of small ncRNAs in sperm (Q. Chen *et al.*, 2015; Peng *et al.*, 2012; U. Sharma *et al.*, 2016). Recent studies reported that tsRNA profiles were altered due to a high fat diet or a low protein diet in mice (Q. Chen *et al.*, 2016; Huypens *et al.*, 2016), a high fat diet or environmental contaminant exposure in rats (de Castro Barbosa *et al.*, 2016; Schuster *et al.*, 2016) as well as in obese men (Donkin *et al.*, 2016). These results highlight the sensitivity of sperm tsRNAs to environmental stressors. Injection of sperm tsRNAs, from mice fed a high fed diet, into zygotes induced metabolic disorders in the offspring (Q. Chen *et al.*, 2015). These recent findings raise exciting new possibilities concerning the potential roles of RNA modifications mediating the sperms' epigenetic memory.

To establish this highly specialized sperm epigenome, male germ cells undergo extensive epigenetic remodelling during a differentiation process, called spermatogenesis, to become mature sperm (Seki et al., 2005).

2.5 The production of mature sperm: spermatogenesis

Spermatogenesis commences in the seminiferous tubules at puberty and refers to the complex *differentiation process of diploid (2n) spermatogonial stem cells (SSCs) into haploid (n) mature spermatozoa* (De Vries *et al.*, 2012). It is tightly controlled by hormones, including testosterone and FSH, and growth factors. Gonadotropin-releasing hormone (GnRH), secreted by the hypothalamus, triggers the production of luteinizing hormone (LH) and

follicle stimulating hormone (FSH) by the pituitary. After, LH is transported to the testis where it stimulates Leydig cells to produce testosterone. Testosterone, LH and FSH act on receptors of the supporting Sertoli cells, which play a key role in testicular function and spermatogenesis.

The process includes both mitotic and meiotic divisions and can be divided into three phases: (1) proliferation and differentiation of spermatogonia, (2) meiosis, and (3) spermiogenesis, the transformation of round spermatids into mature spermatozoa (Figure 7) (De Vries *et al.*, 2012). Throughout these phases, developing germ cells physically interact with Sertoli cells for nourishment and support (Stuppia *et al.*, 2015; Zini *et al.*, 2011). Spermatogenesis takes ~ 70 days in men (Heller *et al.*, 1964), and ~ 50 days in rats (Franca *et al.*, 1998).

One of the principal characteristics of spermatogenesis is the dramatic reorganization of the germ cells' chromatin from a nucleosomal histone-based structure to a densely packed structure using protamines (Rathke *et al.*, 2014). This highly condensed DNA structure protects the paternal genome during transfer from male to oocyte (Oliva, 2006). Compared to an oocyte, a mature human sperm nucleus is $1/13^{th}$ the size (Martins *et al.*, 2007).

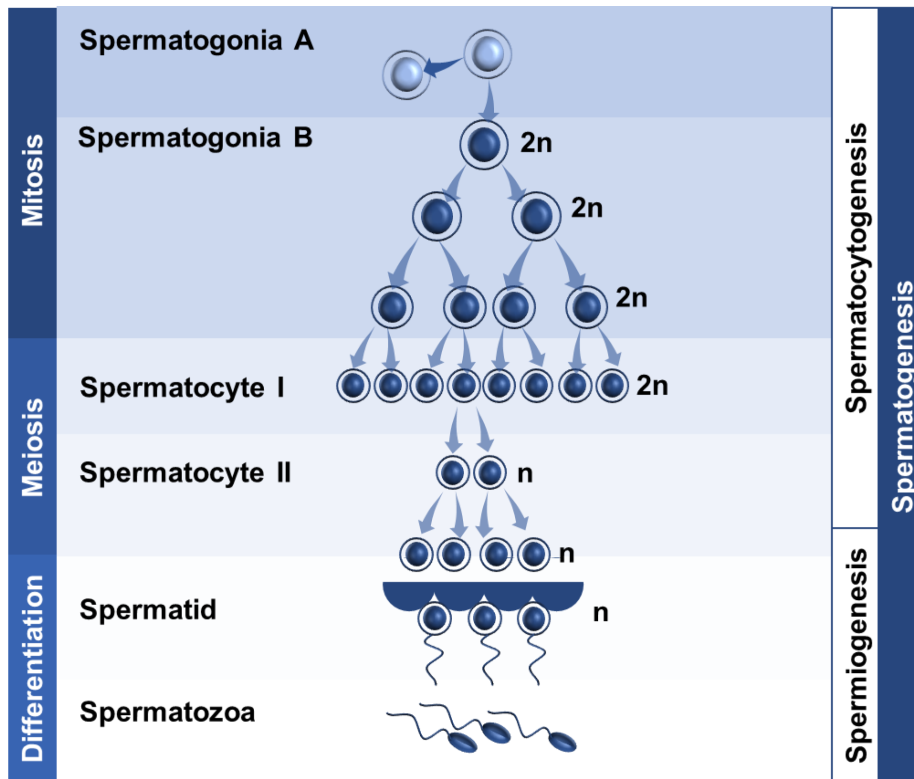

Figure 7. Developmental stages of spermatogenesis. Adapted from (Cheng *et al.*, 2010). © 2019 by P. Herst

2.5.1 Replacement of histones by protamines

As spermatids undergo the final stages of spermatogenesis, the DNA is stripped of *most* nucleosomal packaging and becomes wrapped around transition proteins (TPs), which are later replaced by protamines. Protamine replacement may be required to generate a more hydrodynamic sperm head that accelerates the transit through the female reproductive tract, and protects the genetic material from both physical or chemical damage (R. E. Braun, 2001; Carrell *et al.*, 2007).

The transition of sperm nuclear proteins from histones to protamines is a multistep process that is still poorly understood (Carrell, 2012). First, nucleosomes are destabilized, most likely by hyperacetylation of the somatic histones in the spermatid. Second, the somatic histones are replaced by testis-specific histone variants, which are then replaced by TP1 and TP2. TP1 and TP2 proteins are found in stages 12 and 13 of spermatogenesis. Their importance was confirmed using double knock-out (Tnp1 or Tnp2) mutants which showed a decrease in

normal sperm morphology, motility, chromatin condensation of the remaining transition protein (Shirley *et al.*, 2004). Third, TP1 and TP2 are completely replaced by protamines. Protamine compaction occurs through the formation of disulfide bonds between the protamines and by the formation of toroidal chromatin structures (Ward, 2010).

2.5.2 DNA methylation and histone modifications during spermatogenesis

Alongside the histone removal, several extensive epigenetic changes, including DNA methylation and histone modifications, take place during spermatogenesis. Before meiosis, progressive demethylation-remethylation of DNA takes place (Stuppia *et al.*, 2015). During meiosis, active DNMT3A, DNMT3B, and cofactor DNMT3L regulate the levels of *de novo* DNA methylation. Then, the methylation profile is maintained by active DNMT1 (Oakes *et al.*, 2007).

Concerning the histone modifications, these mainly include acetylation and methylation, which modify the DNA accessibility to transcription factors. Generally, lysine 9 histone H3 (H3-K9) methylation increases during meiosis but is removed after meiosis, thereby promoting gene activation. On the flip side, lysine 4 histone H3 (H3-K4) methylation, decreases during meiosis and thus promotes DNA silencing (Boissonnas *et al.*, 2013). Histone acetyltransferase (HAT) and histone deacetylase (HDAC) play an important role in the histone to protamine transition and in elongating spermatids (Oliva *et al.*, 1986; Sonnack *et al.*, 2002). Only few histones remain to function as epigenetic regulators after completed protamination. However, the few loci that have been shown to retain histones in mature sperm are known to be important in developmental processes, and thus are likely to provide some degree of regulatory competence (Hammoud *et al.*, 2009).

This unique epigenetic blueprint of sperm is rapidly changed, on a genome-wide scale, upon fertilisation and during the formation of primordial germ cells (PGCs)– a process called epigenetic reprogramming (Morgan et al., 2005; Reik et al., 2001). During embryonic gonadal epigenetic reprogramming, paternal and maternal imprints are erased and totipotency is re-established.

2.6 Epigenetic erasure during germline reprogramming

Epigenetic reprogramming involves the erasure of paternal and maternal epigenetic marks such as the demethylation of 5mC which plays a crucial role in genome imprinting, X inactivation, transposon silencing, the stability of centromeric/telomeric structure and gene expression in general (Messerschmidt *et al.*, 2014).

2.6.1 Upon fertilisation

Following successful fertilisation, dynamic changes take place in both paternal and maternal chromatin to transform two haploid cells into a diploid embryo. Throughout this transformation, protamines are removed from the paternal chromatin and nucleosome-bound DNA are re-established with maternally derived histones to give rise to the first cell of the developing embryo, the totipotent zygote. A totipotent zygote is characterized by its ability to give rise to a whole, normal, and fertile individual (Morgan *et al.*, 2005). Totipotency is only displayed by fertilised eggs (zygote) and early embryos in mammals, it spans over few cell cycles as it is lost at the blastocyst stage (Duranthon *et al.*, 2008). As the timescale of these events varies considerably across mammalian species, and as most of the studies have been carried out using mouse models, we will focus on epigenetic reprogramming in mouse in the following sections.

2.6.1.1 Global DNA methylation erasure in pre-implantation embryo

Within the first hour of fertilisation, the paternal genome releases protamine, and is re-packed by maternal nucleosomal histones from the oocyte cytoplasm, forming the paternal pronucleus. Subsequently or synchronously, the paternal pronucleus enlarges as it incorporates more maternal proteins. The paternal and maternal genomes stay separated and undergo differential modifications of their chromatin and epigenetic marks during the first cell cycle (Cantone *et al.*, 2013). The paternal genome shows 80-90% overall CpG methylation which undergoes active genome-wide DNA demethylation before replicating its DNA (Cantone *et al.*, 2013; Popp *et al.*, 2010) (Figure 8). Some specific regions, however, including the paternally methylated imprinting control regions, resist global demethylation. In contrast, the maternal pronucleus undergoes passive replication dependent demethylation during the first few cell cycles. By the blastocyst stage, both parental genomes show low levels of methylation. Then, during the implantation of the embryo, there is a wave of *de*

novo DNA methylation resulting in 70% of the CpGs to be methylated (Popp *et al.*, 2010). DNA methyltransferases (DNMTs) DNMT3A and DNMT3B are responsible for establishing *de novo* methylation marks, whereas, DNMT1 maintains the methylation patterns (Sasaki *et al.*, 2008).

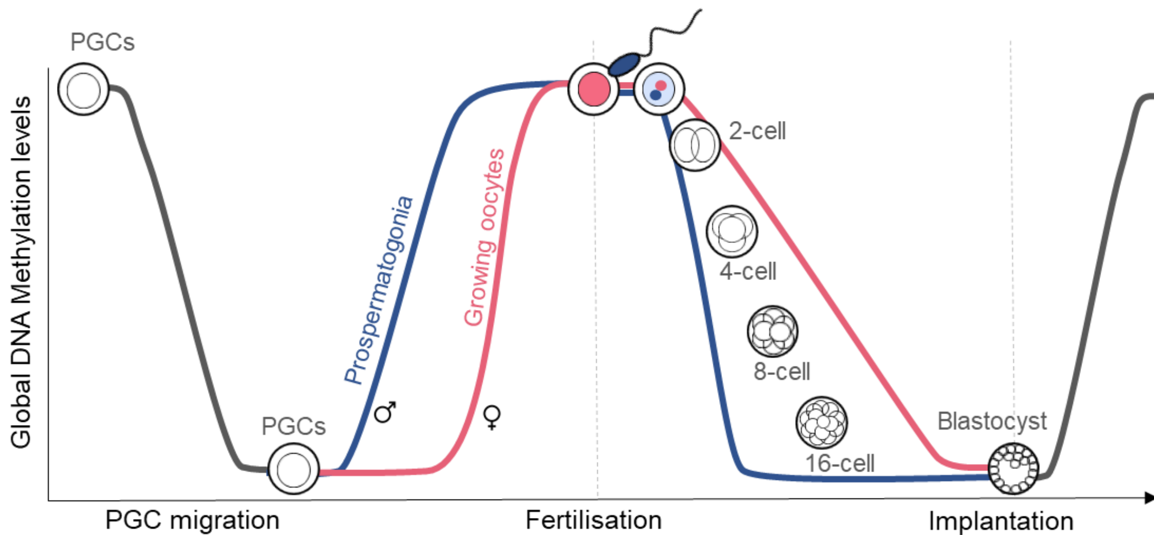

Figure 8. Genome-wide epigenetic reprogramming. Adapted from Smallwood *et al.* (2012) © 2019 by P. Herst

2.6.1.2 Histone composition in pre-implantation embryos

Alongside DNA methylation changes, chromatin organization and histone modifications also play an essential role in establishing a totipotent embryo. Previous studies described that histones incorporated into the paternal pronucleus are hyperacetylated shortly after fertilisation (Adenot *et al.*, 1997; Santos *et al.*, 2002), specifically lysines (K) 9, 14, 18 and 27 of H3 and 5 and 16 of H4 (Adenot *et al.*, 1997; Santenard *et al.*, 2010). This may increase the accessibility of the paternal genome to cellular machinery and allow additional remodeling to occur. In the maternal pronucleus, all forms of H3K4 methylation (me) as well as H3K9me2 and 3 are observed. H3K27me1 is observed in both parental pronuclei (Marcho *et al.*, 2015).

2.6.2 During the formation of primordial germ cells

The second round of epigenetic reprogramming occurs in the primordial germ cells (PGCs), the precursors of sperm and oocytes. It facilitates establishment of sex-specific epigenetic profiles crucial for normal germ-line development (Stringer *et al.*, 2013). At embryonic day

7.25 (E7.25), PGCs appear inside the extra-embryonic mesoderm at the posterior end of the primitive streak, after which they migrate to the genital ridge by E11.5 (Figure 8) (McLaren, 2003; Morgan *et al.*, 2005). During multiple cell divisions and DNA replication cycles, genome-wide DNA demethylation of genic, intergenic and transposon sequences, is completed by E13.5, at which point female PGCs enter meiotic prophase as primary oocytes, whereas male PGCs enter mitotic arrest (Morgan *et al.*, 2005; Seisenberger *et al.*, 2012).

2.6.2.1 Global DNA methylation erasure in PGCs

Migrating PGCs show reduced global levels of DNA methylation (5mC) around E8.0 (Seki *et al.*, 2005) (Figure 8). This DNA demethylation possibly results from the effect of BLIMP1 and PRDM14 which both repress *Dnmt3a*, *Dnmt3b* and ubiquitin-like, containing PHD and RING finger domains 1 (*Uhrf1*), which is essential for *de novo* and maintenance of methylation machinery (Kurimoto *et al.*, 2008; Yamaji *et al.*, 2008). DNA methylation on transposable elements, imprinted loci and single-copy genes is retained at CpG sites until E10.5 (Hajkova *et al.*, 2002; J. Lee *et al.*, 2002).

Concerning the actual DNA demethylation mechanism, studies have identified a rapid loss of 5mC from the male genome and a simultaneous gain of 5hmC between E11.5 and E13.5 (Iqbal *et al.*, 2011; Wossidlo *et al.*, 2011). This time frame is considered to be too short for passive loss of DNA methylation over multiple cell divisions, therefore, DNA demethylation is likely at least in part active. Conversion of 5mC to 5hmC generates unmodified cytosine residues, which could in turn provide a substrate in base excision repair (BER)-mediated active demethylation (S. C. Wu *et al.*, 2010). BER components, including XRCC1 and active poly(-ADP-ribose) polymerase 1 (PARP1) are upregulated in PGCs at E11.5 (Hajkova *et al.*, 2010).

By E13.5, however, the overall methylation level is reduced by more that 90%, resulting in fully stripped parental imprints and promotor CpG methylation at germline-specific genes (Hajkova *et al.*, 2002; Hajkova *et al.*, 2010). To potentially protect against epimutations, retrotransposons such as Intracisternal A particle (IAP) retain methylation (Lane *et al.*, 2003).

2.6.2.2 Histone composition in PGCs

PGCs lose H3K9me2 starting at E7.75, and simultaneously gain H3K27me3 by E9.5 (Saitou *et al.*, 2012). This loss of H3K9me2 is possibly due to the downregulation of a methyltransferase GLP which, together with EHMT2, is required for the deposition of H3K9 mono- and demethylation (Hackett *et al.*, 2012). The repressive chromatin state is hypothesized to be maintained by H3K27me3 upon loss of H3K9me2 (Sasaki *et al.*, 2008). Furthermore, migrating PGCs show upregulated H2A/H4R3me2 which is catalyzed by protein arginine methyltransferase 5 (PRMT5). H2A/H4R3me2 possibly contributes to maintain PGC unipotency and repress the somatic program (Tee *et al.*, 2010).

At approximately E11.5, several other important processes occur. A transient reorganization of linker histone H1, H3K27me3 and H3K9me3 and stable remodeling of global H3K9ac and H2A/H4R3me2 takes place (Hackett *et al.*, 2012). Another important event driven by reprogramming is reactivation of X_i in female PGCs (Heard *et al.*, 2014). This requires the integration of multiple genetic and epigenetic systems, early chromatin reorganization initiating X-reactivation and DNA demethylation, and repression of the long noncoding RNA *Xist* necessary to complete the process (Hackett *et al.*, 2012). The X-chromosome reactivation occurs over a prolonged period and is completed in post-migratory PGCs (Sasaki *et al.*, 2008).

2.7 Other epigenetic events during early mammalian development

Besides genome-wide epigenetic reprogramming, other essential epigenetic events take place during early mammalian development including genomic imprinting and X-chromosome inactivation (Perera *et al.*, 2011).

2.7.1 Genomic imprinting

Throughout mammalian development, genes are usually expressed from both the maternal and paternal allele. Imprinted genes, however, are epigenetically regulated and transcribed in monoallelical parent-of-origin-specific manner (Plasschaert *et al.*, 2014). This means that only the gene of the maternally or paternally inherited chromosome is expressed. There is no substitute allele. Although these imprinted genes represent only small part of the genome, ~100 identified genes, they play an essential role during early mammalian development,

growth and behaviour (Verona *et al.*, 2003). Abnormal imprinting and loss of heterozygosity have been associated with multiple disorders including Beckwith-Wiedemann, Prader-Willi and Angelman syndromes (Horsthemke *et al.*, 2006; van Otterdijk *et al.*, 2016; Weksberg *et al.*, 2005).

Paternal imprints are established during spermatogenesis; whereas, maternal imprints during oogenesis at various time points (Kerjean *et al.*, 2000). Paternal imprints resist genome-wide epigenetic reprogramming after fertilisation. However, once the second wave of epigenetic reprogramming occurs in PGCs, the imprints are erased and are later re-established by *de novo* methylation (Reik *et al.*, 2001). Regulated by imprinting control regions (ICRs), most imprinted genes are found in clusters containing several maternally and paternally expressed imprinted genes. ICRs are differentially methylated regions (DMRs) at which the DNA is only methylated on one allele. Two types of DMRs have been identified including (1) DMRs that become methylated during gametogenesis called “germline DMRs”, and (2) DMRs that acquire methylation after fertilisation called “somatic DMRs” (Lewis *et al.*, 2006).

The best example to explain the influence of ICRs on monoallelic activity and repression of imprinted genes is the insulin-like growth factor 2 (*Igf2)* and *H19* locus (Figure 9). *Igf2* plays an important role in fetal growth and the development of various tissues, particularly the brain (D'Ercole *et al.*, 1996). *Igf2* is a paternally expressed gene located ~80 kb from the maternally expressed *H19* gene, which shares common expression sites and enhancer sequences *(Szabo et al., 2004)*. *Igf2* is methylated on the maternal allele, whereas *H19* is silenced on the paternal allele. The ICR is located upstream from the *H19* promotor and contains multiple binding sites for CCCTC-binding factor (CTCF). When the maternal allele is unmethylated, CTCF binds to the ICR thereby silencing *Igf2* expression and stimulating *H19* transcription (Pidsley *et al.*, 2012). In contrast, on the paternal allele, ICR is methylated, blocking CTCF binding, and stimulates *Igf2* transcription (Bartolomei, 2009; Bartolomei *et al.*, 2011).

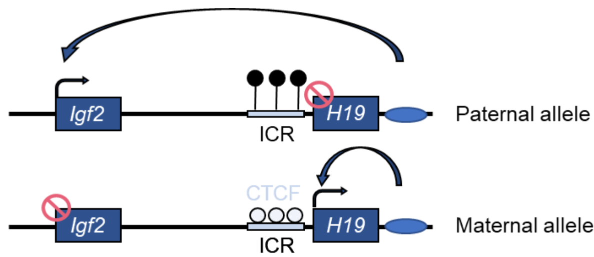

Figure 9. Maternal and paternal regulation of the Igf2-H19 imprinted domain. Adapted from (Bartolomei, 2009). © 2019 by P. Herst

Low methylated or non-methylated *Igf2/H19* has been observed in patients with reduced sperm quality, decreased sperm count and motility compared to fertile men (Boissonnas *et al.*, 2010). Other imprinted genes that play a critical role in male reproduction are *Mest* (mesoderm specific transcript) and *Dazl* (deleted in azoospermia like), which are associated with a low sperm concentration (oligozoospermia) (Marques *et al.*, 2008).

2.7.2 X-chromosome inactivation

X-chromosome inactivation also leads to monoallelic expression, via a different mechanism. Upon fertilisation, female embryos have two active X-chromosomes, of which one is rapidly turned off during early development. The randomly selected silenced X chromosome is densely packaged into a Barr body. This results in equal expression of X-encoded genes between males and females (Dementyeva *et al.*, 2010).

In mice, X-chromosome inactivation takes place in two waves. The first wave commences around the 2-cell to 8-cell embryonic stage, during which the paternal X-chromosome is inactivated in all cells (Pinheiro *et al.*, 2017). At the blastocyst stage, the paternal X-chromosome is reactivated in the inner cell mass. Shortly after, during the implantation of the embryo, a second wave of X-chromosome inactivation occurs randomly in the fetal precursor cells (Barakat *et al.*, 2010; Pinheiro *et al.*, 2017). This final inactivation is inherited throughout cell division. X-chromosome inactivation failure has been associated with various severe genetic disorders including Klinefelter syndrome (XXY), triple X syndrome (XXX) and Turner syndrome (X) causing non-lethal developmental abnormalities (C. Wu *et al.*, 2016).

In summary, the sperm epigenome, established during spermatogenesis, is highly specialized and very different from somatic cells. Besides functioning as a vehicle to transfer their haploid genome to the oocyte, sperm carry important DNA modifications, chromatin proteins and associated marks as well as a wide range of sncRNAs that play critical roles during embryonic development.

3. Paternal Origins of Health and Disease

There are multiple ways through which parents contribute to development and health of their children. Since the introduction of David Barker's hypothesis on Developmental Origins of Health and Disease (DOHaD), countless epidemiological and animal model studies have made significant progress in identifying early life, including *in utero* origins of chronic adult-onset diseases (Barker *et al.*, 1993; Barker *et al.*, 1986; Barker *et al.*, 1989). Initially these studies focussed on the relation between a pregnant mother's lifestyle and the health of her children; whereas the contribution of the father received limited focus. It is now becoming more and more evident that fathers also pass down important life experiences and behaviours to their children.

New light has been shed on the importance of the sperm epigenome in carrying heritable information from one generation to the next. *In vitro*, animal, and human studies have identified several classes of environmental chemicals, including POPs, that modify epigenetic marks. This epigenetic information establishes a memory of past exposures that could be transmitted to subsequent generations, a controversial phenomenon called "trans/inter transgenerational epigenetic inheritance", which led to the new field of Paternal Origins of Health and Disease (POHaD) (Soubry, 2018a).

In this section, a specific focus will be placed on the relation between the environment, particularly environmental pollution, e.g. POPs, and the sperm epigenome and how they impact on the father's heath and his offspring, and thus, POHaD. We discuss the accumulating evidence that epigenetic mechanisms are important in the transfer of information from one generation to the next through the male germ line.

3.1 Epidemiological evidence of POPs affecting the epigenome

An increasing number of studies provide evidence supporting the impact of POPs exposure on the epigenome, particularly DNA methylation.

DNA methylation plays a key epigenetic role during early embryonic development. Increasing evidence has shown associations between global loss of DNA methylation and genomic instability – a common phenotype for cancer and ageing (Fraga *et al.*, 2007; A. S. Wilson *et al.*, 2007). Conversely, an increase in global DNA methylation, especially in the placenta, has been associated with developmental defects (Jin *et al.*, 2013; Reichetzeder *et al.*, 2016).

3.1.1 Relationship between POPs exposure and global DNA methylation in blood

One of the earliest studies documented an association between DNA methylation alterations and POPs exposure in blood plasma samples from Greenland Indigenous men and women (n=71; 14% women). By examining the correlation between the POPs lifetime body burden and percentage of global DNA methylation, researchers found that high levels of POPs exposure are associated with global genomic DNA hypomethylation (Rusiecki *et al.*, 2008); which in turn has also been linked with chromosomal instability and is often found among cancer tissues.

A similar study was conducted in blood plasma samples from healthy Korean men and women (n=86; 60.5% women). Results showed that higher POPs exposure, based on lipid-adjusted serum levels, was also associated with DNA hypomethylation (K. Y. Kim *et al.*, 2010). In contrast to the Greenland Indigenous people, however, the Korean subjects were exposed to lower exposure levels which are commonly seen in the Western World populations.

Since the number of subjects was rather small for both previous studies (K. Y. Kim *et al.*, 2010; Rusiecki *et al.*, 2008), a larger study was conducted including n=524 elderly Swedish people (all aged 70) of which 48% were women (Lind *et al.*, 2013). In opposition to the other studies, high levels of multiple POPs were related to global DNA hypermethylation in serum (lipid-adjusted) using a cross-sectional study design. It is tempting to speculate that this was a result of age, as only elderly Swedish people were included in the study. Abnormal increases of DNA methylation, due to circulating PCBs and DDE, have been associated with several diseases including colorectal cancer (Ehrlich, 2019; T. M. Murphy *et al.*, 2013; Walters *et al.*, 2013). Authors stated, however, to be cautious drawing conclusions about

other ethnic and age groups especially since they investigated only Caucasian individuals from one specific city.

To recognize the sex-specific differences in DNA methylation due to POPs exposure between adult men and women (> 20 years old), M. H. Lee *et al.* (2017) performed a cross-sectional study in Koreans (n=444; 43% women). Authors measured DNA methylation via markers of global DNA methylation (Alu and Long interspersed nuclear elements LINE-1) in peripheral leukocytes. Results showed that various POPs were associated with global DNA hypomethylation in men and global hypermethylation in women (M. H. Lee *et al.*, 2017). Previous studies suggested sex as a confounding variable affecting DNA methylation (Jaffe *et al.*, 2014; S. Shah *et al.*, 2014). Yet, Huen *et al.* (2014), who investigated the sex-specific differences between POPs exposure (PBDE an DDT/E) and DNA methylation in boys and girls (n=358; 50% girls) from California, showed lower levels of DNA methylation in girls compared boys, particularly for LINE-1. Researchers also collected blood samples from mothers during pregnancy (n=14) and/or at delivery (n=40) and discovered a significant association of prenatal DDT/E exposure with hypomethylation of Alu repeats (global DNA methylation marker) (Huen *et al.*, 2014). As the majority of biomedical research is male biased, studies comparing both sexes directly give a deeper understanding of variables controlling physiology, gene networks, cell systems, and the implications of differences in diagnosis and treatment due to sex (Arnold *et al.*, 2012; Wizemann, 2012).

Indeed, these studies have documented associations between POPs exposure and changes in **somatic cell** DNA methylation, yet it was only up till recently that studies started to investigate the impact on the **sperm epigenome** including DNA methylation.

3.1.2 Impact of the POPs exposure on the human sperm epigenome

Various epidemiological studies have addressed the effect of POPs exposure on male reproductive function, including sperm characteristics (motility/concentration/total sperm count), endocrine function and reproductive organ physiology. (Aneck-Hahn *et al.*, 2007; Ayotte *et al.*, 2001; Bush *et al.*, 1986; Y. L. Guo *et al.*, 2000; Richthoff *et al.*, 2003; Toft *et al.*, 2004; Vested *et al.*, 2014). More recently, effects have also been observed in sperm chromatin integrity such as DNA strand breaks or epigenetic changes (Belleau *et al.*, 2018;

Consales *et al.*, 2016; De Jager *et al.*, 2006; Spano *et al.*, 2005). Abnormalities in sperm DNA integrity have a profound impact on the fertility potential of men (Agarwal *et al.*, 2003; G. Y. Kim, 2018).

In a previous Swedish cohort study including 195 healthy adult men, researchers observed a positive association between certain PCB serum levels and sperm DNA integrity using a sperm chromatin structure assay (SCSA) (Rignell-Hydbom *et al.*, 2005). Though, authors stressed that future studies were required to clarify the underlying mechanism.

Another study was conducted investigating the association between similar human dietary POPs exposure and sperm chromatin damage in cohorts including Swedish, Polish, Ukrainian and Greenland Indigenous men (n=707) using SCSA (Spano *et al.*, 2005). A positive association was found for Swedish and Ukrainian men but not in Polish and Greenlandic men. In 2006, the association between POPs and sperm chromatin integrity plus apoptotic biomarkers was investigated, this time, using the Terminal deoxunucleotidyl transferase-driven dUTP Nick End Labelling (TUNEL) assay on ejaculated sperm. Men (n=798) from again Sweden, Poland, Ukraine and Greenland provided fresh semen. Researchers discovered a relationship between exposure to POPs, and DNA integrity, and the anti-apoptotic protein Bcl-xL in all European men but not Indigenous men. Authors highlight the importance of additional issues such as genetic variation and lifestyle as well as all components of the POPs mixture (Spano *et al.*, 2005; Stronati *et al.*, 2006).

While most have been banned, some POPs (e.g. DDT) are still used in certain areas of the world including malaria-endemic countries. In South-Africa, DDT is sprayed indoors on a regular basis (Rogan *et al.*, 2005). A weak yet positive association between non-occupational DDT levels and sperm chromatin integrity was observed in young healthy South African men (n=209) (de Jager *et al.*, 2009).

One of the first the studies to show the impact of POPs exposure on sperm DNA methylation in humans assessed DNA methylation levels on Alu and LINE-1 repeats in sperm from Swedish, Polish, Ukrainian and Greenland Indigenous men (n=607). Both Alu and LINE-1

repeats have been extensively used in population studies to investigate the association between methylation profiles and environmental exposures, especially since Alu and LINE-1 methylation represents up to 50% of global genomic methylation (A. S. Yang *et al.*, 2004) and are thus informative and cost effective. Dependent on the genetic background, study results showed weak yet significant associations between POPs exposure and global DNA methylation, using a flow cytometric fluorescence immunodetection of 5-mC approach. High POPs exposure resulted in decreased methylation (Consales *et al.*, 2016).

Taken together, non-occupational POPs exposure are associated with the sperm chromatin as well as sperm DNA methylation changes dependent on the geographical location. Alongside these studies, various supporting animal studies have shown that these epigenetic changes in sperm may also be transmitted to subsequent generations thereby potentially affecting their health and well-being. This event is also known as inter- and transgenerational epigenetic inheritance.

3.2 Inter vs. transgenerational epigenetic inheritance

Aside from DNA damage and mutations in paternal germ cells, increasing evidence supports the idea that information not directly encoded in the DNA sequence, i.e. epigenetic alterations or "epimutations", acquired during spermatogenesis, may be sustained through mitotic/meiotic cell divisions and from parent to offspring. The epigenome is particularly vulnerable during fertilisation and embryonic development. Epigenetic alternations arising during these stages of life will have a much greater impact on the overall epigenetic status as they amplify through cell division and somatic maintenance, potentially leading to long-lasting phenotypic effects across generations. In contrast, epigenetic alterations occurring in adult quiescent (non-dividing) cells will remain restricted to those cells or tissues.

Inter and transgenerational epigenetic inheritance have been well documented in microorganisms, yeast, plants and in *C. elegans* (Casadesus *et al.*, 2006; Hollick, 2017; Houri-Zeevi *et al.*, 2017; Minkina *et al.*, 2018; Quadrana *et al.*, 2016; Rusche *et al.*, 2003). In mammals, such events seem more of an exception of the rule, especially considering the two waves of genome-wide reprogramming plus external confounding factors. Growing

knowledge of epigenetic reprogramming, however, suggests that epigenetic modifications are not always completely erased between generations, i.e. incomplete erasure, and can be transmitted from one generation to the next through gametes (Anway *et al.*, 2005; Carone *et al.*, 2010; Morgan *et al.*, 1999; S. F. Ng *et al.*, 2010; Radford *et al.*, 2014; Rakyan *et al.*, 2003).

The strongest indication of epigenetic inheritance in mammals is parental imprinting (Hadchouel *et al.*, 1987; Sapienza *et al.*, 1987; Swain *et al.*, 1987). Other early evidence of epigenetic inheritance stems from studying epialleles using for example the Agouti mouse model (Morgan *et al.*, 1999; Rakyan *et al.*, 2002). The Agouti mouse has been used to investigate the impact of nutritional and environmental exposures on the fetal epigenome, as its coat color changes according to the established epigenetic marks during development. The *Agouti* gene codes for a signalling molecule that produces either eumalin (brown/black) or phaeomelanin (yellow/red) pigmentation in the coat (Jirtle, 2014). This gene is metastable due to a retroviral intracisternal A particle (IAP) insertion upstream of the transcription site. IAPs are transposable elements that can change their position within the genome. In the Agouti mouse, the IAP is controlled by DNA methylation. In case IAP is fully unmethylated, the coat color changes to yellow and the mouse develops an obese phenotype which is prone to diabetes and cancer. In contrast, when the IAP is methylated the coat color becomes brown and the mouse has low disease risk (Morgan *et al.*, 1999). A methyl donor-rich maternal diet of Vitamin B9 (folic acid) supplementation, Vitamin B12, choline and betaine shifted the coat color towards a brown pseudoagouti phenotype in the offspring (Wolff *et al.*, 1998), as a result of increased methylation near the IAP transposable element (Waterland *et al.*, 2003). Maternal exposure to bisphenol A, an estrogenic xenobiotic chemical, led to a significant shift in the offspring’s coat color phenotype towards yellow. Along with this shift in coat color, reduced methylation was observed at multiple CpG sites located upstream of the IAP transposable element (Dolinoy *et al.*, 2007). Besides the Agouti model, other rodent studies showed that maternal diet, stress and traumatic exposures during pregnancy influence the offspring’s epigenetic and genetic expression, thereby changing their stress response, glucose metabolism, blood pressure, cholesterol and cardiac energy metabolism (Alkemade *et al.*,

2010; Y. Huang *et al.*, 2014; Maeyama *et al.*, 2015; Pruis *et al.*, 2014; Radford *et al.*, 2014; St-Cyr *et al.*, 2015).

With regard to humans, an increasing number of studies suggests that various contaminants, nutrition, and lifestyle-related conditions can affect the developing male germ cells and influence offspring's future health (Soubry, 2015). To date, **associations** have been made between paternal nutritional and smoking behaviour of cardiovascular and metabolic disorders, yet the exact inter/transgenerational epigenetic mechanisms remain to be deciphered (Figueroa-Colon *et al.*, 2000; Loomba *et al.*, 2008; Pembrey *et al.*, 2006; Sharp *et al.*, 2019; Soubry, 2018b) Though, one would need to understand that epidemiological studies including two or more generations in humans are difficult to realize.

Intergenerational epigenetic inheritance refers to a heritable effect observed in the F1 and F2 generation in case the pregnant female (F0) was exposed (Heard *et al.*, 2014). When a gestating female (F0) is exposed to environmental stressors, it is not only her embryo/fetus (F1) that is directly exposed but also the developing germline (F2) of the embryo/fetus. If the effect persists to the F3 and beyond in the same model, we refer to a transgenerational effect (Heard *et al.*, 2014). This means that epigenetic marks, which potentially influence physiological parameters and disease, are still affected in the absence of direct environmental exposure. Accordingly, if a F0 male or a F0 unpregnant female are exposed, we speak of an intergenerational effect if an effect is observed in the F1, and transgenerational if the effect persisted to F2 and beyond (Figure 9).

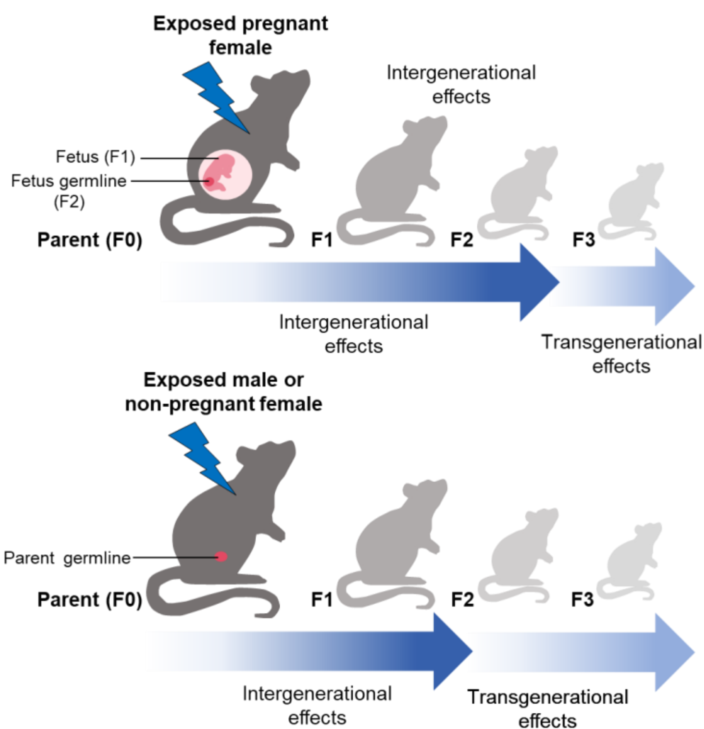

Figure 10. Inter versus transgenerational epigenetic inheritance. © 2019 by P. Herst

3.3 POPs induced paternal inter/transgenerational epigenetic inheritance

Aside from human epidemiological studies, an increasing number of animal studies has provided insight regarding various paternal intern/transgenerational mechanisms of epigenetic inheritance. Since POPs encompass various pollutants, that appear to induce epigenetic inheritance, the following will focus on the effects of the *individual* POPs. The majority of research does not study the impact of POP *mixtures*, therefore, literature on the effects of multiple exposures is limited.

3.3.1 DDT and DDE

DDT exposure has been shown to be associated with toxic effects in humans such as reproductive (including semen quality) and neurological disease, developmental abnormalities, and cancer (Aneck-Hahn *et al.*, 2007; Beard *et al.*, 2006; Cohn *et al.*, 2010; de Jager *et al.*, 2009; Jaga *et al.*, 1999; Longnecker *et al.*, 1997). Causations between environmentally relevant prenatal DDT exposure and offspring obesity and diabetes have been made as well (Valvi *et al.*, 2014; Warner *et al.*, 2017).

To assess whether prenatal DDT exposure caused transgenerational epigenetic inheritance of obesity in the offspring, gestating female rats were intraperitoneally injected with an environmentally relevant dose of DDT (25 mg/kg or 50 mg/kg BW/day) at gestational day eight until 14 (Skinner, Manikkam, *et al.*, 2013). F0 females also received a standard rat diet ad lib. Several F1 males and females were mated with non-littermate females and males to establish the F2 generation. The same was performed for F3. In total four generations were studied. F1 animals did not show an increase of obesity incidence, whereas half of the F3 animals did. Interestingly, more F3 males than F3 females developed obesity. F3 males also developed increased testis disease incidence and lower sperm counts. Kidney disease was observed in both F1 and F3 males and females. Concerning sperm epimutations, F3 males showed differential DNA methylation regions due to ancestral DDT exposure using a methylated DNA immunoprecipitation ChIP (MeDIP-chip) approach (Skinner, Manikkam, *et al.*, 2013).

Several years later, the same group investigated the underlying epigenetic mechanisms of their previous study (Skinner *et al.*, 2018; Skinner, Manikkam, *et al.*, 2013). A similar animal model approach was utilized, though this time, the sperm were collected from each generation (F1-F3) to analyze sperm DNA methylation patters, ncRNA and histone marks. Both DNA methylation and ncRNAs were altered due to prenatal DDT exposure across F1, F2 and F3, yet each generation was differently altered. Most overlap was observed between F1 and F2. A significant number of differential histone retention sites was seen in F3 sperm from the ancestral DDT exposed lineage vs. control (Skinner *et al.*, 2018). Furthermore, the transgenerational impact of prenatal DDT exposure on the repressive histone mark H3K27me3 was investigated using Chromatin Immunoprecipitation Sequencing (ChIP-Seq). Indeed, ancestral DDT exposure induced transgenerational epigenetic inheritance of differential histone retention sites until F3 compared to control (Ben Maamar, Sadler-Riggleman, Beck, & Skinner, 2018). From the same F3 males, several stages of germ cells during spermatogenesis (e.g. prospermatogonia, pachytene spermatocytes, round spermatids, caput epididymal spermatozoa, and caudal sperm) were isolated and subjected to methylated DNA immunoprecipitation sequencing to analyze the differential DNA methylation regions.

In the ancestral DDT exposed lineage, all observed stages of male germ cell development showed unique differential DNA methylation region patterns compared to control (Ben Maamar *et al.*, 2019).

Although performed by the same research group, together these studies show that early life exposure to DDT exposure induces transgenerational epigenetic inheritance in rat male offspring.

3.3.2 Dioxins

Manikkam *et al.* (2012) investigated dioxin induced sperm epimutations in prenatally exposed F3 male rats. Like the DDT studies, pregnant F0 rats were intraperitoneally injected with 0.1% of oral LD50 dose of dioxins (100 ng/kg BW/day) from gestational day eight until 14. Non-littermate females and males were mated to establish the F2 generation and the same was repeated for the F3 generation. Results showed that prenatal exposure leads to kidney disease, pubertal abnormalities and prostate defects (atrophic prostate duct epithelium) in F3 males. In addition, 50 significantly differentially DNA methylated regions in promoters were observed in the same males compared to control (Manikkam *et al.*, 2012). Alterations in *Igf2r* and *Peg3* imprinted gene expression as well as methylation/expression of imprinted genes in both muscle and liver tissues of F1 males have been observed following low intraperitoneal dioxin injection of 2 and 10 ng/kg BW/day (Somm *et al.*, 2013). This is particularly interesting as both genes play an important role in fetal growth (Reik *et al.*, 2003).

3.3.3 PCBs

PCBs have been associated with several adverse health and reproductive health effects (Carpenter, 2011; Faroon *et al.*, 2001; Toft *et al.*, 2004). Dependent on their structure, PCBs can affect different receptors of the thyroid, reproductive and neurotransmitter systems (Topper *et al.*, 2015; Walker *et al.*, 2014).

Besides phenotypic changes, PCBs have also been shown to affect the male sperm and brain epigenome transgenerationally (Gillette *et al.*, 2018). Authors intraperitoneally injected F0 pregnant female rats (n=2) with a low and realistic dose (1 mg/kg) PCB mixture Aroclor 1221 on gestational day eight through 18. F1 males (n=4) were mated with untreated females

and the same approach was repeated for the F3 generation. Using reduced representation bisulfite sequencing (RRBS), authors investigated DNA methylation of CpG islands of F1 and F3 sperm and brain nuclei. Prenatal PCB exposure caused hypermethylation on 74% and 70% of the differentially methylated CpG islands of F1 and F3 brain (specifically the hippocampus and central amygdala) and sperm respectively. Of the significantly differentially methylated CpG islands, 16% were intergenic in sperm (Gillette *et al.*, 2018). These results demonstrate that *in utero* PCB exposure is capable of paternally disrupting sperm DNA methylation across multiple generations.

3.3.4 Other environmental contaminants

Besides POPs, other environmental contaminants have been shown to induce inter/transgenerational epigenetic inheritance of adult onset diseases. Bisphenol A (BPA) and phthalates are abundant endocrine disrupting chemicals in plastics and environmentally relevant doses have been shown to affect male fertility and sperm DNA methylation profiles in rodents across multiple generations (Manikkam *et al.*, 2013; Salian *et al.*, 2009). Vinclozolin, a commonly used fungicide, was reported to transgenerationally affect sperm DNA methylation profiles in rodents as well (Guerrero-Bosagna *et al.*, 2010; Stouder *et al.*, 2010).

Occupational heavy metal exposure, such as mercury, was previously shown to be correlated to altered DNA methylation at imprinted gene *H19* in sperm from reproductive-aged men (Lu *et al.*, 2018). S. K. Murphy *et al.* (2018) observed changes in DNA methylation due to cannabis and tetrahydrocannabinol (THC) in rat sperm using RRBS. The same authors showed associations between cannabis use and DNA methylation in human sperm (S. K. Murphy *et al.*, 2018). Other studies have shown the impact of cigarette smoking on DNA methylation and multiple phenotypic parameters in human sperm (Alkhaled *et al.*, 2018; Laqqan *et al.*, 2017). Negative associations between paternal smoking and sperm counts of the offspring have also been documented (Axelsson *et al.*, 2018). Chemotherapy induced epigenetic alterations in the sperm methylome of adult men who had undergone cancer-related chemotherapy (Shnorhavorian *et al.*, 2017) and an increased risk of congenital abnormalities among the offspring have been documented by others (Stahl *et al.*, 2011).

In addition to environmental contaminants, specific nutrients, can directly affect the epigenome due to their functional roles in DNA methylation and histone modifications. The expression of critical genes associated with physiological and pathological processes, including embryonic development, ageing, and carcinogenesis can thereby be influenced by nutrients (Mazzio et al., 2014). Since this thesis utilizes folic acid as a dietary supplement, the main focus of the upcoming chapter will be on folate and its importance during developmental and cellular processes.

4. Folate

Folate, or vitamin B9, is an essential nutrient that is required for a range of fundamental enzymatic reactions involved in nucleotide biosynthesis, DNA replication, methylation reactions and vitamin metabolism (Crider *et al.*, 2012). Folate, therefore, plays a crucial role in fetal growth and development, as such demands increase during pregnancy. Folate is a member of the 13 essential vitamins as it cannot be synthesized *de novo* in humans (Donnelly, 2001). As a result, it must be obtained from either diet or supplementation. Rich sources of dietary folate are dark leafy greens, legumes, egg yolks, citrus fruits and liver. The synthetic form of folate is folic acid, which is found in fortified foods and vitamin supplements (Crider *et al.*, 2012).

4.1 Folic acid metabolism

Dietary folate usually exists as polyglutamates and needs to be enzymatically converted to monoglutamates by folylpoly-γ-glutamate carboxypeptidase in order to be transported. Folic acid (FA), however, is absorbed by the duodenum and jejunum better (Donnelly, 2001). To become metabolically active, folate and FA are converted to dihydrofolate (DHF) and then to tetrahydrofolate (THF) through reactions that are catalyzed by dihydrofolate reductase (DHFR). Subsequently, THF is transformed into 5,10-methylenetetrahydrofolate (5,10-methylene THF) by the vitamin B6-dependent enzyme serine hydroxymethyltransferase (SHMT) (Crider *et al.*, 2012; Donnelly, 2001). Finally, 5,10-methylene THF is converted by methylenetetrahydrofolate reductase (MTHFR) into the predominant micronutrient 5-methyltetrahydrofolate (5-methylTHF), which acts as a methyl group donor for methionine synthesis from homocysteine (Figure 11).

Methionine is required as a substrate for S-adenosyl methionine (SAM), which plays an essential role in methylation reactions such as DNA, RNA, histone and neurotransmitter methylation (Stover, 2009). SAM also acts as an inhibitor for MTHFR, such that MTHFR is inhibited when SAM concentrations are high which then reduces the synthesis of 5-methylTHF and remethylation of homocysteine. On the contrary, when SAM concentrations are low, remethylation of homocysteine is favored (Crider *et al.*, 2012; Locasale, 2013).

Together with FA, several other dietary nutrients, including vitamin B6, riboflavin, vitamin B12 and choline, are required to maintain 1-carbon flux, homocysteine remethylation, SAM formation and DNA methylation. Improper functioning of folate metabolism, however, affects both methylation and DNA synthesis.

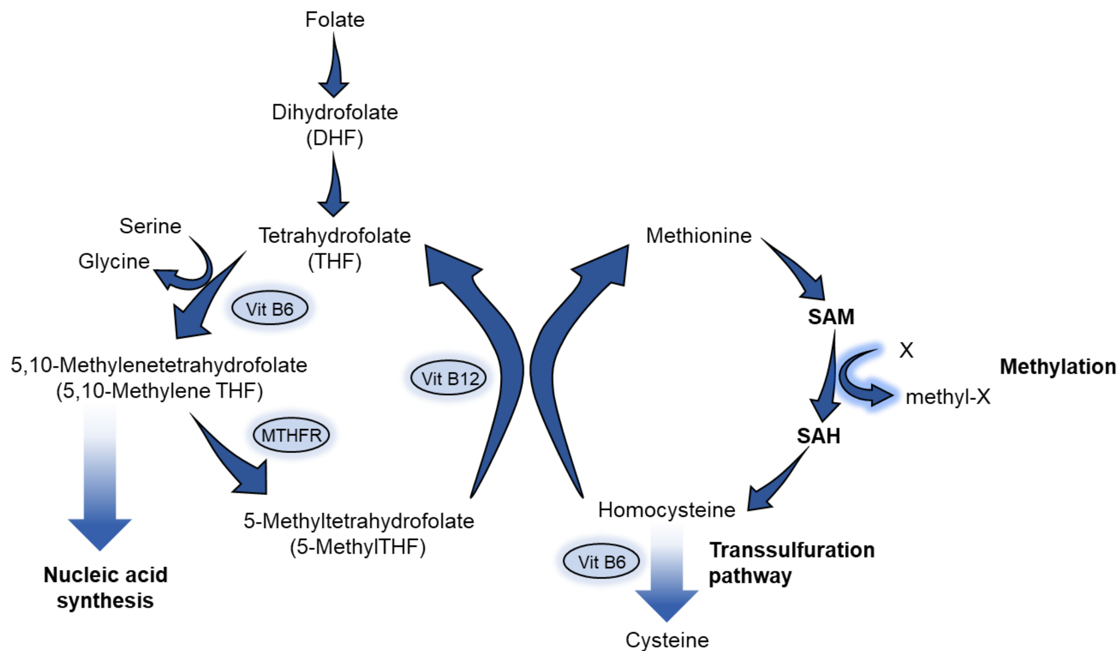

Figure 11. Folate metabolism. Adapted from (Crider *et al.*, 2012). © 2019 by P. Herst

4.2 Folate deficiency

Folate/FA deficiency can be either "absolute" or "functional". Absolute folate deficiency refers to very low folate intake, which can be caused by deficient dietary intake and impaired folate absorption as a result of gastrointestinal disorders or genetic defects in absorption pathways. In contrast, functional folate deficiency is caused by mutations resulting in impaired activity of enzymes involved in folate metabolism.

Well known consequences of maternal folate deficiency are associated with pregnancy affecting the fetus and newborn infant e.g. neural tube defects (NTDs) including spina bifida and anencephaly. NTDs are among the most common category of birth defects worldwide and result from incomplete closure of the neural tube during the fourth week of gestation (Greene *et al.*, 2014). NTDs are fatal or result in life-long disabilities. Embryonic neural crest

cells have a high folate demand as they highly express folate receptors. Folate accommodates the rapidly dividing and developing embryo by regulating (1) cell division and maintenance via *de novo* DNA synthesis and repair, (2) epigenetic regulation via DNA methylation and (3) re-methylation of plasma homocysteine to methionine (Safi *et al.*, 2012).

During pregnancy, erythrocyte levels increase through erythropoiesis, which requires adequate levels of folate, vitamin B12 and iron. Folate deficiency can lead to decreased impaired DNA synthesis thereby inhibiting nuclear division and thus erythrocyte production resulting in anemia in mothers (Koury *et al.*, 2004).

Folate deficiency can also affect the conversion of nucleic acids specifically, deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). Misincorporation of uracil instead of thymidine can potentially cause DNA strand breaks and mutagenesis. Repetitive uracil misincorporation leads to insufficient cycles of DNA repair, ultimately causing chromosome breakage, which further worsen/intensifies chromatin changes. Normal methylation of RNA, histones and phospholipids can also be affected (Lamprecht *et al.*, 2003).

4.3 Folic acid fortification: a public health success story

In the 1960's, researchers became aware of the relationship between apparent folate deficiency and NTDs (Bower *et al.*, 1989; Hibbard *et al.*, 1965; Mulinare *et al.*, 1988; Smithells *et al.*, 1983). Subsequently, the British Medical Research Council initiated an randomized control trial to investigate the impact of FA supplementation as a preventive measure for recurrent NTDs ("Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group," 1991). Women with a history of conceiving children with NTDs had a 70% reduced risk aby taking a 4000 µg FA supplement daily. In a following study, an 800 µg daily FA supplement reduced the risk of having a first child with NTDs by 100% (Czeizel *et al.*, 1992). As a result, the Centers for Disease Control and Prevention, U.S. Public Health Service and the U.S. Preventive Services Task Force encouraged pregnant women or women childbearing age to consume a daily FA supplement (~ 400 µg) ("Recommendations for the use of folic acid to reduce the

number of cases of spina bifida and other neural tube defects," 1992; Centers for Disease, 1991; Force, 2009).

Although this supplementation approach is a step in the right direction, many pregnancies are unplanned and thus many women do not take FA during such critical periods (Tam *et al.*, 2005). The neural tube closure takes place only at 28 days after conception; therefore, women should preferably take the supplement before becoming pregnant. Consequently, regulations for mandatory FA fortification in wheat flour were introduced in 53 countries (Centers for Disease *et al.*, 2010). In Canada, mandatory FA fortification of cereal grain products (150 µg/100 g) was fully implemented in 1998 (Ray, 2008). Since then, a pronounced reduction of NTDs has been reported (Ray *et al.*, 2002). De Wals *et al.* (2007) showed an overall of 46% reduction of NTD cases post mandatory fortification across seven Canadian provinces between 1993 and 2002. Other countries such as South Africa, Costa Rica, Chili, Argentina, Brazil and the United States observed similar and/or stronger trends (L. T. Chen *et al.*, 2004; Hertrampf *et al.*, 2004; Lopez-Camelo *et al.*, 2010; Williams *et al.*, 2002).

4.4 Concerns about adverse effects of folic acid overconsumption

Although mandatory FA fortification reduced the number of NTD cases in many countries, safety concerns have arisen about the effects of FA overconsumption (Y. I. Kim, 2004; O'Neill *et al.*, 2014; A. D. Smith *et al.*, 2008). In Canada, the estimated intake of FA from fortified foods is ~ 100 to 200 µg per day and pregnant women are advised to take an additional multivitamin containing 400 µg daily. Aside fortified foods, many supplements contain > 400 µg of FA leading to FA doses above the tolerable upper intake level of 1000 µg per day (Allain-Doiron *et al.*, 2009).

Higher doses of FA have been shown to mask symptoms of vitamin B12 deficiency which could lead to a missed diagnosis and sub-acute degeneration of the spinal cord, particularly in the elderly (MacFarlane *et al.*, 2011). High folate status in vitamin B12 deficient individuals was also associated with further impairment of vitamin B12 metabolism including decreased activity of two vitamin B12 dependent enzymes and increased circulating methylmalonic acid and tHcy, a biomarker of numerous chronic diseases (Selhub

et al., 2007; Selhub *et al.*, 2009). Several European countries decided to not adopt mandatory FA fortification. The Dutch Health Council and United Kingdom's Food Standards Agency Board even recommended against it, partly due to the potential masking the diagnosis of vitamin B12 deficiency.

Wien *et al.* (2012) performed a systematic review and meta-analysis of (randomized) controlled studies of FA supplementation and cancer incidence/mortality and observed a "borderline significant increase" in the frequency of total cancer risk in the FA group compared to control. Two other independent studies found similar results (Qin *et al.*, 2013; Vollset *et al.*, 2013).

4.5 Effect of paternal folate deficiency and supplementation

Recent advances in research show that adequate paternal folate levels may be as important to the development and health of their offspring as those of the mother. Lambrot *et al.* (2013) investigated the effect of a folate deficient and sufficient diet on DNA methylation in spermatogenesis and pregnancy outcomes in mice. Male mice received a control FA-sufficient (2 mg/kg diet) or a FA-deficient (0.3 mg/kg diet) diet starting *in utero* until testes were collected at postnatal day 6, 10, 12, 14 and 18. To assess reproductive fitness, multiple adult mice of both treatment groups were kept for two or four months. Indeed, adult males receiving a FA deficient diet showed a significantly reduced pregnancy rate compared to control FA sufficient mice. Offspring from FA deficient males had more developmental abnormalities including craniofacial and musculoskeletal malformations. Using a genome-wide methylation analysis approach named methylated DNA immunoprecipitation (MeDIP), 57 differentially methylated genomic regions in sperm of mice receiving FA deficient diet were observed. Reduced levels of sperm H3K4 and H3K9 monomethylation were also detected due to the FA deficiency diet (Lambrot *et al.*, 2013). These research outcomes highlight environmentally sensitive regions of the sperm epigenome in response to diet and ultimately stress the importance of adequate paternal folate levels with regard to reproductive success i.e. healthy pregnancy outcomes.

Ly *et al.* (2017) studied the intergenerational effect of paternal folate deficiency and supplementation on the sperm epigenome, reproductive outcomes and offspring health. F0 dams were fed either a control FA sufficient (2 mg/kg diet), a 7-fold FA deficient (0.3 mg/kg diet), a 10-fold high FA supplemented (20 mg/kg diet) or a 20-fold high FA supplemented (40 mg/kg diet) diet, starting four weeks before gestation and throughout mating, parturition and lactation. The F1 male offspring were given the same as their respective prenatal diets until day of sacrifice at postnatal day 200. At 18 weeks old, F1 males were mated with untreated females. These pregnant females were given a chow diet (18% protein) also throughout gestation and lactation. The resultant F2 males were kept on a regular mouse chow diet. Plasma and red blood cell (RBC) total folate concentrations showed that 7-fold FA deficient F0 dams and F1 males had significantly lower RBC and plasma folate concentrations relative to control. The 10- and 20-fold FA supplemented F0 dams and F1 males had significantly higher RBC and plasma folate concentrations. No differences were observed in F2 males. In contrast to other functional health parameters, F1 sperm counts were significantly lower in the FA deficient and 20-fold FA supplemented groups. No congenital malformations, including craniofacial/ limb abnormalities and cleft palate were observed in any of the treatment groups. Litters sired by 20-fold FA supplemented F1 males, however, showed an increase number of resorptions and abnormalities in the embryos. F1 males from all treatment groups showed similar sperm DNA methylation levels of 70-75%. The paternally imprinted *H19* gene showed high levels of methylation in all F1 males and were not affected by the diets. Increased DNA methylation on several imprinted genes were observed in the F2 placentas of the 20-fold FA supplementation group. F2 sperm counts did not differ between treatment groups (Ly *et al.*, 2017). These results highlight the impact of FA deficiency and supplementation on the reproductive fitness and epigenetic phenotype of the exposed F1 developing male germ cells and its subsequent generation F2. It is likely, however, that other epigenetic mechanisms, that were not assessed in this study including histone methylation, are also affected by the diets.

Various studies in men have shown the impact of paternal FA on semen parameters. Two observational studies conducted by Boxmeer *et al.* (2009) and Wallock *et al.* (2001) showed that both low serum and seminal folate levels are inversely related to semen quality. A few

randomized trials have shown a positive impact of high FA supplementation (5 mg daily or >10 times the daily recommended allowance of 400 µg) on semen concentration/quality in infertile men (Boonyarangkul *et al.*, 2015; Ebisch *et al.*, 2007; Wong *et al.*, 2002). On the contrary, Aarabi *et al.* (2015) showed that six months of high FA supplementation (5 mg daily) caused an unexpected significant global DNA methylation loss across different regions of the sperm epigenome of normozoospermic men with idiopathic male infertility using RRBS. DNA methylation loss was exacerbated in sperm of individuals with the MTHFR 677T polymorphism (genotype TT). Therefore, recommending high-dose FA supplementation to infertile men should be taken with caution. A recent prospective cohort study investigated the association between periconceptional paternal FA status and embryonic growth trajectories in early pregnancy (Hoek *et al.*, 2019). The study included 511 pregnancies of which 202 were conceived naturally and 208 pregnancies by in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI). According to their RBC folate concentrations, fathers were assigned into four groups including, quartile (Q)1: 525-874 nmol/l, Q2: 875-1018 nmol/l, Q3:1019-1195 nmol/l (reference level) and Q4: 1196-4343 nmol/l. The study showed that low (Q1 and Q2) and high (Q4) long-term paternal FA status are associated with reduced embryonic growth trajectories between gestational age seven and eleven weeks of natural pregnancies compared with Q3. Particularly longitudinal crown-rump length was significantly reduced in group Q2 and Q4 of natural pregnancies. Similar results were found in Q4 for embryonic volume. In the IVF-ICSI groups no significant associations were found between FA and embryonic growth trajectories (Hoek *et al.*, 2019). Authors speculate that IVF-ICSI hormone therapy overrules the influence of paternal folate status on the epigenetic reprogramming of the embryo and receptivity status of the endometrium (Hoek *et al.*, 2019; Lonergan *et al.*, 2006). Together, these results suggest that both low and high paternal periconceptional folate status are associated with reduced embryonic growth trajectories in natural pregnancies.

Taken together, these studies highlight the role of folate/FA on the epigenome, specifically DNA methylation, and potential effects on sperm parameters and the health of subsequent generations.

5. Project design

5.1 Main hypotheses and objectives

1. ***In utero* exposure to an environmentally relevant Arctic mixture of POPs perturbs the sperm epigenome, thereby affecting embryonic development of subsequent generations in a transgenerational fashion via the paternal germline.**

    a. We aim to determine the molecular underpinnings of how exposure to an environmentally relevant Arctic mixture of POPs perturbs the sperm epigenome and affects the embryonic development of the next and subsequent generations in a paternally-mediated manner using a proven rat model.

Rationale: Environmental POPs exposure poses a health risk to the Northern Canadian Indigenous populations, due to direct consumption of contaminated country food including fish and marine mammals as well as terrestrial animals such as caribou, geese in addition to eggs and berries (Kuhnlein *et al.*, 2000). Over past few years, significant declines of POPs concentrations have been observed in maternal blood within three Arctic regions, yet, Northern Canadian Indigenous populations continue to have the higher levels of POPs compared to Southern Canadians (Donaldson *et al.*, 2010). Substituting nutrient-dense country food to energy-dense and nutrient-poor processed food, however, may have a negative health impact e.g. obesity, type 2 diabetes and cardiovascular disease (Donaldson *et al.*, 2010; Kuhnlein *et al.*, 2004). Northern Canadian Indigenous populations already have a ten-year shorter life expectancy, with more chronic diseases, increased rates of neo/postnatal deaths, adverse birth outcomes as well as preterm births and infant mortality compared to the rest of Canada (Donaldson *et al.*, 2010; Sheppard *et al.*, 2017; Wilkins *et al.*, 2008). It is tempting to speculate that POPs exposure takes part in promoting some of these adverse health effects, especially considering that epidemiological studies have associated (maternal) POPs exposure with shorter pregnancy durations, affected neurodevelopment, endocrine function, and immune function in Arctic populations (Dallaire *et al.*, 2013; Weihe *et al.*, 2016). POPs exposure has also been shown to alter the sperm epigenome, e.g. DNA methylation (Consales *et al.*, 2016; Rignell-Hydbom *et al.*,

2005). Furthermore, animal studies have provided a thorough insight in the possibility and mechanisms of inter/transgenerationally inherited epimutations induced by environmental contaminant exposure, including POPs (Ben Maamar *et al.*, 2019; Gillette *et al.*, 2018; Manikkam *et al.*, 2012; Mennigen *et al.*, 2018). Nonetheless, we observed multiple reoccurring limitations in these studies such as:

- Intraperitoneal injections containing pharmacological doses of POPs (Anway *et al.*, 2005)
- POPs exposure only during several days of gestation (Ben Maamar, Sadler-Riggleman, Beck, & Skinner, 2018; Gillette *et al.*, 2018; Skinner *et al.*, 2018)
- Results are based on the effect of one POP contaminant, rather than a mixture (Manikkam *et al.*, 2012)
- The sperm epigenome is monitored throughout F1 – F3 and not further (Q. Chen *et al.*, 2015; Gillette *et al.*, 2018)
- Both the dam and sire are exposed (Anway *et al.*, 2005)
- The impact on the early embryonic gene expression is unknown (Mennigen *et al.*, 2018)
- No nutritional intervention against contaminants investigated (Anway *et al.*, 2005; Guerrero-Bosagna *et al.*, 2010; Manikkam *et al.*, 2012; Nilsson *et al.*, 2018)

As such, in the present thesis, we converted these limitations into strengths which will discussed starting in *section 5.2*.

2. **An early life nutritional intervention of FA supplementation reduces or counteracts the POP induced sperm epimutations and associated transgenerational phenotypes.**

    b. We aim to determine whether early life FA supplementation can protect the sperm epigenome from any damage induced by the environmentally relevant

Arctic mixture of POPs and improve the embryonic development of future generations

Rationale: It is well known that maternal supplementation with folic acid protects the fetus against many developmental abnormalities (Y. M. Chan *et al.*, 2015; S. Liu *et al.*, 2016). Folic acid functions as a methyl donor in the methyl cycle, a cycle that is of high importance during prenatal development as rapid cell division and epigenetic reprogramming occur. For this reason, an embryo developing under an insufficient folic acid status is particularly vulnerable to epigenetic errors (Lambrot *et al.*, 2013). FA's potential protective role against environmental pollutants, such as BPA, DDT and air pollutants, has been demonstrated multiple times (Dolinoy *et al.*, 2007; Goodrich *et al.*, 2018; Minguez-Alarcon *et al.*, 2016; Ouyang *et al.*, 2014; Schmidt *et al.*, 2017; Zhong *et al.*, 2017).

As mentioned in *section 5.4*, FA supplementation has also been shown to alter the sperm epigenome (Aarabi *et al.*, 2015; Lambrot *et al.*, 2013; Ly *et al.*, 2017). FA supplements are already prescribed to pregnant women and is readily accessible in fortified foods, FA is therefore an appealing candidate for a nutritional intervention against POPs.

5.2 Animal model

Rats (*Rattus norvegicus*) where chosen as a model because they are a classical organism to study reproductive and developmental toxicology (Zimmermann *et al.*, 2000b). Some of the unique advantages of the rat model include their relatively small size, yet they are bigger than mice, which allows researchers to harvest larger sized organs and tissue volumes, they are easy to manipulate and have a short life-cycle (Zimmermann *et al.*, 2000a). Their gestational length is approximately 22 days and litter sizes range between 6 and 12 (Sengupta, 2013). Rats provide larger sperm samples than mice and, considering the number of analyses to be performed on the same sperm samples to test the thesis hypotheses, this is important. Furthermore, previous studies conducted by our laboratory identified a positive response to environmental contaminants using the same model (Anas *et al.*, 2005; Maurice *et al.*, 2018). We specifically selected the outbred Sprague-Dawley rat line, as outbred models preserve the broad genetic variety / heterogeneity and thus mimic the genetic situation in human populations (Zimmermann *et al.*, 2000b).

5.3 Pollutant mixture

The POPs mixture utilized represents the contaminant composition found in ringed seal (*Pusa hispida*) blubber from the Canadian Arctic, a traditional Northern Canadian Indigenous food (D. Muir *et al.*, 1999). The mixture compromises more than 15 POPs including its major component, a custom Arochlor and congener neat mix containing multiple PCBs congeners (Table 5.1).

An earlier study, conducted by our laboratory, tested multiple doses of this mixture on male rat reproductive development (Anas *et al.*, 2005). F0 Sprague-Dawley dams were exposed to either a low (50 µg/kg BW), medium (500 µg/kg BW) or high (5000 µg/kg BW) dose of PCBs (incl. other POPs) thrice weekly, five weeks before gestation and until parturition. F0 dams were mated to unexposed males. Although no F0 dams exposed to the high-dose treatment died, they did show several signs of systemic acute systemic toxicity (Anas *et al.*, 2005). High-dose POPs exposure also led to a high mortality rate in the F1 male offspring. Low- and medium-dose treatments did not show such or any signs of toxicity. F1 male offspring descendent from medium-dose exposed F0 dams, showed altered testes weight at PND 21 and lower ventral prostate weights at PND 60. At PND 90, the epididymal, ventral prostate, and seminal vesicle weights were lower compared to control. Sperm motility parameters were significantly altered at PND 90 comparing the medium-dose treatment group to control. Perhaps most important, in contrast to low-dose, the medium-dose treatment resulted in comparable concentrations that were detected in maternal plasma of Northern Canadian Indigenous women who gave birth (Butler Walker *et al.*, 2003).

Considering these results, the medium-dose was utilized throughout the whole course of the present study as it generates comparable plasma POPs concentrations to those observed in Arctic populations and are, therefore, environmentally relevant (Anas *et al.*, 2005; Bjerregaard *et al.*, 2001; Butler Walker *et al.*, 2003). Furthermore, POPs were dissolved in corn oil to deliver the treatment by gavage.

Compound	CAS no.	Origin[a]	% Weight
Aroclor and congener neat mix[b]		AccuStandard	32.40
Technical chlordane	57-74-9	AccuStandard	21.40
p,p′-Dichlorodiphenyldichloroethylene (DDE)	72-55-9	Sigma-Aldrich	19.30
p,p′-Dichlorodiphenyltrichloroethane (DDT)	50-29-3	Sigma-Aldrich	6.80
Technical toxaphene	8001-35-2	AccuStandard	6.50
α-Hexachlorocyclohexane (HCH)	319-84-6	Sigma-Aldrich	6.20
Aldrin	309-00-2	Sigma-Aldrich	2.50
Dieldrin	60-57-1	Sigma-Aldrich	2.10
1, 2, 4, 5-Tetrachlorobenzene	95-94-3	Sigma-Aldrich	0.90
p,p′-Dichlorodiphenyldichloroethane (DDD)	72-54-8	Sigma-Aldrich	0.50
β-Hexachlorocyclohexane (HCH)	319-85-7	Sigma-Aldrich	0.40
Hexachlorobenzene	118-74-1	AccuStandard	0.40
Mirex	2385-85-5	Sigma-Aldrich.	0.20
γ -hexachlorocyclohexane or Lindane *(γ*-HCH)	58-89-9	Sigma-Aldrich	0.20
Pentachlorobenzene	608-93-5	Sigma-Aldrich	0.20

Table 5-1. Composition of environmentally relevant POPs mixture (Anas et al. 2005 Biol Reprod). [a]AccuStandard Inc. (New Haven, CT); Sigma-Aldrich Inc (St Louis, Missouri). [b]Aroclor and congener neat mix contains 2,4,4′-trichlorobiphenyl (PCB 28, 1%), 2,2′,4,4′-tetrachlorobiphenyl (PCB 47, 0.80%), 3,3,4,4′-tetrachlorobiphenyl (PCB 77, 0.0044%), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126, 0.02%), Aroclor 1254 (39.30%), and Aroclor 1260 (58.90%).

5.4 FA doses

FA doses were designed to mimic two different scenarios after mandatory FA fortification was introduced in Canada. (1) The "1X" FA experimental diet represents the recommended daily allowance (RDA) of 0.4 mg FA, from fortified foods, in humans consuming a daily average of 2000 kcal. In rats, this FA dose would approximate their basal required FA intake of 2 mg/kg diet FA. (2) The "3X" FA experimental diet represents the FA intake from fortified foods plus a daily FA vitamin supplement of ~ 1.0 mg FA (3-fold the RDA). In rats, this equals an intake of 6 mg/kg diet FA (B. G. Swayne *et al.*, 2012).

For the 1X FA experimental diet, AIN-93 food pellets, containing 2 mg/kg diet FA, were used (Reeves *et al.*, 1993). For the 3X experiment diet, AIN-93 food pellets were modified to contain 6 mg/kg diet FA. Both diets contained the same choline and methionine levels (Reeves *et al.*, 1993).

5.5 Experimental design

F0 founder females (n=8) were divided into four treatment groups:

- Control (0 µg/kg BW POPs; 1X FA)
- POPs (500 µg/kg BW POPs; 1X FA)
- FA (0 µg/kg BW POPs; 3X FA)
- POPs+FA (500 µg/kg POPs; 3X FA)

Treatments were administered 5 weeks before reproduction and until parturition. F1 males were bred with untreated females to obtain F2 offspring. Likewise, F3 and F4 generation offspring were generated. Only F0 founder females were directly exposed to the POPs mixture and/or 3X FA diet (Figure 12). Subsequent F1-F4 offspring received a 1X FA diet. At each generation, caudal epididymal sperm were collected from at least 12 male rats per treatment group at PND 150. To investigate early development, we collected two-cell embryos from the untreated females that were mated with “ancestrally treated” males in generation F1-F2.

Sperm were subjected to miRNA, ChIP and RRBS sequencing analyses whereas two-cell embryos were subjected to transcriptomic analyses to determine which paternal epigenetic components were affected by the developmental exposures. Due to limited amount of sperm, and many parameters to investigate, we randomly pooled sperm ($5 \cdot 10^6$ cells per male) of three to four individuals, descendent from different F0 dams. In total, three pools per treatment were established, representing nine to twelve individuals. The same “pooling” was done for two-cell embryos.

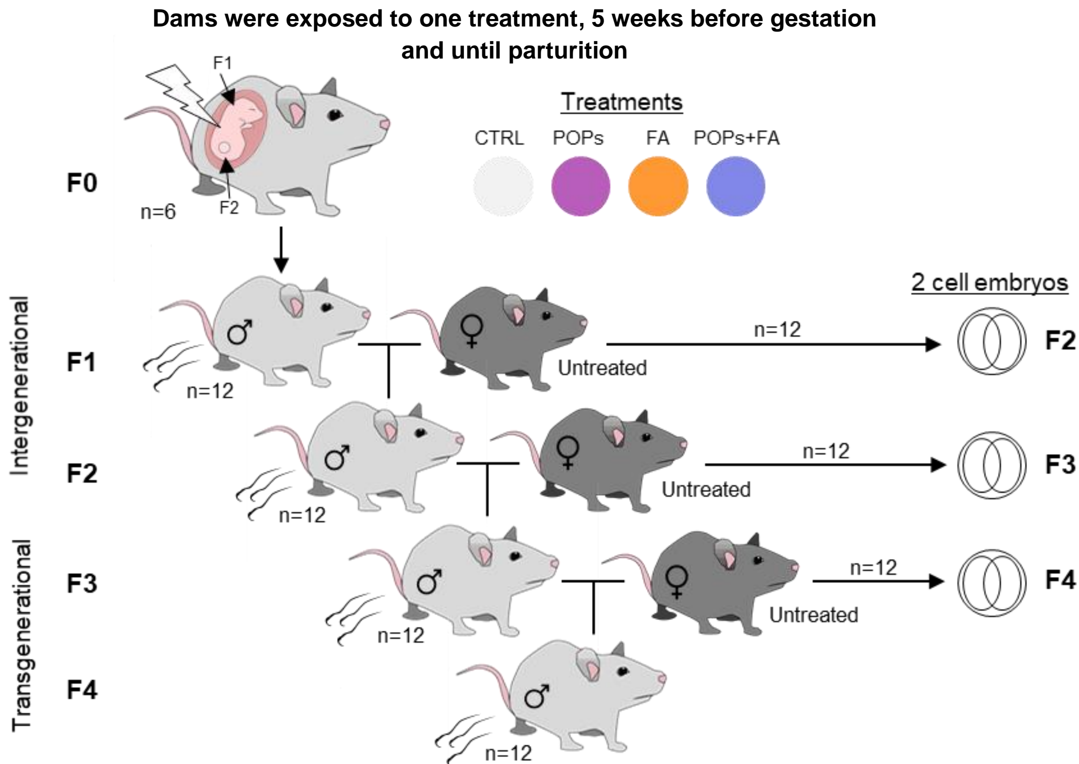

Figure 12. *Experimental design.* © 2019 by P. Herst

5.5.1 Paternal miRNA expression

As described in *section 2.4*, sperm carry various types of sncRNAs such as miRNAs, piRNAs and tsRNAs. Sperm RNAs play a critical role during early embryonic development, as removal has been shown to decrease blastocyst formation and live birth rate in embryos. Body weight of the F1 offspring was also lower compared to control (L. Guo *et al.*, 2017). Among the sncRNAs, miRNAs have been the most studied. Changes in sperm miRNA expression may affect spermatogenesis, sperm maturation and male fertility (Gunes *et al.*, 2013; Hayashi *et al.*, 2008; Miska *et al.*, 2016).

Previous studies, utilizing *in vitro* fertilization or experimental manipulation of total sperm content, demonstrated offspring phenotypes associated with the paternal environment including behavioral changes, obesity and altered glucose metabolism (Q. Chen *et al.*, 2015; Dupont *et al.*, 2019; Gapp *et al.*, 2014; Gapp *et al.*, 2018; Grandjean *et al.*, 2015).

Given the evidence that sperm-born sncRNAs, including miRNAs, may serve a role in epigenetic inheritance, we hypothesize that *in utero* exposure to POPs alters miRNA expression in sperm transgenerationally. In addition, POPs-induced dysregulation of miRNA expression is reduced or prevented by FA supplementation. Therefore, total miRNA content was extracted from sperm and subjected to sequencing using the Illumina HiSeq 2500 system at the Next-Generation Sequencing Platform.

5.5.2 Histone activation mark H3K4me3 in sperm

Although, the majority of histones is removed during spermatogenesis, as described in *section 2.5*, multiple studies have shown the importance of such retained histones in the transmission of paternal epigenetic information to the next generation. Hammoud *et al.* (2009) showed strong enrichments of H3K27me3, H3K4me2 and H3K4me3 at certain developmental promotors in sperm from fertile donors. Shortly after, Brykczynska *et al.* (2010) demonstrated that sperm nucleosomes particularly contain H3K27me3 and H3K4me2, which are primarily expressed during spermatogenesis and/or during later stages of embryogenesis in mouse and human sperm. The early embryo shows low, yet detectable levels of paternal H3K4me3 marks (X. Liu *et al.*, 2016; Zheng *et al.*, 2016). Overexpressing H3K4 demethylase KDM1A during spermatogenesis in mice reduces H3K4me2 in sperm and impairs the development and survivability of the offspring (Siklenka *et al.*, 2015). These studies suggest that these H3 lysine methylation marks could transmit a paternal epigenetic memory (Lambrot *et al.*, 2013; Zhang *et al.*, 2016).

Here, we hypothesize that that *in utero* POPs exposure alters the paternal histone H3K4me3 levels transgenerationally. FA supplementation prevents or reduces these H3K4me3 alterations. We performed chromatin immunoprecipitation (ChIP) followed by next generation sequencing to measure histone H3K4me3 levels in F1-F4 sperm.

The success of ChIP is highly dependent on the specificity of the antibody. R. N. Shah *et al.* (2018) recently examined the performance of 52 commercially available H3K4me antibodies and showed that many commonly used antibodies poorly distinguish between H3K4me1, me2 and me3, leading to different results/interpretations between studies (R. N. Shah *et al.*, 2018). These data were carefully taken into consideration for the choice of antibody in the

present study especially since only ~1% of the histones are retained in rat sperm (Brykczynska *et al.*, 2010; Hammoud *et al.*, 2009). The ChIP protocols from Hisano *et al.* (2013) and Siklenka *et al.* (2015) were customized accordingly.

5.5.3 The sperm methylome

The sperm methylome is also sensitive to the environment and transgenerational alterations have been observed previously, as seen in *section 3*. Within the present study, we hypothesize that DNA methylation at specific loci of the paternal epigenome is modified by *in utero* POPs exposure and thereby compromises the health of the offspring and subsequent generations. FA supplementation prevents/reduces the transgenerational effects induced by POPs in sperm.

We used Reduced Representation Bisulfite Sequencing (RRBS) to generate methylation profiles of the F1-F4 sperm. In contrast to whole genome bisulfite sequencing (WGBS), RRBS includes digestion of the input DNA using a methylation-insensitive restriction enzyme (e.g. *Msp1*) that specifically cleaves at CpG-rich fragments, after which the DNA is bisulfite converted and sequenced (Meissner *et al.*, 2008). Subsequently, using bisulfite conversion, the unmethylated cytosines are converted into uracil, whereas methylated cytosines remain unchanged. Lastly, the bisulfite converted DNA is amplified by PCR followed by sequencing. Generally, between 0.01-0.03 µg of high-quality DNA is required for RRBS, while WGBS requires 5 µg (Gu *et al.*, 2011). As such, RRBS has been widely used due to its low DNA requirements.

The RRBS protocol has been successfully used and validated previously in our laboratory. The sequencing results were validated using pyrosequencing. Importantly, somatic cell contamination, in all sperm samples, was measured before the RRBS protocol was carried out.

5.5.4 Two-cell embryo transcriptome

As described in *section 2.6*, parental epigenetic information, particularly DNA methylation, is rapidly erased upon fertilisation, and during PGC migration (Figure 8). Furthermore, histone mark H3K4me3 is generally depleted in the paternal genome of the zygote. At the

later two-cell embryo stage, H3K4me3 is rapidly re-established and its levels become comparable to the maternal genome after implantation (Legoff *et al.*, 2019; Zhang *et al.*, 2016). Few studies have focused on the inter/transgenerational effect of paternal stressors on the early-embryo transcriptome (Siklenka *et al.*, 2015).

Here, were hypothesize that *in utero* paternal POPs exposure alters the two-cell embryo transcriptome transgenerationally (F2-F4). Prenatal FA supplementation protects and/or reduces the transcriptomic dysregulation caused by POPs in two-cell embryos. Paternal epigenetic marks escaping reprogramming will presumably be first observed in the two-cell embryo stage. Therefore, two-cell embryos were specifically chosen as their gene expression pattern is more related to the paternal genome. We used RNA-sequencing to map the embryonic genomes.

6. Wildlife and environmental pollution

A complementary study to the main project was also carried out. We were fortunate to collaborate with the Norwegian Polar Institute in Tromsø, Norway, to investigate the impact of POPs on the fat metabolism of wild roaming polar bear mothers and her cubs.

The main scope of the thesis is about the impact of the paternal environment on the offspring using an animal model, therefore, investigating the effect of POPs on Arctic apex predators is pertinent and adds value to the research. Furthermore, with this complementary project, we stretched our scientific knowledge base towards fat and energy metabolism, a novel aspect of POPs toxicity.

6.1 Main hypotheses and objectives

1. **Exposure to POPs alters the adipose tissue gene transcription of polar bear dams and their cubs from Svalbard, Norway.**

    a. We aim to determine the molecular underpinnings of how exposure to POPs perturbs the adipose tissue transcriptome and affects the health of the next generation in a maternally-mediated manner using RNA sequencing.

Rationale: Polar bears (*Ursus maritimus*) are on top of the Arctic food chain and consequently display some of the highest POPs concentrations (Andersen *et al.*, 2001; Letcher *et al.*, 2010; M. A. McKinney *et al.*, 2011; D. C. Muir *et al.*, 2006; Routti *et al.*, 2019; Verreault *et al.*, 2005). Their lipid rich diet mostly consists out of ringed seals (*Pusa hispida*) and bearded seals (*Eringnathus barbatus*) (Derocher *et al.*, 2002; M.A. McKinney *et al.*, 2017; Thiemann *et al.*, 2008).

Pregnant polar bear females retreat to their dens for up to eight months during which they depend almost entirely on energy stored in white adipose tissue which contains over 90% of the POPs body burden (Atkinson *et al.*, 1996; Gebbink *et al.*, 2008; Ramsay *et al.*, 1988). During fasting, these energy stores are mobilized increasing circulating POPs concentrations in the blood (Tartu, Bourgeon, *et al.*, 2017). Besides endocrine disruptive effects, previous

studies suggest that liver and fat extracts of contaminants affect adipogenesis, i.e. differentiation of pre-adipocytes into mature fat cells, in cells originating from both polar bears and mice (Routti *et al.*, 2016). Furthermore, individual PCBs and organochlorine pesticides (OCPs) as well as POP mixtures, reflecting concentrations in polar bears, modulated polar bear peroxisome proliferator-activated receptor gamma (*PPARG*), a key regulator of adipogenesis (Routti *et al.*, 2016).

Major routes of POPs excretion are via gestation and lactation. As a result, cubs are exposed to high levels of POPs during critical stages of development, especially since these POPs levels are tightly related to their mother's (Bytingsvik, Lie, *et al.*, 2012; Bytingsvik *et al.*, 2013; Bytingsvik, van Leeuwen, *et al.*, 2012). Since polar bear cubs are expected to have a high proportion of proliferating preadipocytes as a result of rapid fat deposition (Shimba *et al.*, 2011), they are likely to be more sensitive to POPs induced alterations to the adipose tissue. No studies, however, have focussed on the overall impact of POPs exposure on the adipose tissue gene expression of wild roaming polar bears, especially not mothers and cubs.

6.2 Experimental design

We received adipose tissue biopsies of mother polar bears (n=13) with each a cub pair (♂-♂n=5 | ♂-♀n=4 | ♀-♀n=4) that were sampled in Spring of either 2001, 2012 or 2013. All cubs were approximately 4 months old. ΣPOPs lipid weight concentrations in plasma (ng/g) of mother bears was measured by our collaborators as described previously (Tartu, Lille-Langoy, *et al.*, 2017) (Figure 13). The fat biopsies of the cubs were too scant to assess ΣPOPs lipid weight concentrations. POPs concentrations in cubs, however, have been shown to be highly correlated to their mothers (Bytingsvik, Lie, *et al.*, 2012; Bytingsvik, van Leeuwen, *et al.*, 2012).

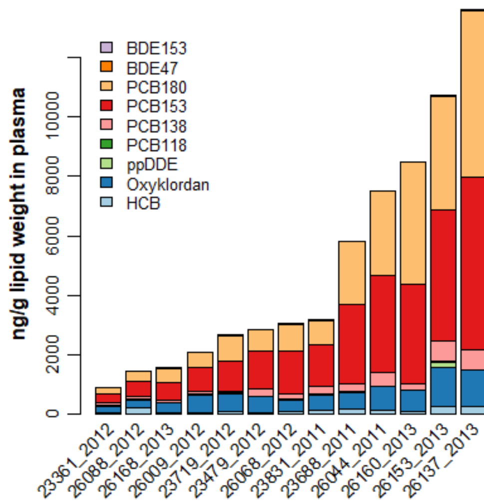

Figure 13. ΣPOPs lipid weight concentrations in plasma of all biopsies sampled from mother bears.

Transcriptomes were analyzed using RNA sequencing and results were validated using PCR. We assessed the correlation between POPs exposure and adipose tissue gene expression in both mothers and cubs.

Taken together, it is essential to investigate the impact of such pollutants on human and wildlife health considering their persistence in the environment and long-term effects.

Chapter 1

Folic acid supplementation reduces multigenerational sperm miRNA perturbation induced by *in utero* environmental contaminant exposure.

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1.1 Resume

Les polluants organiques persistants (POPs) peuvent induire des changements épigénétiques dans la lignée germinale paternelle. Ici, nous rapportons que la supplémentation en acide folique (AF) atténue les profils de miARN spermatiques de manière transgénérationnelle après une exposition *in utero* paternelle aux POPs dans un modèle de rat. Les mères fondatrices gestantes ont été exposées à un mélange de POPs (ou à de l'huile de maïs) représentatif de l'environnement ± supplémentation en AF. Les descendants mâles F1-F4 ultérieurs n'ont pas été exposés aux POPs et ont été nourris avec le régime de contrôle de l'AF. Les profils de miARN spermatiques des lignées intergénérationnelles (F1, F2) et transgénérationnelles (F3, F4) ont été étudiés en utilisant un séquençage en profondeur des miARN. À travers les générations F1-F4, les profils de miARN spermatiques étaient moins perturbés par les POPs + FA que les spermatozoïdes de descendants de mères traitées avec des POPs seuls. L'exposition aux POPs a systématiquement conduit à l'altération de trois miARN spermatiques sur deux générations, et similairement, un miARN spermatique a été altéré en raison de l'exposition aux POPs + AF. Ce dernier était commun avec un des miARN spermatiques modifiés de manière intergénérationnelle par les POPs. Les miARN spermatiques affectés par les POPs seuls sont connus pour cibler les gènes impliqués dans le développement des glandes mammaires et des organes embryonnaires en F1, la différenciation sexuelle et le développement du système reproducteur en F2 et le développement de la cognition et du cerveau en F3. Cependant, lorsque le traitement aux POPs a été combiné avec une supplémentation en AF, ces mêmes voies génétiques ciblées par les miARN ont été perturbées dans une moindre mesure et uniquement dans les spermatozoïdes des F1. Ces résultats suggèrent que l'AF atténue partiellement l'effet des POPs sur les miARN dérivés paternellement d'une manière intergénérationnelle.

1.2 Abstract

Persistent organic pollutants (POPs) can induce epigenetic changes in the paternal germline. Here we report that folic acid (FA) supplementation mitigates sperm miRNA profiles transgenerationally following *in utero* paternal exposure to POPs in a rat model. Pregnant founder dams were exposed to an environmentally-relevant POPs mixture (or corn oil) ± FA supplementation and subsequent F1-F4 male descendants were not exposed to POPs and were fed the FA control diet. Sperm miRNA profiles of intergenerational (F1, F2) and transgenerational (F3, F4) lineages were investigated using miRNA deep sequencing. Across the F1-F4 generations, sperm miRNA profiles were less perturbed with POPs+FA compared to sperm from descendants of dams treated with POPs alone. POPs exposure consistently led to alteration of three sperm miRNAs across two generations, and similarly one sperm miRNA due to POPs+FA; which was in common with one POPs intergenerationally altered sperm miRNA. The sperm miRNAs that were affected by POPs alone are known to target genes involved in mammary gland and embryonic organ development in F1, sex differentiation and reproductive system development in F2 and cognition and brain development in F3. When the POPs treatment was combined with FA supplementation, however, these same miRNA-targeted gene pathways were perturbed to a lesser extend and only in F1 sperm. These findings suggest that FA partially mitigates the effect of POPs on paternally-derived miRNA in a intergenerational manner.

Keywords: Organochlorine, transgenerational epigenetic inheritance, micro RNA, prenatal exposure, folate.

1.3 Introduction

Environmental pollutants, including persistent organic pollutants (POPs), pose ongoing threats to global ecosystems (Landrigan *et al.*, 2018). POPs are synthetic organic compounds that resist environmental degradation and are distributed via long-distance atmospheric transport mechanisms to deposit in colder regions, notably the Arctic (Hung *et al.*, 2016; Mackay *et al.*, 1995). International restrictions have decreased POPs over the past decade; however, because of climate change some POPs are released back into the environment (Hung *et al.*, 2010). Due to their lipophilic characteristics, POPs bio-accumulate in adipose tissues putting human and wildlife health at risk (Landrigan *et al.*, 2018).

The male gamete has been shown to be susceptible to damage caused by environmental toxicants such as dichlorodiphenyltrichloroethane (DDT) (De Jager *et al.*, 2006); furthermore, studies have shown that POPs exposure impairs sperm parameters (Anas *et al.*, 2005; Maurice *et al.*, 2018; Mumford *et al.*, 2015), DNA integrity (Spano *et al.*, 2005) and chromatin condensation (De Jager *et al.*, 2006). With respect to the sperm epigenome, DNA methylation can be altered by POPs as was showed previously by a permutation analysis in rats; and flow cytometric immunodetection and PCR pyrosequencing in men (Belleau *et al.*, 2018; Consales *et al.*, 2016). Furthermore, experience-dependent information may potentially be transmitted via sperm small noncoding RNA, such as microRNA (miRNA), from the father to offspring (Rodgers *et al.*, 2015; U. Sharma *et al.*, 2018).

Most studies focus on the effects of individual POPs; however, humans and wildlife are exposed to complex POPs mixtures. We hypothesized that *in utero* exposure to an environmentally-relevant Artic POPs mixture alters the sperm epigenome, specifically miRNA expression, across multiple, unexposed generations (F1 through F4).

Additionally, we investigated whether a nutritional intervention, folic acid (FA), could counteract these multigenerational epigenetic changes. Folate functions as a methyl donor in the methyl cycle, which is vital during prenatal development when epigenetic reprogramming occurs; an embryo developing under an insufficient folate status may be vulnerable to methylation-dependent epigenetic errors (Crider *et al.*, 2012; K. C. Kim *et al.*, 2009).

Therefore, we hypothesized that FA supplementation moderates the POPs-induced dysregulation of sperm miRNA expression in F1 through F4 generations. Using a four-generation rat model (Figure 1.10.1), we analyzed the paternal lineage of sperm (F1-F4) derived from treated F0 dams by miRNA deep sequencing (miRNA-seq).

1.4 Methods

1.4.1 Persistent Organic Pollutant (POPs) mixture.

The POPs mixture (Table 1.10.1) represents the pollutant composition found in Ringed seal blubber of Northern Quebec which is a traditional food of Inuit people in that region (Bengston Nash *et al.*, 2013; D. Muir *et al.*, 1999). Mixture components were dissolved in corn oil (Aldrich-Sigma, Oakville, ON, Canada) to obtain a stock solution of 5 mg polychlorinated biphenyls (PCBs)/ml corn oil including remaining POPs, that was kept in the dark at room temperature (Table 1.10.1). The experimental dose, which is considered environmentally relevant, was made by diluting the stock solution with corn oil to a concentration of 500 µg PCBs/kg body weight as described previously (Anas *et al.*, 2005); concentrations of the other POPs can be calculated from proportions listed in Table 1.10.1.

1.4.2 Animal studies and breeding.

Animal care and all treatment procedures were in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Université Laval animal Research Ethics Committee (certificate # 2015010-2). Forty-five-day-old female outbred Sprague-Dawley rats (Charles Rivers Laboratories, Saint Constant, QC, Canada) were housed two per cage in standard rat cages under controlled lighting (12 h light-dark cycle), humidity (46 ± 10%) and temperature (22 ± 1°C). After 10 days of acclimatization, female rats (F0 founder dams) were randomly assigned to four treatment groups (n=6) designated 'Control (CTRL)', 'Persistent Organic Pollutants (POPs)', 'Folic Acid supplementation (FA)' and 'Persistent Organic Pollutants + Folic Acid supplementation (POPs+FA)' (Figure 1.10.1). F0 founder dams were gavaged with the POPs mixture corresponding to 500 µg PCBs /kg body weight (Anas *et al.*, 2005); or corn oil (CTRL) thrice weekly and were fed the AIN-93G diet (Reeves *et al.*, 1993) containing either 2 mg/kg (1X) or 6 mg/kg (3X) of FA (#110700 and #117819 Dyets Inc., Bethlehem, PA) ad libitum. Experimental diets represent the North American FA intake in the post-fortification era (1X) and in combination with a daily 1 mg FA prenatal multivitamin (3X) (B. G. Swayne *et al.*, 2012). Treatments were only administered to F0 founder dams for 9 weeks in total; 5 weeks before mating to untreated males at postnatal day

(PND 90) and until parturition. Subsequent lineages, F1 through F4, were neither exposed to POPs nor 3X FA – instead they received 1X FA diet ad libitum.

To maximize genetic diversity, F1 male offspring, descendent from different litters, were randomly selected (two per litter) to establish subsequent generations for each treatment lineage. At PND 90, F1 males (n=12) from CTRL, POPs, FA and POPs+FA were bred with untreated females (PND 70) to obtain the F2 lineage. Likewise, F3 and F4 generation lineages were generated. At PND 150, F1-F4 males were anesthetized using 3% isoflurane and sacrificed by exsanguination via cardiac puncture followed by CO_2 asphyxiation.

1.4.3 Sperm isolation.

Sperm were recovered from the caudal epididymides of F1-F4 Control, POPs, FA and POPsFA male rats (n=12) as follows: dissected caudal epididymides were placed into prewarmed Gibco®Medium-199 without phenol red (Life Technologies, Burlington, ON, Canada), nicked several times using a scalpel, and incubated at 37°C while gently agitating to allow sperm to diffuse from the epididymis. After 30 min, diffused sperm were centrifuged at 2,500 xg for 10 min at 4°C. Supernatant was removed, somatic cell contamination was avoided by washing the sperm pellet twice with hypotonic buffer (0.45% NaCl w/v) and centrifuged at 2,500 xg for 5 min at 4°C. Subsequently, the sperm pellet was washed twice with cold 1X phosphate-buffered saline (PBS) and centrifuged at 2,500 xg for 5 min at 4°C. After the second PBS wash, the pellet was resuspended in 500 µl Freezing Medium Test Yolk Buffer with gentamicin sulfate (Irvine Scientific, Edmonton, AB, Canada) and incubated for 10 min at room temperature. Last, collected sperm were stored at -80°C.

1.4.4 RNA extraction.

To minimize the impact of individual variation within treatment lineages, sperm were pooled from four males, with each descendant from different F0 founder dams, to provide a total of ~20 x 10^6 sperm per pool; CTRL, POPs, FA, POPs+FA (n = 3 pools composed of four individuals per pool; Figure 1.10.1). Total RNA was extracted from pooled sperm using mirVana™ miRNA Isolation Kit (Life Technologies) according to the manufacturer’s instructions and eluted in 50 µl of the provided elution buffer.

1.4.5 Reverse transcriptase PCR.

To validate F1-F4 miRNA sequencing results, reverse transcriptase PCR was conducted using sperm from the same individuals that were also pooled for miRNA sequencing. Multiple miRNAs (>1000 normalized read counts) with a fold-change (CTRL/treatment) of >1.5 were selected for validation. Several other miRNAs with a fold change of ~ 1 or -1 were considered as an endogenous control. We normalized using the same miRNA (miR-99a-5p) for all treatments and all generations. Extracted RNA was reverse transcribed with provided primers using the miScript II RT Kit (QIAGEN, Toronto, ON, Canada) according to manufacturer's instructions. cDNAs were subjected to Real Time PCR using the miScript SYBR® Green PCR , Kit (QIAGEN) and the following primers (QIAGEN):
Rn_miR-34c*_1 miScript Primer Assay, Rn_miR-16_2 miScript Primer Assay, Rn_miR-340-5p_2 miScript Primer Assay, Rn_miR-30b_1 miScript Primer Assay, Rn_miR-1_2 miScript Primer Assay, Rn_miR-547_2 miScript Primer Assay, Rn_miR-489*_1 miScript Primer Assay, Rn_miR-429_1 miScript Primer Assay, Rn_miR-471_1 miScript Primer Assay, Rn_miR-125a_1 miScript Primer Assay and Rn_miR-101a_3 miScript Primer Assay. miR_99a_5p was used as endogenous control, miR_99a_3' (5'-CTG CCA CAG ACC CAT AGA AAC-3') and miR_99a_5' (5'-ATC CGA TCT TGT GGT GAA GTG-3'). The PCR protocol was carried out using the LightCycler® 480 (Roche Life Science) with the following program: pre-denaturation of one cycle at 95°C for 15 min, followed by PCR amplification for 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and elongation at 70°C for 30 s.

1.4.6 miRNA sequencing.

NEBNext Multiplex Small RNA (New Englands Biolabs Inc., Ipswich, MA) was used to prepare miRNA sequencing libraries according to manufacturer's instructions. Twenty-four libraries were prepared using 30 ng miRNA purified using mirVana miRNA isolation kit (Thermo Fisher Scientific, Mississauga, Canada). 3' SR adaptors were ligated to the 3' end of miRNA followed by ligation of SR RT primers to the 5'end of miRNA-3' adaptors, which was further used for the reverse transcription step. Subsequently, 5' SR adaptors were ligated to the 5' end of miRNA. Following reverse transcription, an amplification for 13 cycles was performed to incorporate specific indexes for multiplexing. After purification using GenElute

PCR clean-up kit (Sigma-Aldrich, St-Louis, MO), the appropriate range of cDNA fragments (120-150 bp) was extracted on a 3% gel using a Pippin Prep instrument (Sage Science, Beverly, MA). Samples were quantified using a QBit 3.0 fluorometer (Thermo Fisher Scientific, Mississauga, Canada). miRNA libraries were pooled in equimolar ratio and the quality was examined with a DNA screentape D1000 HS on a TapeStation 2200 (Agilent Technologies, Santa Clara, CA). The final length range of libraries was verified and contained only the fraction of miRNAs. Subsequently, miRNA libraries were sequenced using two lanes of a rapid run flowcell on an HiSeq 2500 system at the Next-Generation Sequencing Platform, Genomics Center, CHU de Québec Research Center, Quebec City, Canada for single read 50 bp sequencing.

1.4.7 Bioinformatic analysis.

The raw sequence quality was validated using FastQC v0.11.4 (Andrews, 2010). Quality filtration of fastq reads and adaptor removal were carried out using Trimmomatic v0.35 (Bolger *et al.*, 2014) with the following options: ILLUMINACLIP:2:30:10, TRAILING:3, LEADING:3, SLIDINGWINDOW:4:15 and MINLEN:16. Trimmed sequences were converted to fasta format using custom bash script. Blast alignment was performed using blast v2.2.31+ against the *Rattus norvegicus* sequences extracted from the miRBase database release 21 with the blastn-short algorithm, a word size of 4 and a maximal E-value of 0.01 (Altschul *et al.*, 1997; Griffiths-Jones *et al.*, 2006). Blastn results were aggregated and counts were normalized using R v3.2.0 (Team, 2013). The FactoMineR package was used to produce the Principal Component Analysis (PCA) plots. Differential expression analysis was performed using the DESeq2 v1.20.0 package (Lê *et al.*, 2008; Love *et al.*, 2014).

For subsequent analysis, a statistical significance for differential expression was set to *p-value* ≤ 0.05, FDR $\leq 5\%$ and miRNAs were considered significantly differentially expressed when the difference was 0.58 on the Log2 scale ($-1.5 \geq |\text{fold change}| \geq 1.5$) (CTRL vs Treatment). Gene-ontology analysis (GO) was performed using Ingenuity® Pathway Analysis (IPA®, Ingenuity Systems Inc., Redwood City, CA) and Metascape (Tripathi *et al.*, 2015) to identify gene targets that were experimentally validated by TarBase and miRecords pathways and highly predicted gene targets by TargetScan.

1.5 Results and Discussion

In support of our initial hypothesis, we first demonstrate that *in utero* exposure to POPs altered intergenerational sperm miRNA profiles. A total of 747 different miRNAs was detected in the sperm of rats from each of the CTRL, POPs, FA and POPs+FA lineages in F1-F4 generations (Figure 1.10.2), of which a total of 91 miRNAs were significantly differentially expressed compared to CTRL (*p-value* ≤ 0.05; FDR ≤ 5%; -1.5 ≥ |fold change| ≥ 1.5). *In utero* exposure to POPs dysregulated 10 miRNAs (10↑, 0↓) by 1.5-fold in F1 (F1 CTRL vs. F1 POPs; Figure 1.10.2A, B). Micro RNA dysregulation due to POPs exposure persisted across his subsequent unexposed generations, 10 miRNAs in F1, 37 miRNAs in F2 and 10 miRNAs in F3 and 1 miRNA in F4. The profile of small RNAs, including miRNAs, can be altered by environmental events and subsequently persist to modulate gene expression over multiple generations (Houri-Zeevi *et al.*, 2017). In our model, since F1 males and their developing germline were directly exposed to POPs, perturbation of F1 sperm was predicted. As the F2 sons were derived from the exposed F1 paternal germline, perhaps sperm produced by the F2 sons contain upstream chromatin and/or DNA methylation changes that escaped remodelling during development and spermatogenesis that could impact sperm miRNA profiles (Donkin *et al.*, 2018). For instance, altered RNA profiles, including noncoding RNA, were observed as a result of reduced sperm H3K4me2 in F1 transgenic mice, compared to control (Siklenka *et al.*, 2015).

Although others have reported inter- and transgenerational perturbation of non-coding RNAs in sperm following intraperitoneal injections of pharmacological levels of pesticides (Ben Maamar, Sadler-Riggleman, Beck, McBirney*, et al.*, 2018; Skinner *et al.*, 2018), ours is the first to demonstrate that environmentally-relevant ancestral contaminant exposures disrupt the sperm miRNA profile. *In utero* exposure to POPs+FA supplementation altered fewer miRNAs 1 (1↑, 0↓) in F1 sperm compared to POPs (10; 10↑, 0↓). It is tempting to speculate that maternal consumption of 3X FA diets may have partly protected her offspring's sperm epigenome from toxicant-induced perturbation.

Interestingly, various developmental and disease conditions induced by POPs (e.g. neurodevelopmental deficits, altered reproductive functions and immunotoxicity) are related

to oxidative stress–mediated cellular damage (Betteridge, 2000; Pham-Huy *et al.*, 2008; Wells *et al.*, 2009). Studies in humans reported oxidative stress after accidental polychlorinated biphenyl (PCB) poisoning or occupational POPs exposures (W. Guo *et al.*, 2016; Wen *et al.*, 2008). Furthermore, oxidative stress has been shown to alter small non-coding RNA (including miRNA) expression in somatic cells and sperm (Engedal *et al.*, 2018; Mostafa *et al.*, 2016). The protective role of FA supplementation in the F1 sperm may be partly explained by its antioxidant activity if the miRNA changes are caused by oxidative stress induced by POPs exposure (i.e. F1 POPs males) (X. Guo *et al.*, 2015; Joshi *et al.*, 2001; S. J. Lee *et al.*, 2011). If, however, the miRNA changes in POPs exposed sperm are due to an altered methylation capacity or dysregulated nucleotide synthesis or mutations, then the increased availability of methyl groups provided by FA supplementation may mitigate the POPs effect by supporting DNA repair through nucleotide synthesis. Additional studies of the interaction between POPs and FA are required.

With respect to the interaction between FA and POPs, a previous cross-sectional study observed an inversed relationship between folate concentration and dichlorodiphenyltrichloroethane (DDT) isomers including metabolites in the blood of healthy women; the authors proposed that (1) folate may increase DDT (including metabolites) metabolism and excretion, and (2) DDT decreases the levels of folate in the body (Arguelles *et al.*, 2009). This provides insight to possible similar events taking place in directly exposed sperm.

Similar to the POPs treatment, the number of altered miRNAs unexpectedly increases from F1 to F2 due to ancestral POPs+FA (1 miRNA in F1 and 12 miRNAs in F2; Figure 1.10.2A). Based on previous findings in animal studies, *in utero* FA supplementation alters the sperm epigenome via DNA methylation and chromatin structure/histone modifications over multiple generations (Bernal *et al.*, 2010; Lambrot *et al.*, 2013; Ly *et al.*, 2017). In turn, these FA induced epigenetic changes in sperm could alter the methyl donor pool and subsequently impact gene expression during spermatogenesis including expression of miRNA.

Concerning the intergenerational effect observed in F1 and F2 due to POPs and POPs+FA, it remains puzzling how environmentally-perturbed paternal miRNAs can persist across multiple generations (Yuan *et al.*, 2016). To become heritable, parts of the sperm chromatin must escape reprogramming, leading to the possibility that sperm miRNA profiles are subsequently modified by environmental factors (Houri-Zeevi *et al.*, 2017). There are clear examples of sperm DNA methylation that escape reprogramming and histones can be involved (Siklenka *et al.*, 2015).

We performed gene-ontology analysis (GO) to identify gene targets of the significantly dysregulated miRNAs by 1.5-fold in F1-F4 (Figure 1.10.2C). Previous studies have shown that some POPs are endocrine disrupters and interfere with hormone-regulated processes including genital development, puberty onset and sperm production (Gregoraszczuk *et al.*, 2013; Vested *et al.*, 2014). Interestingly, here we show that *in utero* exposure to POPs particularly affected miRNAs implicated in mammary gland development (p = 1.97E-14) and embryonic organ development (p = 1.62E-11) in F1, whereas POPs+FA did not (Figure 1.10.2C, left). In fact, only a few similar pathways were significantly affected by POPs and POPs+FA, such as cancer pathways (POPs p = 1.04E-31; POPs+FA p = 9.92E-05), PI3K-Akt signalling pathway (POPs p = 2.21E-21; POPs+FA p = 0.004) and blood vessel morphogenesis (POPs p = 1.41E-27; POPs+FA p = 3.99E-05) in F1. Although similar pathways were perturbed, POPs+FA appeared to affect fewer genes compared to POPs in F1. With regard to FA treatment alone, altered sperm miRNAs were specifically implicated in brain development (p = 8.53E-08) and developmental growth (p = 3.31E-07) in F1.

In F2, *in utero* exposure to POPs affected miRNAs in cancer (p = 6.76E-31), sex differentiation (p = 1.29E-13), brain development (p = 7.28E-23) and reproductive system development (p = 1.02E-29) (Figure 1.10.2C, middle), similar to F1. Not seen in F1, however, blood vessel (p = 7.18E-27), heart (p = 3.98E-26) and kidney (p = 2.37E-09) development were affected by *in utero* POPs exposure in F2. Several similarities were observed between POPs and POPs+FA, though, again fewer genes seemed to be affected by POPs+FA and FA alone in F2.

In F3, ancestral exposure to POPs disrupted pathways involved in response to oxidative stress (p = 2.61E-10) cognition (p = 5.65E-10) and brain development (p = 1.44E-08) (Figure 1.10.2C, right). Also in F3, four pathways were affected by both ancestral POPs and POPs+FA, which was similar to in F2. Again, fewer genes were altered due ancestral POPs+FA compared to POPs alone.

No significantly affected pathways were observed due to all treatments in F4 ($p \leq 0.05$). Taken together, each treatment affected the sperm miRNA profile differently in each generation, implying different multigenerational signatures mediated by miRNAs.

Next, we identified several treatment-specific dysregulated miRNAs compared to CTRL in F1-F4 generations that were unique to POPs exposure and/or FA supplementation (Figure 1.10.2A). In F1, 9 miRNAs were treatment-specific for POPs (purple), 5 miRNAs for FA and 0 miRNAs for POPs+FA (blue). Also in F1, POPs and POPs+FA shared 1 miRNA independently from FA (Figure 1.10.2A). POPs and POPs+FA consistently shared miRNAs until F2.

To further explore whether dietary FA supplementation in F0 dams can correct the dysregulated miRNA expression induced by ancestral POPs exposure, we compared the fold change of treatment-specific miRNAs due to POPs versus POPs+FA (Figure 1.10.3A, B). Consistent with our hypothesis, we repeatedly observed that the dysregulated miRNA fold-changes due to POPs+FA in F1-F3 generations were corrected or shifted towards CTRL levels (Figure 1.10.3A).

As examples, in F1, besides rno-miR-6334, the majority of miRNAs are up regulated due to POPs (purple) and restored or close to restored by FA supplementation (POPs+FA, blue; Figure 1.10.3B left). This effect was more profound in F2 as 25 out of 29 miRNAs were restored by POPs+FA. Even in F3, we observed a mitigating effect by the FA supplementation on POPs dysregulated miRNAs, as six out of ten miRNAs were brought back towards to control levels. Regardless of treatment, when compared to control, the Fold

change intensity of the significantly altered sperm miRNAs lessened across generations, particularly after F2.

To further investigate whether *in utero* exposure to POPs and POPs+FA supplementation alters sperm miRNA expression transgenerationally, we identified overlapping and non-overlapping dysregulated miRNAs between generations per treatment lineage (Figure 1.10.4 A, B). Several studies have shown altered sperm miRNA expression profiles due to paternal diet/lifestyle (de Castro Barbosa *et al.*, 2016; Fullston *et al.*, 2013; Paris *et al.*, 2015; Rodgers *et al.*, 2013); however, few reported transgenerational inheritance of sperm miRNAs (Grandjean *et al.*, 2015; Rodgers *et al.*, 2015). Here, we found 3 intergenerational (between F1 and F2 generations) dysregulated miRNAs due to POPs exposure including rno-miR-6334, rno-miR-19b-3b and rno-miR-30b-5p (Figure 1.10.4A). Interestingly, previous studies showed that the miR-30 family plays an important regulatory role in tissue and organ development, more specifically, and pertinent to our study, reproductive development (Mao *et al.*, 2018). MiR-30 is highly expressed in both mouse and human testis tissue and is associated with the Homeobox protein and Zn transport, which are critical for male fertility (Madison-Villar *et al.*, 2011). MiR-19 has been previously shown to be implicated in intergenerational inheritance as microinjection of either testis or sperm miR-19b of male mice fed a Western-like diet, into native one-cell embryos, lead to a Western-like diet-induced metabolic phenotype in his offspring (Grandjean *et al.*, 2015). No significantly differentially expressed miRNAs were altered beyond F2, therefore, no transgenerational epigenetic inheritance was induced by ancestral POPs exposure.

In utero exposure to POPs+FA supplementation intergenerationally (F1 versus F2) dysregulated one miRNA (Figure 1.10.4A). In contrast to the POPs lineage, the miR-30 and miR-19 families were not affected. Only miR-6334 was intergenerationally affected due to POPs+FA until F2. Little is known about the role of miR-6334, and no experimentally validated gene targets have been detected so far.

Using real-time PCR, three miRNAs were validated in sperm from the same individuals that had been previously pooled for miRNA sequencing (Figure 1.10.4C, D). To be detectable

using qPCR, miR34c-5p, 340-5p and 471-5p were selected based on their normalized read counts of > 1000 and > 1.5 fold change (CTRL/treatment). Nonetheless, we observed comparable results between miRNA sequencing data and qPCR data.

Lastly, concerning the phenotypic outcomes of the current study, our team previously described in a corresponding, complementary study subtle but significant deleterious effects of prenatal exposure to POPs on male reproductive function and early embryo gene expression across at least three generations (Lessard *et al.*, 2019). In that study, sperm quality and fertility were reduced in F2 and F3 males, respectively. Further, the poorest pregnancy outcomes were observed in F3 males and F4 two-cell embryos had the highest number of significantly differentially expressed genes compared to untreated control animals (Lessard *et al.*, 2019).

In conclusion, this is a unique demonstration of the vulnerability of the paternal epigenome to the ancestral environment. We show that *in utero* exposure to environmentally-relevant contaminants perturbs sperm miRNAs intergenerationally, but that the severity of perturbation decreases after the F2 generation. Moreover, this is the first report of a nutritionally-pertinent intervention that can mitigate the effect of such contaminants.

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1.7 Author Contributions

Authors contributed to initial conception or design (JLB, JMT, SK, AJM); final design (PMH, MD, JLB); data acquisition, analysis, or interpretation (PMH, ML, PLC, PN, MV, AD, MD, JLB), drafting the manuscript (PMH); and critically revising the manuscript (PMH, ML, PLC, PN, MV, AD, JMT, SK, AJM, MOBB, MD, JLB). All authors gave final approval and agreed to be accountable for all aspects of work in ensuring that questions relating the accuracy or integrity of any part of the work are appropriately investigated and resolved.

1.8 Competing Interests Statement

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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1.10 Figures and tables

Table 1.10.1. Composition of environmentally relevant POPs mixture (Anas et al. 2005 Biol Reprod). [a]AccuStandard Inc. (New Haven, CT); Sigma-Aldrich Inc (St Louis, Missouri). [b]Aroclor and congener neat mix contains 2,4,4′-trichlorobiphenyl (PCB 28, 1%), 2,2′,4,4′-tetrachlorobiphenyl (PCB 47, 0.80%), 3,3,4,4′-tetrachlorobiphenyl (PCB 77, 0.0044%), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126, 0.02%), Aroclor 1254 (39.30%), and Aroclor 1260 (58.90%).

Compound	**CAS no.**	**Origin[a]**	**% Weight**
Aroclor and congener neat mix[b]		AccuStandard	32.40
Technical chlordane	57-74-9	AccuStandard	21.40
p,p′-Dichlorodiphenyldichloroethylene (DDE)	72-55-9	Sigma-Aldrich	19.30
p,p′-Dichlorodiphenyltrichloroethane (DDT)	50-29-3	Sigma-Aldrich	6.80
Technical toxaphene	8001-35-2	AccuStandard	6.50
α-Hexachlorocyclohexane (HCH)	319-84-6	Sigma-Aldrich	6.20
Aldrin	309-00-2	Sigma-Aldrich	2.50
Dieldrin	60-57-1	Sigma-Aldrich	2.10
1, 2, 4, 5-Tetrachlorobenzene	95-94-3	Sigma-Aldrich	0.90
p,p′-Dichlorodiphenyldichloroethane (DDD)	72-54-8	Sigma-Aldrich	0.50
β-Hexachlorocyclohexane (HCH)	319-85-7	Sigma-Aldrich	0.40
Hexachlorobenzene	118-74-1	AccuStandard	0.40
Mirex	2385-85-5	Sigma-Aldrich.	0.20
γ -hexachlorocyclohexane or Lindane (*γ*-HCH)	58-89-9	Sigma-Aldrich	0.20
Pentachlorobenzene	608-93-5	Sigma-Aldrich	0.20

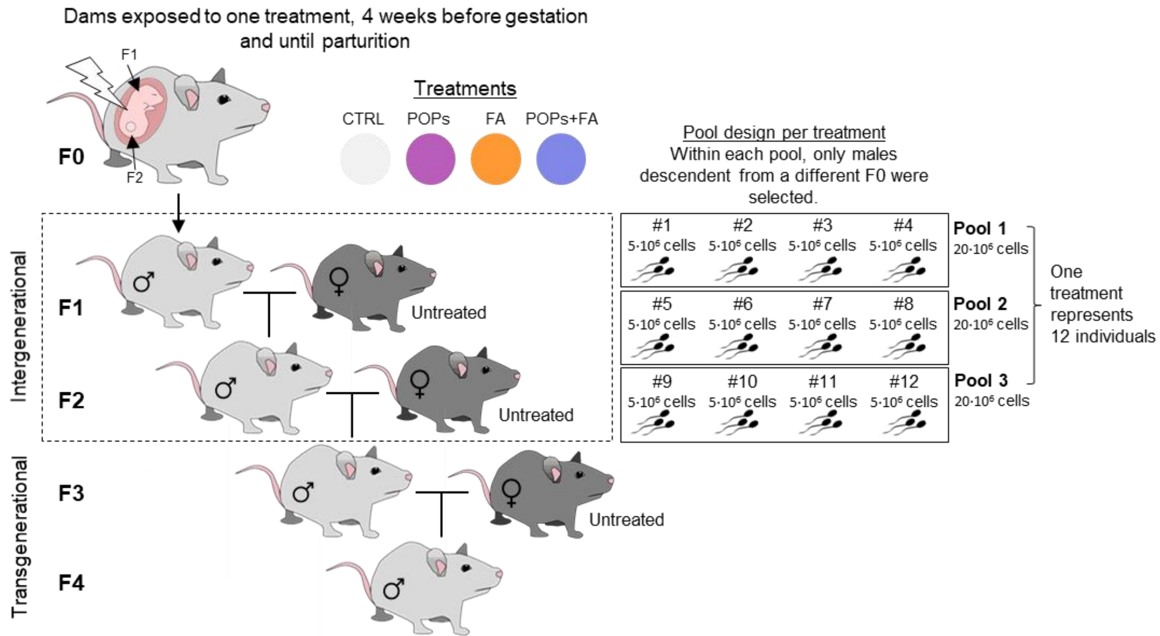

Figure 1.10.1. Experimental design. Four treatment groups of Sprague-Dawley F0 founder females (n=6) were gavaged with either an environmentally-relevant POPs mixture (500 µg PCBs plus remaining POPs/kg body weight) or corn oil (control); in addition, the F0 females received diets ad libitum containing 2 mg/kg diet (1X) or 6 mg/kg diet (3X) FA representing the North American FA intake in the post-fortification era (1X) and with a daily 1 mg FA prenatal multivitamin (3X) respectively. Treatments were administered 5 weeks before reproduction (x untreated males) and until parturition. After birth of the F1, all F0 founder dams and subsequent generations received 1X ad libitum. F1 males were bred with untreated females to obtain F2 offspring. Likewise, F3 and F4 generation lineages were produced. During the establishment of each generation, sperm were collected from 12 males per treatment group at PND 150. Since F0 dams were exposed, an intergenerational effect can be observed from F1 and a transgenerational effect starting from F3.

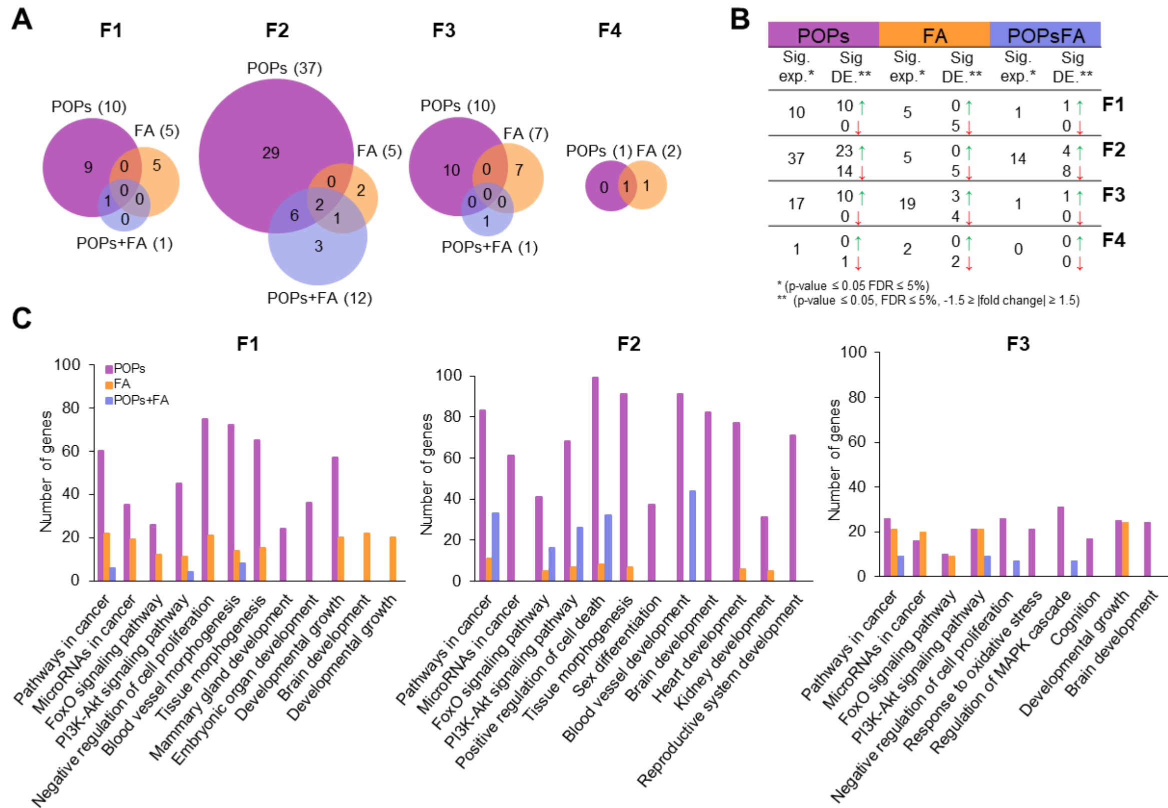

Figure 1.10.2. *In utero* exposure to POPs or POPs+FA alters sperm miRNA expression differentially. (A) *In utero* exposure to POPs and POPs+FA exposure display altered miRNA expression profiles, as revealed by miSeq analyses. Venn diagrams comparing the number and overlap of significantly differentially expressed (*p-value* ≤ 0.05; FDR ≤ 5%; -1.5 ≥ |fold change| ≥ 1.5) miRNAs in POPs (purple), FA (orange) or POPs+FA (blue) compared to CTRL sperm in F1-F4 sperm. **(B)** Table including total number of significantly expressed (Sig. exp.) genes (*p-value* ≤ 0.05, FDR ≤ 5%) and the number of significantly differentially expressed (Sig. DE) genes (*p-value* ≤ 0.05; FDR ≤ 5%; -1.5 ≥ |fold change| ≥ 1.5) that are up- or downregulated indicated by ↑ and ↓ respectively due to POPs, FA or POPs+FA in F1-F4. **(C)** Gene ontology and pathway analysis based on miRNA-targeted genes, that were experimentally validated by TarBase and miRecords, plus highly predicted gene targets by TargetScan, for POPs (purple), FA (orange) and POPs+FA (blue). Top significant ($p < 0.05$) GOs and KEGG enriched pathways predicted by dysregulated miRNAs in F1-F3 are presented. Pathways are ranked by number of miRNA-targeted genes. Consistently, POPs targeted a higher number of genes for listed pathways in F1 and F2. FA and specifically POPs+FA targeted nearly as many genes implicated in similar pathways.

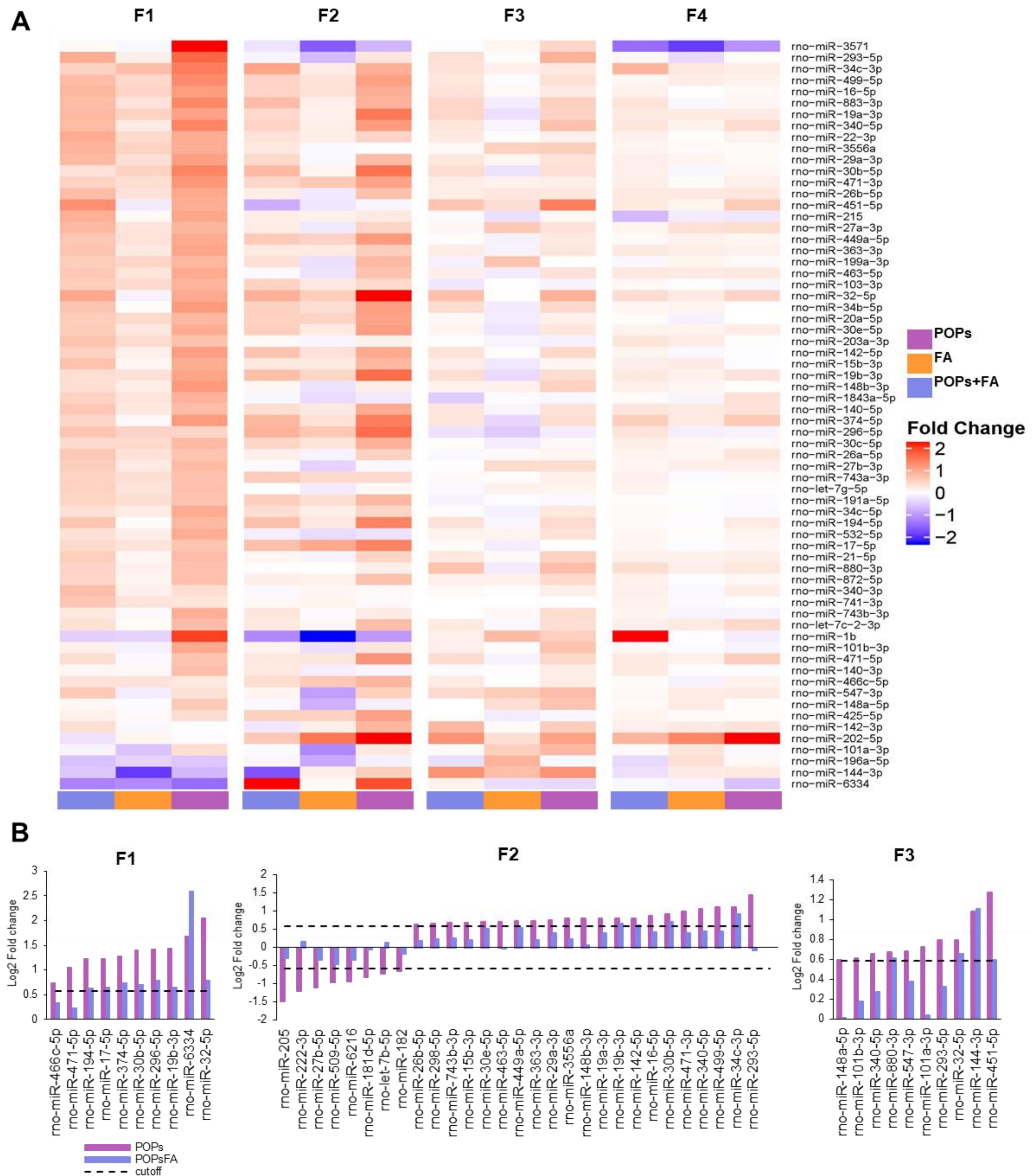

Figure 1.10.3. Combining FA with POPs counteracts the effect of POPs on sperm miRNA expression in F1-F3. (A) Sperm microRNA dynamics of all significant coexpressed sperm miRNAs (*p-value* ≤ 0.05; FDR ≤ 5%) across treatments, POPs (purple), FA (orange), POPs+FA (blue) in F1-F4. A clear dilution effect can be observed after F2 until F4. Additionally, compared to POPs, POPs+FA seems to alter similar sperm miRNAs but to a lesser extent, particularly in F1. **(B)** Graphs illustrating the Log2 Fold change of all sperm miRNAs specifically altered due to POPs (9 in F1, 29 in F2 and 10 in F3) compared to POPs+FA. Dashed line represents Log2 Fold change of 0.58 which equals a fold change of 1.5. All sperm miRNAs with a Log2 Fold change

below 0.58 was considered as “no change” thus control level. In F1-F3, we repeatedly observed the Log2 Fold change of POPs altered sperm miRNAs to be brought back towards control level by POPs+FA.

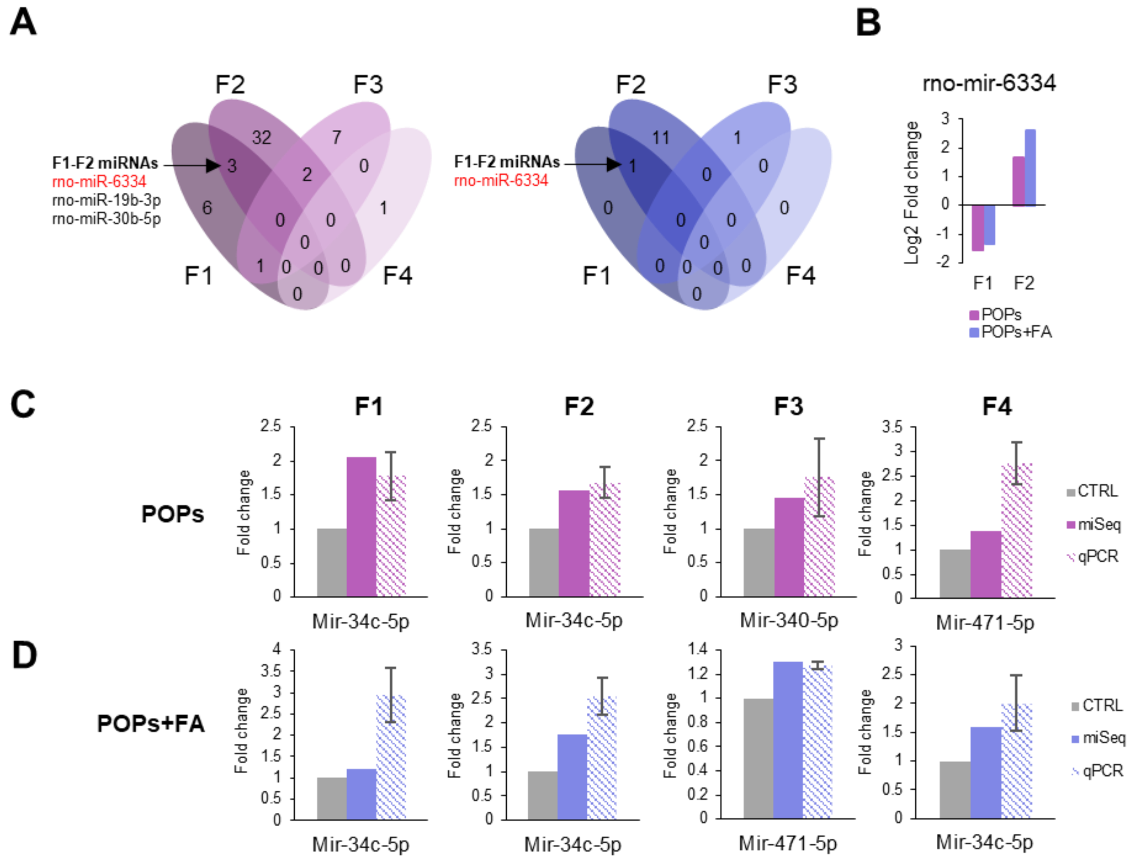

Figure 1.10.4. *In utero* exposure to both POPs and POPs+FA affects sperm miRNA expression intergenerationally (F1-F2). (A) Venn diagrams depicting the overlap of significantly differentially expressed (*p-value* ≤ 0.05; FDR ≤ 5%; -1.5 ≥ |Fold change| ≥ 1.5) miRNAs due to POPs (purple) and POPs+FA (blue) respectively. Three miRNAs were intergenerationally altered due to *in utero* POPs exposure and one miRNA due to POPs+FA. One intergenerational miRNA (rno-miR-6334) was conserved between POPs and POPs+FA. **(B)** The expression of rno-miR-6334 in Log2 Fold change due to POPs (purple) and POPs+FA (blue). Rno-miR-6334 is altered in similar direction due to both treatments. **(C, D)** Validation of POPs (purple) and POPs+FA (blue) miRNA sequencing data using real-time PCR. Total RNA was extracted from CTRL, POPs and POPs+FA sperm. The expression of miRNAs relative to endogenous control RNA was determined by real-time PCR. The results are expressed as a fold change of POPs or POPs+FA to CTRL. Data are presented as means ± S.D. from 3-5 rats, each assay performed in triplicate.

Chapter 2

Folic acid supplementation partially rescues alterations in sperm DNA methylation and histone retention associated with *in utero* environmental pollutant exposure multigenerationally.

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2.1 Resume

Les polluants organiques persistants (POPs) peuvent induire des épimutations dans la lignée germinale paternelle. Nous avons émis l'hypothèse que l'exposition *in utero* à un mélange de POPs représentatif de l'environnement modifie l'expression du méthylome spermatique et de H3K4me3, sur plusieurs générations (non) exposées (F1 à F4). L'acide folique (AF) annule l'effet hypométhylant du BPA sur l'ADN, ainsi, nous avons aussi émis l'hypothèse qu'une supplémentation nutritionnelle en acide folique (AF) empêche ou atténue les effets spermatiques induits par les POPs sur plusieurs générations. Seules les femelles Sprague-Dawley gestantes ont été traitées avec un mélange de POPs représentatif de l'environnement ou de l'huile de maïs ± une supplémentation en AF. Les descendants mâles F1-F4 ultérieurs n'ont pas été exposés aux POPs et ont été nourris avec le régime contrôle d'AF. La méthylation de l'ADN spermatique et les profils d'expression H3K4me3 des lignées intergénérationnelles (F1, F2) et transgénérationnelles (F3, F4) ont été étudiés en utilisant le séquençage au bisulfite à représentation réduite (RRBS) et le séquençage par immunoprécipitation de la chromatine (ChIP-Seq). L'exposition *in utero* aux POPs a affecté la méthylation de l'ADN des spermatozoïdes et l'expression de H3K4me3 de manière transgénérationnelle. La plupart des changements dans la méthylation de l'ADN ont été observés dans les régions distales intergéniques et de haute mer. Contrairement aux sites hyperméthylés différentiellement méthylés (SDM), les SDM hypométhylés, associés à l'exposition aux POPs, étaient principalement impliqués dans les voies de développement neurologique, en particulier en F1. La supplémentation alimentaire en AF en combinaison avec les POPs a affecté les SDM hypométhylés associés au développement osseux en F2 et les voies neurodéveloppementales en F3 et F4. De plus, la supplémentation en AF a partiellement sauvé les méthylations de l'ADN des spermatozoïdes et les changements d'expression de H3K4me3. Nous avons constamment observé un effet de dilution du traitement pour tous les traitements jusqu'à F3, mais pas F4. Ces résultats soulignent le rôle du père dans la transmission des informations environnementales acquises à sa progéniture et aux générations suivantes.

2.2 Abstract

Persistent organic pollutants (POPs) can induce epimutations in the paternal germline. We hypothesized that *in utero* exposure to an environmentally-relevant POPs mixture alters the sperm methylome and H3K4me3 expression, across multiple, (un)exposed generations (F1 through F4). Folic acid (FA) negates the DNA hypomethylating effect of BPA, thus, we further hypothesized that a nutritional intervention of dietary folic acid (FA) prevents or mitigates these POP-induced effects in sperm. Only pregnant Sprague-Dawley founder dams were treated with an environmentally-relevant POPs mixture or corn oil ± FA supplementation. Subsequent F1-F4 male offspring were not exposed to POPs and were fed the FA control diet. Sperm DNA methylation and H3K4me3 expression profiles of intergenerational (F1, F2) and transgenerational (F3, F4) lineages were investigated using reduced representation bisulfite sequencing (RRBS) and chromatin immunoprecipitation sequencing (ChIP-Seq). *In utero* POPs exposure affected sperm DNA methylation and H3K4me3 expression transgenerationally. Most changes in DNA methylation were observed in distal intergenic and open sea regions. In contrast to hyper-methylated differentially methylated sites (DMSs), hypo-methylated DMSs, associated to POPs exposure, were mostly involved in neurodevelopmental pathways, particularly in F1. Dietary FA supplementation in combination with POPs affected hypo-methylated DMSs associated with bone development in F2, and neurodevelopmental pathways in F3 and F4. Furthermore, FA partially rescued POPs induced sperm DNA methylation and H3K4me3 expression changes. We consistently observed a treatment dilution effect in DNA methylation for all treatments until F3, but not F4. These results stress the role of the father in the transmission of acquired environmental information to his offspring and subsequent generations.

Keywords: Organochlorine, transgenerational epigenetic inheritance, DNA methylation, histone retention, prenatal exposure, folate.

2.3 Introduction

Persistent organic pollutants (POPs) pose an ongoing threat to human health and the environment (Landrigan *et al.*, 2018). Their semi-volatile character and high environmental half-lives result in long-range atmospheric transport and global planetary distribution (Hung *et al.*, 2010). POPs bioaccumulate and biomagnify in the food chain and are particularly abundant in the Arctic. Consequently, Indigenous populations encounter elevated exposures to POPs through their reliance on the traditional diet comprised of fish and marine mammals (Laird *et al.*, 2013). Many adverse health effects have been linked to POPs exposure, including impaired neurodevelopment, immune and reproductive function (Carpenter, 2011). Sperm have been shown to be susceptible to damage induced by environmental pollutants such as dichlorodiphenyltrichloroethane (DDT) (De Jager *et al.*, 2006); other studies reported that POP exposure promotes impaired sperm parameters (Anas *et al.*, 2005; De Jager *et al.*, 2006; Maurice *et al.*, 2018; Mumford *et al.*, 2015), DNA integrity (de Jager *et al.*, 2009; Spano *et al.*, 2005) and chromatin condensation (De Jager *et al.*, 2006) in men. The impact of paternal POP exposure, however, can span multiple generations via epigenetic signals inherited by the sperm, and ultimately affect offspring health (Ben Maamar *et al.*, 2019; Ben Maamar, Sadler-Riggleman, Beck, & Skinner, 2018; Herst *et al.*, 2019; Skinner *et al.*, 2018). Identifying the molecular mechanisms of POP induced epigenetic inheritance in sperm is essential to fully understand how a father's environment can impact the health and development of his subsequent generations.

We previously demonstrated that *in utero* exposure to POPs perturbs sperm microRNA (miRNA) profiles over multiple generations in rats (Herst *et al.*, 2019). Furthermore, we observed that POP affected sperm leads to aberrant lipid homeostasis, male reproductive parameters, pregnancy outcomes and early embryonic gene expression in the offspring (Lessard *et al.*, 2019; Navarro *et al.*, 2019). Besides small non-coding RNAs, including miRNAs, experience-dependent information may also be transmitted via other sperm born epigenetic components, including DNA methylation and chromatin packaging mediated by post-translational histone modifications (Ben Maamar *et al.*, 2019; Lambrot *et al.*, 2013; Ly *et al.*, 2017; Skinner *et al.*, 2018).

Thus far, DNA methylation is the most studied epigenetic component in relation to environmental exposures. In sperm, DNA methylation plays an important role in various biological processes including silencing transposable elements, DNA compaction, paternal genomic imprinting, and X-chromosome inactivation (Larson *et al.*, 2016; D. Miller *et al.*, 2010; Stewart *et al.*, 2016; Zamudio *et al.*, 2015). Abnormal DNA methylation is associated with impaired male fertility, affected embryo quality and increased disorder susceptibility in the offspring (Aston *et al.*, 2015; Jenkins *et al.*, 2014). Previous studies have shown that endocrine disrupting chemicals, including certain POPs, can induce DNA methylome alternations in the male reproductive system and disrupt male germ cell epigenomic reprogramming in rodents (Belleau *et al.*, 2018; Consales *et al.*, 2016; Guerrero-Bosagna *et al.*, 2010; Guerrero-Bosagna *et al.*, 2012). Sperm DNA methylation alterations may also persist into the early embryo and influence the transcriptome and epigenome of somatic tissues (Ben Maamar *et al.*, 2019; Wei *et al.*, 2014).

In contrast to somatic cells, most histones are replaced by protamines to allow the condensed genomic structure into the sperm head. Depending on the species, 1-15% of the mammalian genome remains bound to histones (Balhorn *et al.*, 1977; Brykczynska *et al.*, 2010; Gatewood *et al.*, 1990). Several laboratories have demonstrated that histone-bound chromatin regulate embryonic development and facilitate the regulation of early embryonic transcription (Arpanahi *et al.*, 2009; Casas *et al.*, 2014; Gatewood *et al.*, 1987; Hammoud *et al.*, 2009). Histone H3 lysine 4 di-/trimethylation (H3K4me2/3) is of particular interest considering its enrichment at multiple promotors of genes that are highly expressed during spermatogenesis, and localization to HOX gene clusters, noncoding RNAs and paternally expressed imprinted loci (Brunner *et al.*, 2014; Brykczynska *et al.*, 2010; Hammoud *et al.*, 2009). Overexpressing H3K4 demethylase KDM1A during spermatogenesis impairs embryonic gene expression, fetal development and survivability over multiple generations in mice (Siklenka *et al.*, 2015). Furthermore, early embryos show low, yet detectable levels of paternal H3K4me3 marks (X. Liu et al., 2016; Zheng et al., 2016). These studies suggest that these H3 lysine methylation marks could transmit a paternal epigenetic memory (Lambrot et al., 2013; Zhang et al., 2016).

Most studies focus on the effects of individual POPs, the possibility that H3K4me2/3 sites and DNA methylation are altered due an environmentally-relevant POP mixture, in a paternal

manner, over four generations has not been reported (Ben Maamar, Sadler-Riggleman, Beck, & Skinner, 2018; Skinner *et al.*, 2018). Here, we hypothesize that *in utero* exposure to an environmentally-relevant POPs mixture alters the sperm methylome and H3K4me3 expression, across multiple, (un)exposed generations (F1 through F4). We further assessed whether a nutritional intervention of dietary folic acid (FA) prevents or mitigates these POP-induced effects.

2.4 Methods

2.4.1 Persistent Organic Pollutant (POPs) mixture

The POPs mixture (Table 2.12.1) represents the pollutant composition found in Ringed seal blubber of Northern Quebec which is a traditional food of Inuit people in that region (Bengston Nash *et al.*, 2013; D. Muir *et al.*, 1999). Mixture components were dissolved in corn oil (Aldrich-Sigma, Oakville, ON, Canada) to obtain a stock solution of 5 mg polychlorinated biphenyls (PCBs)/ml corn oil including remaining POPs (Table 2.12.1). The experimental dose, was made by diluting the stock solution with corn oil to a concentration of 500 µg PCBs/kg body weight as described previously (Anas *et al.*, 2005); concentrations of the other POPs can be calculated from proportions listed in Table 2.12.1.

2.4.2 Animal studies and breeding

Animal care and all treatment procedures were in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Université Laval animal Research Ethics Committee (certificate # 2015010-2). Forty-five-day-old female outbred Sprague-Dawley rats (Charles Rivers Laboratories, Saint Constant, QC, Canada). After 10 days of acclimatization, female rats (F0 founder dams) were randomly assigned to four treatment groups (n=6) designated 'Control (CTRL)', 'Persistent Organic Pollutants (POPs)', 'Folic Acid supplementation (FA)' and 'Persistent Organic Pollutants + Folic Acid supplementation (POPs+FA)' (Figure 2.11.1). F0 founder dams were gavaged with the POPs mixture corresponding to 500 µg PCBs /kg body weight (Anas *et al.*, 2005); or corn oil (CTRL) thrice weekly and were fed the AIN-93G diet (Reeves *et al.*, 1993) containing either 2 mg/kg (1X) or 6 mg/kg (3X) of FA (#110700 and #117819 Dyets Inc., Bethlehem, PA) ad libitum. Experimental diets represent the North American FA intake in the post-fortification era (1X) and in combination with a daily 1 mg FA prenatal multivitamin (3X) (B. G. Swayne *et al.*, 2012). Treatments were only administered to F0 founder dams for 9 weeks in total; 5 weeks before mating to untreated males at postnatal day (PND 90) and until parturition. Subsequent lineages, F1 through F4, were neither exposed to POPs nor 3X FA – instead they received 1X FA diet ad libitum.

To maximize genetic diversity, F1 male offspring, descendent from different litters, were randomly selected (two per litter) to establish subsequent generations for each treatment lineage. At PND 90, F1 males (n=12) from CTRL, POPs, FA and POPs+FA were bred with untreated females (PND 70) to obtain the F2 lineage. Likewise, F3 and F4 generation lineages were generated. At PND 150, F1-F4 males were sacrificed.

2.4.3 Sperm isolation

Sperm were recovered from the caudal epididymides of F1-F4 Control, POPs, FA and POPsFA male rats (n=12) as described previously (Lessard *et al.,* 2019).

2.4.4 DNA extraction

To minimize the impact of individual variation within treatment lineages, sperm were pooled from three males, with each descendant from different F0 founder dams, to provide a total of ~ $5 \cdot 10^6$ or $16 \cdot 10^6$ sperm per pool; CTRL, POPs, FA, POPs+FA (n = 3 pools composed of three individuals per pool) dependent on the type of analysis (Herst *et al.*, 2019). For DNA extraction, $16 \cdot 10^6$ sperm, from three individuals, were resuspended in 180 µl lysis buffer (1.5 ml 0.5M EDTA pH 8.0, 50 µl 1M Tris pH 8.0, 200 µl 1 M DTT completed to 5 ml ddH_2O) and incubated overnight at 37^oC with 20 µl Proteinase K and 6.5 µl Sarkosyl 30%. Sperm DNA was isolated using the QIAmp DNA Micro Kit (Qiagen, Canada)

2.4.5 Bisulfite pyrosequencing

Prior to DNA methylation analyses, somatic cell contamination was assessed by bisulfite pyrosequencing of multiple imprinted genes. In somatic cells, the average methylation of imprinted genes is approximately 50% as both parental alleles are present and only one is completely methylated. In sperm, however, only one parental allele is present and thus paternally and maternally methylated loci are expected to have a 100% or 0% methylation level, respectively. In the present study, sperm germline differentially methylated regions (DMRs) *DLK1* (paternal) and *PLAGL1* (maternal) were assessed. Isolated sperm DNA was subjected to bisulfite conversion using the EpiTect® Fast DNA Bisulfite Kit (Qiagen #59104, Canada). Next, the bisulfite converted products were amplified by PCR using the PyroMark® PCR Kit (Qiagen #978703, Canada) accompanied by predesigned PyroMark® CpG Assays for *DLK1* and *PLAGL1*. Pyrosequencing was performed using PyroMark® Advanced CpG

reagents (Qiagen #970902, Canada) and the PyroMark® Q24 Vacuum Workstation. Everything was conducing using the manufacturer's instructions.

2.4.6 DNA methylation analyses followed by sequencing

500 µg isolated DNA was digested using Msp1 (New England Biolabs #R0106M, Canada) at 37°C overnight. Next, digested DNA was subjected to DNA end repair and A-tailing using Klenow fragment (3'→ 5' exo, New England Biolabs #M0212M, Canada). AMPure XP magnetic beads (Beckman Coulter, Brea, CA, #A63881, USA), in 2x ratio, were used to clean up end-paired / A-tailed DNA. Prior to bisulfite conversion, cleaned up DNA was subjected to adapter ligation using T4 DNA ligase enzyme and buffer 10X (New England Biolabs # M0202M, Canada) and NEB methylated adaptors (New England Biolabs # E7535L, Canada) followed by bead cleanup with AMPure XP magnetic beads. Purified DNA was bisulfite converted using the EpiTect® Fast DNA Bisulfite kit (Qiagen #59104, Canada). All steps were performed according to the accompanying manufacturer's instructions.

The NEBNext Ultra II DNA library prep kit for Illumina (New Englands Biolabs Inc., Ipswich, MA, USA) was used to prepare 48 DNA sequencing libraries, according to manufacturer's instruction. Briefly, libraries were prepared using the fragmented genomic DNA treated with bisulfite. Following ligation of NEBNext adaptors, treatment with USER® Enzyme and purification step with AxyPrep Mag PCR Clean-up kit (Axygen, Big Flats, NY, USA), a PCR enrichment step of 13 cycles was performed to incorporate specific indexed adapters for the multiplexing. The quality of final amplified libraries was examined with a DNA screentape D1000 on a TapeStation 2200 and the quantification was done on the QuBit 3.0 fluorometer (ThermoFisher Scientific, Canada). Subsequently, RRBS-seq libraries with unique index were pooled together in equimolar ratio and sequenced for single read 50 bp sequencing using eight lanes of a high output flow cell on an Illumina HiSeq 2500 V4 system at the Next-Generation Sequencing Platform, Genomics Center, CHU de Québec-Université Laval Research Center, Québec City, Canada. The average insert size for the paired-end libraries was 300 bp.

2.4.7 Chromatin immunoprecipitation followed by next generation sequencing

A total of $16 \cdot 10^6$ sperm, from three different individuals, were used for chromatin immunoprecipitation (ChIP). Pooled sperm were prepared as previously described (Hisano *et al.*, 2013), though, with multiple modifications. Pooled sperm were pre-treated with 50 mM DTT for 2h at room temperature (RT). DTT was quenched using NEM. Prior to MNase digestion, eight aliquots each of $2 \cdot 10^6$ sperm were treated with fresh non-ionic detergents in lysis buffer (0.5% nonident-P40, 1% sodium deoxycholate, 0.3 M sucrose, 0.5 M DTT, 15 mM Tris-HCl pH 7.5, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA) on ice. Next, the chromatin was digested using 15 Units of MNase (Roche, cat. no. 10107921001) at 37°C for exactly 5 min. The reaction was put to a stop using 0.5 M EDTA. Soluble chromatin was recovered by centrifugation at 17,000 xg for 10 min at RT. Subsequently, mono-nucleosomal chromatin was incubated with 0.5 µg primary ChIP grade Rabbit Anti-H3K4me3 antibody (Abcam ab8580, Canada) and aside, 50 µl beads, per IP, were blocked (Dynabeads™ Protein A Invitrogen, Canada) with 0.5% BSA in 1X PBS on a rotator at 4°C overnight. The next morning, blocked beads were washed trice with 0.5% BSA in 1X PBS and combined buffer (15 mM Tris-HCl pH 7.5, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 85 mM Tris-HCl pH 7.5,3 mM $MgCl_2$, 2 mM $CaCl_2$ and 0.3 M sucrose). The beads were resuspended in 50 µl combined buffer. The blocked bead slurry was incubated with the chromatin-antibody complex on a rotator at 4°C for 3 h. After incubation, the bead-antibody-chromatin complex was retrieved using a magnetic rack and washed twice with Wash Buffer A (50 mM Tris-HCL pH 7.5, 10 mM EDTA and 75 mM NaCl) and Wash Buffer B (50 mM Tris-HCl pH 7.5, 10 mM EDTA and 125 mM NaCl) and put on the rotator at 4°C for 5 min each. The bead-complex was transferred to a 1.5 ml low-bind tube and subsequently eluted twice using 125 µl elution buffer (0.1 M $NaHCO_3$, 0.2% SDS and 5 mM DTT) at 65°C for 10 minutes while shaking. RNaseA and Proteinase K were added to the pooled elutes as described by Hisano *et al.* (2013). Lastly, immunoprecipitated chromatin was recovered using the Zymogen ChIP-Clean and Concentrator Kit (Zymo Research # D5205, USA) and the DNA quality was assessed using the TapeStation High Sensitivity DNA ScreenTape Analysis (Agilent #5067-5585, USA) according to manufacturer's instructions.

The NEBNext Ultra II DNA library prep kit for Illumina (New Englands Biolabs Inc., Ipswich, MA, USA) was used to prepare 48 DNA sequencing libraries, according to manufacturer's instruction. Briefly, libraries were prepared using 500 pg of enriched fragmented DNA following ChIP experiment. Following ligation of NEBNext adaptors, treatment with USER® Enzyme and purification step with AxyPrep Mag PCR Clean-up kit (Axygen, Big Flats, NY, USA), a PCR enrichment step of 18 cycles was performed to incorporate specific indexed adapters for the multiplexing. The quality of final amplified libraries was examined with a DNA screentape D1000 on a TapeStation 2200 and the quantification was done on the QuBit 3.0 fluorometer (ThermoFisher Scientific, Canada). Subsequently, ChIP-seq libraries with unique index were pooled together in equimolar ratio and sequenced for single read 50 bp sequencing using seven lanes of a high output flow cell on an Illumina HiSeq 2500 V3 system at the Next-Generation Sequencing Platform, Genomics Center, CHU de Québec-Université Laval Research Center, Québec City, Canada. The average insert size for the paired-end libraries was 160 bp.

2.4.8 Bioinformatic analyses

Reads were trimmed using Trimmomatic v0.39 with the following options: TRAILING:30, SLIDINGWINDOW:4:20 and MINLEN:30 (Bolger *et al.*, 2014). All other options used the default values. Quality check was performed on raw and trimmed data to ensure the quality of the reads using FastQC v0.11.8 and MultiQC v1.8. Reads were aligned using Bismark v0.22.3 against the Rattus norvegicus genome (Rnor_6.0) (Andrews, 2010; Ewels *et al.*, 2016). The methylation calls for every single C analyzed were extracted using the bismark_methylation_extractor script provided with Bismark (Krueger *et al.*, 2011). The heatmaps were produced using the pheatmap package in R-3.6.0 (Gentleman *et al.*, 2004; Kolde, 2019; R.Team, 2013). Permutation analysis RRBS sequencing data. Using the R package methylKit we have looked for differentially methylated site (DMS) between control and treatment for the F1, F2, F3 and F4 generations separately. Significant DMS were identified as sites having false discovery rates lower than 1% and with a minimum methylation difference of 20%. Annotation of these sites was performed using ChIPseeker v1.20.0. Finally, methylInheritance R package was used to assess the relation between the number of conserved DMS from one generation to the next using permutation analysis

(Belleau *et al.*, 2018). The observed number of conserved DMS was compared to the random distribution obtain from 1000 permutations. All the analysis was performed using R-3.6.0.

Region selection for sperm H3K4me3 ChIP-Seq dataset. Regions enriched for H3K4me3 in sperm were identified using the R/Bioconductor package csaw (Lun *et al.*, 2016). Rn6 blacklisted regions were excluded and sequencing reads were counted in 150 bp sliding windows for each library across the genome. Reads were then counted in 2000 bp contiguous bins for each library across the genome to estimate background signal. We then filtered windows with background to identify regions enriched for H3K4me3 in sperm, and merged contiguous 150 bp windows that remained. Windows with a log2 fold change over 8 from the level of background enrichment were kept. Windows that were 200 bp apart were merged. Maximum region size was set at 6000 bp. Parameters were visualized and optimized using Integrative Genome Viewer. This conferred a total of three.

Normalization for sperm H3K4me3 ChIP-Seq dataset. MA-plots were used to compare the log2 ratio of counts per filtered windows (M) against the average abundance (A) of the window across all libraries, before and after normalization. M value comparison at high abundance showed a non-linear distribution for samples, entailing a slight immunoprecipitation efficiency bias. Consequently, to correct for non-linear biases, loess normalization was used for all libraries, which assumes that most windows are not differentially enriched (Ballman *et al.*, 2004; Robinson *et al.*, 2010). The counts per million normalization was also used for all libraries to correct for differences in library size. PCA plots were then generated to visualize the normalized data after library size normalization (counts per million), composition bias normalization (loess), and batch correction (using the ComBat function from the sva R/Bioconductor package).

2.4.9 Gene ontology and pathways analysis

Hyper- and hypo-methylated genic DMSs affected by *in utero* POPs, FA and POPs+FA exposure were analyzed using Metascape online software version 3.0 (http://metascape.org) for functional enrichment using ontology terms collected from gene ontology (GO) for biological processes, cellular components, and molecular functions (http://geneontology.org), Reactome Gene Set pathways (http://portal.genego.com), and

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (http://www.genome.jp/kegg). For subsequent analyses, the top 40 most significant biological pathways with a *p-value* cutoff of 0.01 were included (Figure S2.13.1-3) of which only ones with the lowest *p-value* are displayed.

2.5 Results

2.5.1 Changes in sperm DNA methylation following in utero POPs exposure

To identify differentially methylated sites (DMSs) between CTRL versus POPs, FA and POPs+FA F1-F4 sperm, we first established genome-wide DNA methylation profiles using RRBS. The minimum sequencing depth was 14X. *In utero* exposure to POPs significantly altered 295 DMSs (*p-value* 0.05; FDR ≤ 1%; absolute mean methylation ≥ 20%) of which 161 were hyper-methylated (gain of methylation = ↑) and 134 hypo-methylated (loss of methylation = ↓) compared to CTRL in F1 (Figure 2.11.2A). In subsequent POP generations, 271 (164↑; 107↓), 205 (102↑; 103↓) and 407 (230↑; 177↓) DMSs were detected in respectively F2, F3 and F4 (Figure 2.11.2A). Most hyper- and hypo-methylated DMSs overlapped with distal intergenic (56.5%; n=665) and intronic (28%; n=330) regions compared to other genomic annotations across four generations (Figure 2.11.2C; Table S2.13.1). As for *in utero* FA exposure, 464 (275↑, 189↓), 400 (181↑, 219↓), 241 (149↑, 92↓) and 433 (299↑,134↓) DMSs were detected in respectively F1, F2, F3 and F4 sperm (Figure 2.11.2D). Of all genomic annotations, the majority of hyper- and hypo-methylated DMSs also associated with distal intergenic (57%; n=877) and intronic regions (27%; n=416) in F1-F4 (Figure 2.11.2F; Table S2.13.1). In contrast to POPs and FA, the combined treatment POPs+FA altered 408 DMSs in F1, of which most were hypo-methylated i.e. 161↑ and 247↓ (Figure 2.11.2G). A similar pattern was observed in F2 and F4 with respectively 420 (94↑, 326↓) and 456 (179↑, 277↓) detected DMSs (Figure 2.11.2G). In F3, however, the number of hyper- versus hypo-methylated sites were nearly similar with 211 (99↑; 112↓) DMSs observed (Figure 2.11.2G). POPs+FA sperm lineage genomic annotations associated with 57.2% (n=855) distal intergenic and 26% (n=389) intronic regions compared to other genomic annotations (Figure 2.11.2I; Table S2.13.1). We consistently observed a lower total number of detected DMSs in F3 across all treatments, for instance 271 to 205 DMSs due to POPs, but particularly 400 to 241 DMSs due to FA and 420 to 211 DMSs due to POPs+FA in F2 and F3 (Figure 2.11.2A, D, G). This predicted treatment dilution effect did not persist until F4. Instead, all treatments showed a minimum increase of 1.8-fold in total DMSs by F4 compared to F3 (Figure 2.11.2A, D, G).

2.5.2 CpG content and neighborhood

Complementary to genic region characterization, we assessed the distribution of DMSs based on their proximity to CpG content and neighborhood context, i.e. island, shore, shelf and open sea. CpG shores lie within the 2 kb up- and downstream of a CpG island, whereas CpG shelves are defined as 2 to 4 kb outside of a shore (Sandoval *et al.*, 2011). Regions beyond CpG shores are known as CpG open sea regions. Across all generations, most DMSs were located in CpG open sea regions due to POPs (88.5%; n=1043), FA (86.1%; n=1324), and POPs+FA (83%; n=1246) (Figure 2.11.2B, E, H). The lowest percentage of DMSs were found in CpG shelves, shores and islands.

2.5.3 Gene enrichment analysis

We performed gene-ontology analysis (GO), using Metascape, to characterize biological pathways associated to the genes with DMSs (*p-value* 0.05; FDR ≤ 1%; absolute mean methylation ≥ 20%) in POPs, FA and POPs+FA sperm across F1-F4. Distal intergenic regions were excluded resulting in 134, 115, 49 and 93 DMSs due to POPs; 197, 181, 110 and 123 DMSs due to FA; 161, 184, 107 and 188 DMSs due to POPs+FA in respectively F1, F2, F3 and F4 (Table 2.12.2). GO analyses were run on both hyper- and hypo-methylated DMSs to display the top 40 most significant biological pathways with a *p-value* cutoff of 0.01 (Figure S2.13.1-3). As methylation status influences gene expression differently, however, genes were separated based on their DMSs hyper- and hypo-methylated status for subsequent GO analyses (Figure 2.11.3). For visualization purposes, up to seven unique pathways with the highest enrichment are depicted per treatment and generation. In POPs lineage sperm, genes associated with hyper-methylated DMSs were particularly enriched in social learning pathways e.g. social behavior (p = 6.80E-04), intraspecies interaction between organisms (p = 6.80E-04), multi-organism behavior (p = 1.82E-03) and learning (p = 2.17E-03) in F1. In subsequent F2, F3 and F4 generations, fundamental pathways were mostly enriched ($p \leq$ 8.46E-03) (Figure 2.11.3A). Genes targeted through hypo-methylated DMSs were specifically involved in locomotory and walking behavior ($p \leq$ 2.41E-04) as well as neurodevelopmental pathways such as axonogenesis (p = 2.42E-05), axon development (p = 5.02E-05) and neuron projection morphogenesis (p = 3.13E-04) in F1 (Figure 2.11.3B). In F2, 50 hypo-methylated DMSs were detected with no associated biological pathways (Table 2.12.2). Moreover, no hypo-methylated DMSs were detected in F3, nor F4 of ancestrally

POP exposed sperm (Table 2.12.2). Concerning FA, hyper-methylated DMSs targeted genes associated with Ras and MAPK signaling ($p \leq$ 8.20E-05), which is involved in cell proliferation, differentiation, angiogenesis and migration (Chung *et al.*, 2011) in F1 (Figure 2.11.3C). In F2, multiple pathways implicated in neurotransmission ($p \leq$ 1.33E-03), adult behavior ($p =$ 2.22E-05) and skeletal muscle cell differentiation ($p =$ 1.66E-03) were affected (Figure 2.11.3C). Then, in F4, additional neurodevelopmental pathways were affected including neuronal system ($p =$ 1.02E-05), positive regulation of nervous system development ($p =$ 5.92E-05), glutamatergic synapse ($p =$ 1.99E-04) and neuromuscular process ($p =$ 4.02E-04) (Figure 2.11.3C). In contrast to hyper-methylated DMSs, hypo-methylated DMSs affected genes involved in synaptic processes and multiple cellular responses to fluid shear stress, zinc ion and gamma radiation ($p \leq$ 2.05E-03) in F1 (Figure 2.11.3D). In F2, the 'AGE-RAGE signaling pathways in diabetic complications' ($p =$ 6.41E-04) was the strongest enriched pathway. We did not observe any hypo-methylated DMSs in F4 ancestrally FA exposed sperm (Table 2.12.2). In contrast to POPs and FA, *in utero* POPs+FA exposure lead to mostly hypo-methylated DMSs associated with genes particularly enriched in bone developmental pathways ($p \leq$ 1.60E-03) in F2; and neurodevelopmental pathways ($p \leq$ 3.90E-03) in F3 and F4 (Figure 2.11.3F; Table 2.12.2). Hyper-methylated DMS genes were implicated in various fundamental cellular pathways (Figure 2.11.3E). No pathways were associated to altered methylation in promotor regions for POPs, FA, and POPs+FA at F1-F4.

2.5.4 Partial rescue of the sperm methylome by dietary FA supplementation

Next, we explored the hypothesis whether dietary FA supplementation in F0 dams can correct the dysregulated DNA methylation expression induced by *in utero* POPs exposure. As such, we compared the percentage of DNA methylation of overlapping genes associated to treatment-specific DMSs (including distal intergenic regions) due to POPs and POPs+FA (Figure 2.11.4). Across generations, we observed a partial rescuing or correcting effect, as overall DNA methylation was brought towards CTRL levels by 56%, 39%, 65% and 46% of the overlapping genes associated to POPs- and POPs+FA-specific DMSs in respectively F1, F2, F3 an F4 (Figure 2.11.4).

2.5.5 In utero FA and POPs+FA exposure conserve DMSs transgenerationally

To further investigate whether *in utero* exposure to POPs, FA, and POPs+FA supplementation alters sperm DMSs transgenerationally, we identified overlapping and non-overlapping dysregulated DMSs between generations per treatment lineage (Figure 2.11.5). To demonstrate that the DMSs, found across generations, were not due to random change, we performed permutation analysis as described by Belleau *et al.* (2018). *In utero* POP exposure did not significantly alter the same DMSs inter- or transgenerationally. FA, however, consistently affected 76 (36↑, 40↓) DMSs intergenerationally from F1-F2 (Figure 2.11.5A, B). The majority of these 76 DMSs were associated to distal intergenic regions (56.5%, n=43) and 70 unique genes of which four (*Extl3, Asb2, Nphp4, Rexo2*) were located in promotor regions (Table S2.13.2). In F3 and F4 we detected respectively 14 (8↑, 6↓) and 3 (↑) DMSs that were transgenerationally affected (Figure 2.11.5C; Table S2.13.2). In F3, we solely observed genes associated with transgenerational hypo-methylated DMSs of which 50% was associated with distal intergenic regions. Promotor regions for *Nphp4* and *Rexo2* were detected in F3. In F4, no promoter regions were affected (Table S2.13.2). *In utero* POPs+FA, significantly altered 54 hypo-methylated DMSs and 35 unique associated genes between F1 and F2 (Figure 2.11.5B, C; Table S2.13.3). We observed multiple transgenerationally affected hypo-methylated DMSs in F3 (8↓) and F4 (7↓) with respectively four and three associated genes (Figure 2.11.5B, C; Table S2.13.3).

2.5.6 Changes in histone methylation due to in utero POPs exposure (preliminary)

Aside from sperm DNA methylation, we analyzed the impact of *in utero* POPs, FA and POPs+FA exposure on sperm histone methylation, particularly H3K4me3. After library size normalization (counts per million), composition bias normalization (loess), and batch correction, normalized data were plotted by the first two principal components in a principal component analysis (PCA) (Figure 2.11.6; Figure S2.13.4). Both components explain 39.9%, 35.1%, 32.7% and 35.5% in respectively F1, F2, F3 and F4. PCA plots demonstrated a general clustered separation of CTRL versus POPs, FA and POPs+FA. Through F1, F2 and F3, specifically FA and POPs+FA cluster together (Figure 2.11.6). Except F1, consistent variation between POPs replicates is observed in all generations. In F4, CTRL, POPs and FA generally cluster in a similar trend, whereas a combination treatment of POPs+FA does not (Figure 2.11.6). Concerning generational effects, we also performed PCA analyses per

treatment across all generations (Figure S2.13.5). Each treatment showed a unique clustering different from CTRL across generations (Figure S2.13.5).

2.6 Discussion

An increasing number of human and animal studies provide evidence supporting the impact of POPs exposure on the somatic and spermatic cell epigenome (Ben Maamar, Sadler-Riggleman, Beck, & Skinner, 2018; Consales *et al.*, 2016; K. Y. Kim *et al.*, 2010; Lind *et al.*, 2013; Skinner *et al.*, 2018). In the present study, we demonstrate that *in utero* exposure to environmentally relevant POP mixture, intended to approximate body burdens in Northern Indigenous populations (Anas *et al.*, 2005), affects sperm DNA and histone H3K4me3 methylation over multiple (un)exposed generations (F1-F4) using a Sprague-Dawley rat model. We observe that concomitant FA supplementation only partially brings DNA methylation towards CTRL levels consistently across generations. On the contrary, preliminary ChIP sequencing results demonstrate a potential protective effect induced by FA, particularly in F2 but perhaps not F1, F3 and F4.

A combined analysis of DNA methylation and histone methylation retention of sperm purified from individuals of the same study provides a comprehensive analysis of epigenetic alterations associated to environmentally induced epigenetic multigenerational inheritance of sperm epimutations induced by *in utero* POPs, FA and POPs+FA exposure. A comparison of F1, F2, F3 and F4 generations allowed us to assess the differences between direct and indirect exposure and thereby observe intergenerational (F1-F2) versus transgenerational effects (F3-F4). The F2 phenotype, however, may be the result of the combination of direct and indirect exposure, yet it is not possible to distinguish between both.

As we used a mixture of contaminants, the cumulative effect may differ from effects observed using single contaminants (Ben Maamar *et al.*, 2019; Skinner *et al.*, 2018). Furthermore, FA diets were designed to mimic physiological levels. The FA control diet represents the recommended daily allowance (RDA) of 0.4 mg FA, from fortified foods, in humans consuming a daily average of 2000 kcal. The supplemented diet represents the FA intake from fortified foods <u>plus</u> a daily FA vitamin supplement of ~ 1.0 mg FA (3-fold the RDA) (B. G. Swayne *et al.*, 2012).

DNA methylation changes, due to all treatments, occurred mostly in distal intergenic regions and CpG open-sea. Although major alterations in DNA methylation of promotors clearly can alter gene expression, previous work showed that differentially methylated regions (DMRs) located in distal intergenic areas play an important role in genome regulation (Delaval *et al.*, 2004; Stadler *et al.*, 2011). One of the best examples of such mechanisms involves imprinted genes H19 and IGF2 (Bartolomei *et al.*, 2011). Sperm exhibit a high density of methylation at intergenic regions (H. Guo *et al.*, 2014; Z. D. Smith *et al.*, 2012). Previous evidence showed that *in utero* PCB and vinclozolin exposure alters F1 and F3 sperm DNA methylation of intergenic regions in Sprague-Dawley rats. These intergenic differentially methylated CpG islands overlapped with repetitive elements from retrotransposons that were hypo-methylated due to the treatments (Gillette *et al.*, 2018). In sperm, CpG islands near promotors are typically hypo-methylated, whereas repetitive sequences and transposable elements in intergenic regions are generally hyper-methylated (Molaro *et al.*, 2011; Schilling *et al.*, 2007). Here, we observed that, in contrast to *in utero* POPs and FA, POPs+FA exposure resulted in mostly hypo-methylated distal intergenic regions in F1, F2 and F4. It is tempting to speculate that environmental contaminant exposure to POPs and/or POPs+FA, may increase the vulnerability to transposable elements, which could point toward altered genomic stability.

Various POP contaminants have structural similarities with naturally occurring hormones and may interfere with normal homeostasis, reproduction, and developmental processes (Gregoraszczuk *et al.*, 2013). *In utero* POPs exposure affected hyper-methylated DMSs associated to various general biological processes between F1 and F4. Hypo-methylated DMSs, however, were mostly involved in neurodevelopmental pathways in only F1. Interestingly, in a parallel study, we previously demonstrated that *in utero* POP exposure altered sperm miRNAs associated with various developmental pathways including brain development and cognition in the same animal model (Herst *et al.*, 2019). Furthermore, an increasing number of epidemiological studies have associated environmental *in utero* POP exposure to negative impacts on neurodevelopment, cognitive functioning and memory in children (Berghuis *et al.*, 2015; Jacobson *et al.*, 1996; Longnecker *et al.*, 2003; Patandin *et al.*, 1999). Recent evidence suggests that *in utero* contaminant exposure, i.e. DDT and vinclozolin, is associated to alterations in the brain methylome (Gillette *et al.*, 2018). Though,

whether there is a direct link between information transferred via the germline to brain somatic tissue is unknown. Like POPs, i*n utero* POPs+FA exposure affected hyper-methylated DMSs involved in various fundamental, yet different biological processes. Hypo-methylated DMSs were mostly involved in bone developmental pathways in F2, and neurodevelopmental pathways in F3 and F4. *In utero* POPs and FA exposure affected DMSs, however, were not involved in bone development. Interestingly, only POPs+FA exposure resulted in hypo-methylated DMSs that were associated to biological processes throughout all generations. POPs exposure has been previously associated with bone weakness in postmenopausal women (Paunescu *et al.*, 2013), (penile) bone density in polar bears (Daugaard-Petersen *et al.*, 2018; Tenenbaum, 2004), and skeletal/dental effects in male and female rats (Romero *et al.*, 2017). Adverse skeletal effects, as a result of POP exposure, are potentially mediated via the aryl hydrocarbon receptor (AHR), particularly as osteoclasts, osteoblasts, ameloblasts, odontoblasts, and chondrocytes express AHR (Gierthy *et al.*, 1994; Ilvesaro *et al.*, 2005; Ryan *et al.*, 2007; Sahlberg *et al.*, 2002; J. H. Yang *et al.*, 2010). FA supplementation seems to affect the rate of bone metabolism shown by a randomized double blind clinical trial (Salari *et al.*, 2014). A relation between bone development and a combined POPs and FA exposure, has not yet been established.

We recently demonstrated that FA supplementation partially mitigates the effects of POPs on paternally-derived miRNA and male reproductive parameters in a multigenerational manner (Herst *et al.*, 2019; Lessard *et al.*, 2019). Here, we observed a partial protective effect of FA supplementation against ancestral POP exposure in sperm DNA methylation. Preliminary sperm H3K4me3 results indicate a potential protective effect of FA particularly in F2, as POPs+FA replicates cluster towards CTRL replicates presented by PCA plots. Further data analysis, using pairwise comparison between samples and 5% extraction of the regions corresponding to the main source of variability i.e. FA supplementation/POPs exposure, will determine which genomic sites across generations are rescued by FA supplementation. Peptide assays have previously shown a negative correlation between H3K4me3 and DNA methylation (Ooi *et al.*, 2007); therefore, low levels of H3K4me3 may result in elevated levels of DNA methylation. We selected FA supplementation as a nutritional intervention, as some bioactive compounds, such as methyl donors may counteract or prevent the pollutant induced epigenetic damage (S. Li *et al.*, 2019). FA

supplementation was previously shown to reverse DNA hypomethylation induced by early developmental BPA exposure (Dolinoy *et al.*, 2007). Furthermore, FA supplementation is well known to reduce the incidence of congenital birth anomalies (Lopez-Camelo *et al.*, 2010; R. D. Wilson *et al.*, 2015). It should be emphasized, however, that adverse health outcomes as a result of high dose FA supplementation have been observed (L. Deng *et al.*, 2008; Y. Deng *et al.*, 2017).

We further observed an overall decrease in the number of significantly DMSs due to all investigated treatments between F1-F3. This dilution effect supports the rationale that reprogramming will minimize generational transmission. In F4, however, the number of significantly DMSs substantially increased. These results are in conjunction with previously obtained observations from a parallel study, for instance, the F3 paternal lineage showed the worst pregnancy outcomes among all generations even though the F3 males did not display any altered sperm parameters (Lessard *et al.*, 2019). In addition, transcriptomic analyses of the F4 two-cell embryos revealed a surprisingly high number of significantly differentially expressed genes due to all treatments (Lessard *et al.*, 2019). We initially postulated that the sperm produced by the F3 paternal lineage contained accumulated upstream epimutations including perturbed DNA methylation, histone modifications, or noncoding RNAs, that escaped remodeling during development and spermatogenesis. Compared to other generations, however, the F3 paternal lineage showed the lowest number of significant DMSs. Furthermore, preliminary data obtained by ChIP sequencing analysis, PCA plots showed the lowest percentage of total variation between samples in the F3 generation.

Interestingly, only ancestrally FA and POPs+FA exposed sperm retained a subset of differentially methylated sites between F1 to F4, suggesting a common mechanism of environmental exposure and germline epigenome interaction. *In utero* POPs exposure, however, affected the F2, F3 and F4 generation via non inter/transgenerationally retained DMSs.

2.7 Conclusion

The present study confirms our hypothesis that *in utero* exposure to an environmentally-relevant POPs mixture significantly alters the sperm DNA methylome and H3K4me3 expression across multiple (un)exposed generations (F1 through F4) using a rodent model. These results stress the role of the father in the transmission of acquired environmental information to his offspring and subsequent generations, thus the Paternal Origins of Health and Disease (POHaD) paradigm. Dietary FA supplementation, however, only partially rescued the POPs induced effects in the sperm epigenome. To our knowledge, this is the first evidence of transgenerational epigenetic inheritance induced by an environmentally relevant contaminant mixture and nutritional intervention across four generations.

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2.9 Author contributions

Authors contributed to initial conception or design (JLB, JMT, SK, AJM); final design (PMH, MD, JLB); data acquisition, analysis, or interpretation (PMH, ML, PLC, PN, MV, AD, MD, JLB), drafting the manuscript (PMH); and critically revising the manuscript (PMH, ML, PLC, PN, MV, AD, JMT, SK, AJM, MOBB, MD, JLB). All authors gave final approval and agreed to be accountable for all aspects of work in ensuring that questions relating the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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2.11 Figures

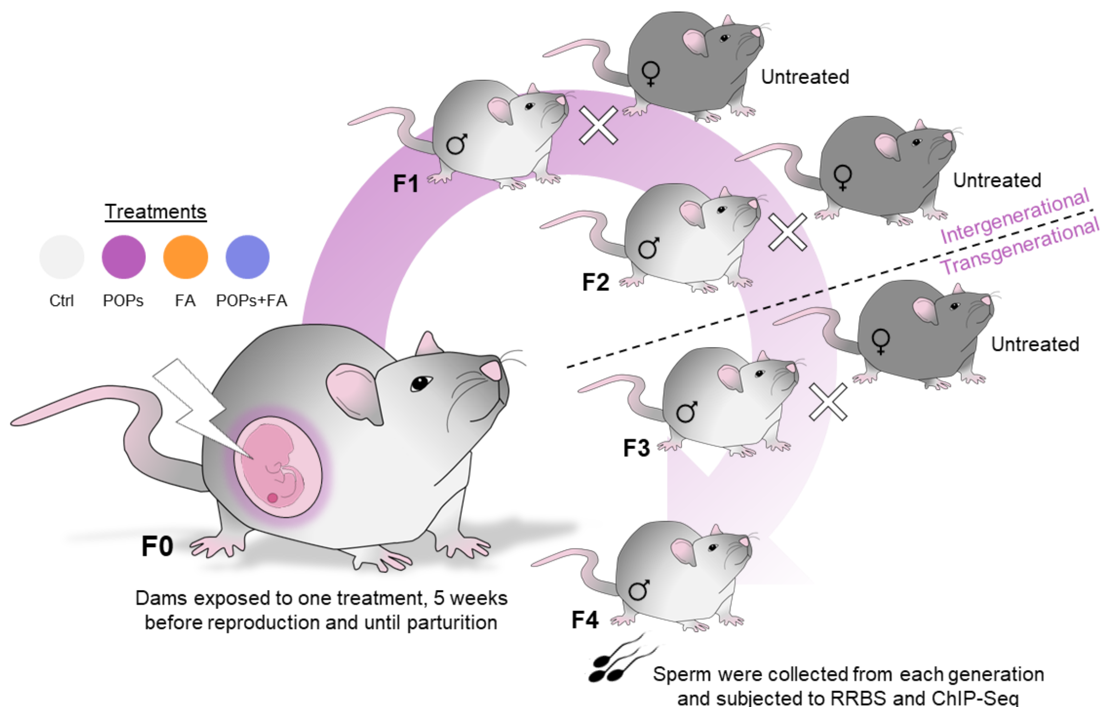


Figure 2.11.1. Experimental design. Four treatment groups of Sprague-Dawley F0 founder females (n=6) were gavaged with either an environmentally-relevant POPs mixture (500 µg PCBs plus remaining POPs/kg body weight) or corn oil (control); in addition, the F0 females received diets ad libitum containing 2 mg/kg diet (1X) or 6 mg/kg diet (3X) FA representing the North American FA intake in the post-fortification era (1X) and with a daily 1 mg FA prenatal multivitamin (3X) respectively. Treatments were administered 5 weeks before reproduction (x untreated males) and until parturition. After birth of the F1, all F0 founder dams and subsequent generations received 1X ad libitum. F1 males were bred with untreated females to obtain F2 offspring. Likewise, F3 and F4 generation lineages were produced. During the establishment of each generation, sperm were collected from 12 males per treatment group at PND 150. Since F0 dams were exposed, an intergenerational effect can be observed from F1 and F2 a transgenerational effect starting from F3.

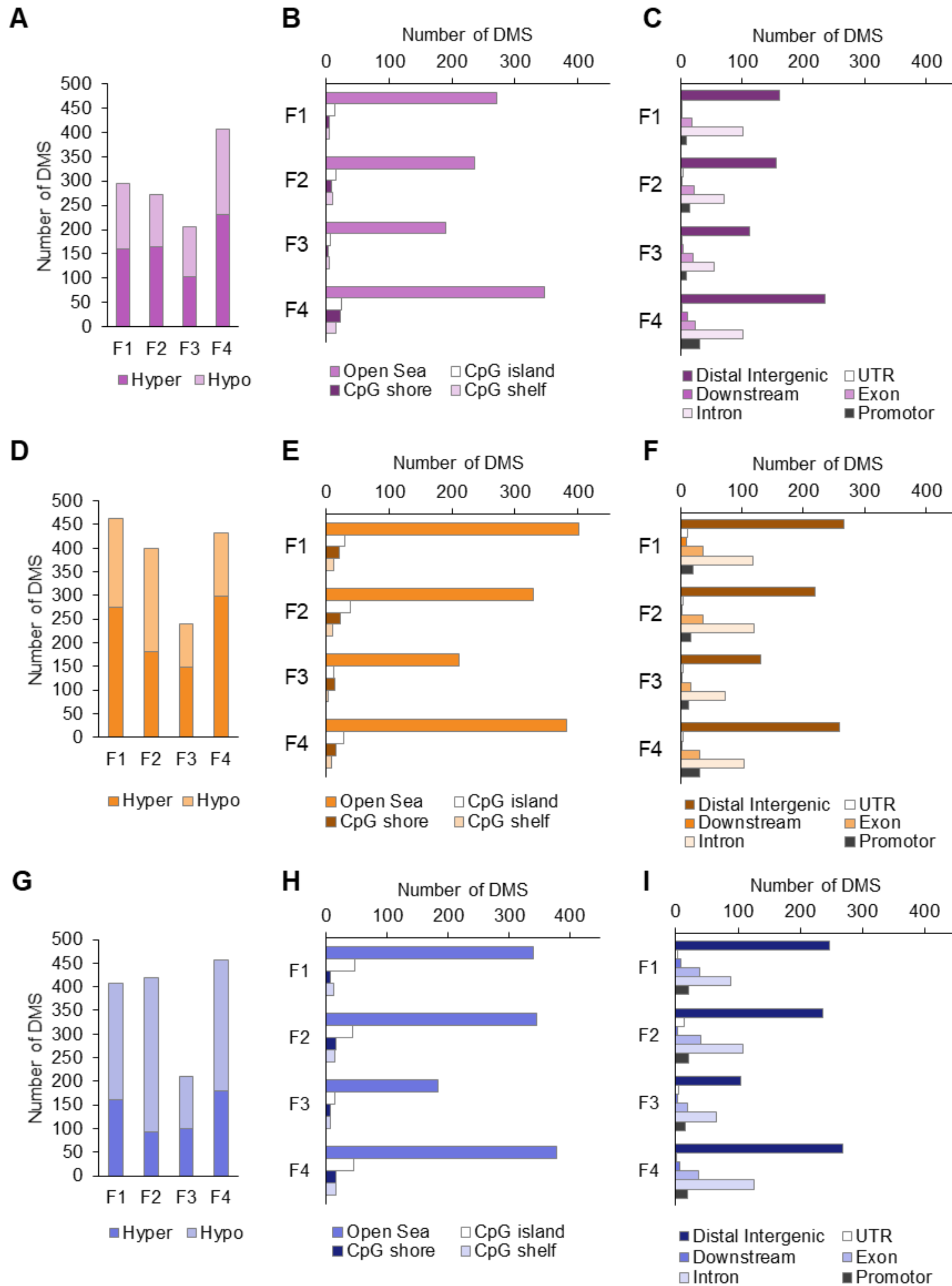


Figure 2.11.2. The number of hyper- and hypo-methylated DMSs and associated CpG and genomic annotations detected in F1-F4 POPs, FA and POPs+FA sperm. A consistent dilution effect of total detected DMS persisted until F3, but not F4 in all treatments. Only significant DMSs (*p-value* 0.05; FDR ≤ 1%; absolute mean methylation ≥ 20%) were included in presented analyses. **(A)** Hyper- and hypo-methylated DMSs due to POPs (purple) **(B)** CpG annotations due to POPs **(C)** Genomic annotations due to POPs **(D)** Hyper- and hypo-

methylated DMSs due to FA (orange) **(E)** CpG annotations due to FA **(F)** Genomic annotations due to FA **(G)** Hyper- and hypo-methylated DMSs due to POPs+FA (blue) **(H)** CpG annotations due to POPs+FA **(I)** Genomic annotations due to POPs+FA.

A

Hyper DMS

Biological process POPs

F1
Protein localization to synapse (4)
Import across plasma membrane (4)
Social behavior (3)
Intraspecies interaction between organisms (3)
Multi-organism behavior (3)
Learning (4)
Regulation of striated muscle contraction (3)

F2
Carbohydrate derivative transport (4)
Epithelial cell morphogenesis (3)
Adenylate cyclase-inhibiting G protein-coupled receptor signaling pathway (3)

F3
Organophosphate catabolic process (4)
Phospholipid metabolic process (5)
Hemostasis (5)
Microtubule-based movement (4)
Mitochondrion organization (5)
Phospholipid biosynthetic process (3)
Alcohol metabolic process (4)

F4
Epidermal cell differentiation (5)
Sensory perception of mechanical stimulus (6)
Epidermis development (5)
Keratinocyte differentiation (3)

0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0
-Log(p-value)

B

Hypo DMS

Biological process

F1
Locomotory behavior (7)
Lysosomal protein catabolic process (3)
Protein catabolic process in the vacuole (3)
Axonogenesis (8)
Ionotropic glutamate receptor signaling pathway (3)
Axon development (8)
Cell morphogenesis involved in neuron differentiation (8)
Walking behavior (3)
Cellular response to amyloid-beta (3)
Neuron projection morphogenesis (8)

0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0
-Log(p-value)

C

Biological process FA

F1
Positive regulation of hydrolase activity (14)
Ras signaling pathway (8)
Vascular endothelial growth factor signaling pathway (4)
RAF/MAP kinase cascade (7)
MAPK1/MAPK3 signaling (7)
FLT3 Signaling (7)
MAPK family signaling cascades (7)

F2
Adult behavior (6)
Regulation of synaptic vesicle endocytosis (3)
Adult locomotory behavior (4)
Mitochondrial fission (3)
Synaptic vesicle endocytosis (3)
Presynaptic endocytosis (3)
Skeletal muscle cell differentiation (3)

F3
Epithelial cell morphogenesis (3)
Epithelial cell development (5)
Response to nutrient levels (7)
Establishment or maintenance of cell polarity (4)
Response to extracellular stimulus (7)
Autophagy - animal (3)
Positive regulation of cell-substrate adhesion (3)

F4
Neuronal System (9)
Positive regulation of nervous system development (12)
Glutamatergic synapse (5)
Calcium signaling pathway (6)
Positive regulation of cell-substrate adhesion (5)
Neuromuscular process (5)
Activation of NMDA receptors and postsynaptic events (3)
Mitochondrial fission (3)

0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0
-Log(p-value)

D

Biological process FA

F1
Glutamatergic synapse (5)
Response to fluid shear stress (3)
Response to zinc ion (3)
Response to gamma radiation (3)
Regulation of postsynaptic membrane potential (4)
Aldosterone synthesis and secretion (3)
Calcium signaling pathway (4)

F2
AGE-RAGE signaling pathway in diabetic complications (4)
Phosphatidylinositol-mediated signaling (4)
Inositol lipid-mediated signaling (4)
Phosphatidylinositol signaling system (3)
T cell receptor signaling pathway (3)
Th17 cell differentiation (3)

F3
Protein localization to cell periphery (5)
Protein localization to plasma membrane (4)
Positive regulation of cell death (5)

0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0
-Log(p-value)

E

Biological process POPs+FA

F1
Organic hydroxy compound biosynthetic process (5)
Alcohol biosynthetic process (4)
Phospholipid biosynthetic process (4)
Protein localization to synapse (3)
Organophosphate biosynthetic process (6)
Phospholipid metabolic process (5)
Response to metal ion (5)

F2
Epithelial cell morphogenesis (3)
Establishment of protein localization to plasma membrane (3)
Post-Golgi vesicle-mediated transport (3)
Golgi vesicle transport (4)
Cell-substrate adhesion (4)
Regulation of JNK cascade (3)
Actin filament organization (4)
Eye development (4)

F3
Organelle disassembly (3)
Response to nutrient (4)
Cellular component disassembly (4)

F4
Calcium-mediated signaling using intracellular calcium source (3)
Actin filament organization (8)
Actin cytoskeleton organization (10)
Negative regulation of lipid biosynthetic process (3)
Sensory organ development (8)
Positive regulation of phosphatidylinositol 3-kinase signaling (3)

0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0
-Log(p-value)

F

Biological process POPs+FA

F1
Fat cell differentiation (6)
Positive regulation of MAPK cascade (7)
Neutrophil degranulation (6)
Positive regulation of gliogenesis (3)
Positive regulation of kinase activity (7)
Organ growth (4)
Multicellular organismal homeostasis (6)

F2
Chondrocyte development (4)
Growth plate cartilage chondrocyte differentiation (3)
Growth plate cartilage development (3)
Gland morphogenesis (5)
Endochondral bone growth (3)
Bone growth (3)

F3
Axon regeneration (3)
Neuron projection regeneration (3)
Lysosomal transport (3)
Vacuolar transport (3)
Interaction with host (3)
Protein localization to plasma membrane (4)
Hematopoietic progenitor cell differentiation (3)

F4
Glutamatergic synapse (4)
Synaptic transmission, glutamatergic (4)
Synthesis of substrates in N-glycan biosythesis (3)
Regulation of synaptic transmission, glutamatergic (3)
Regulation of exocytosis (3)
Carbohydrate derivative transport (3)
Positive regulation of cell migration (7)

0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0
-Log(p-value)

Figure 2.11.3. Most enriched biological pathways identified following GO analyses without distal intergenic DMSs for hyper-methylated DMSs due to **(A)** POPs (purple) **(C)** FA (orange), and **(E)** POPs+FA (blue); and hypo-methylated DMSs due to **(B)** POPs, **(D)** FA, and **(F)** POPs+FA across generations F1-F4. The number of genes associated to each pathway is displayed in parentheses.

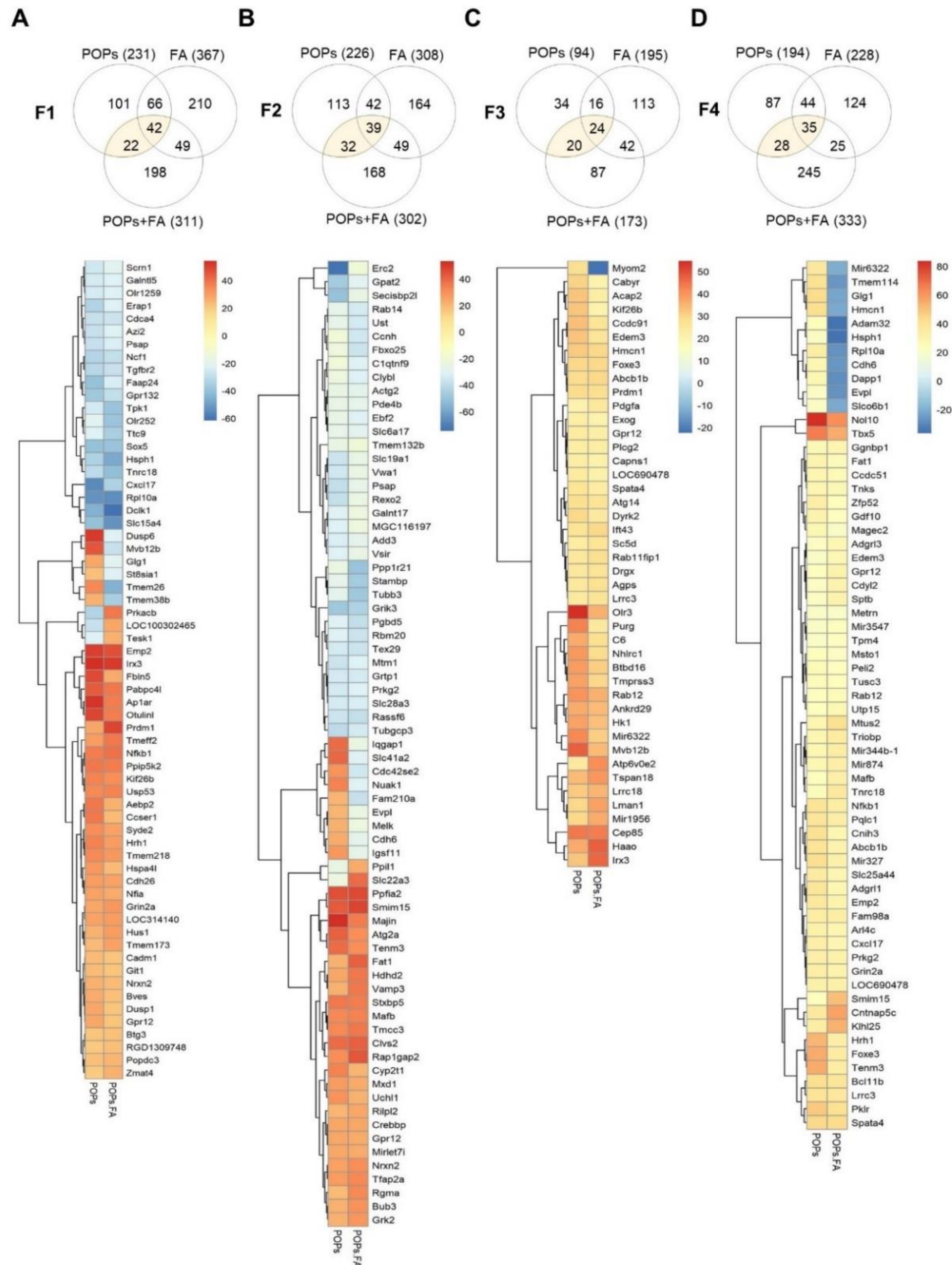
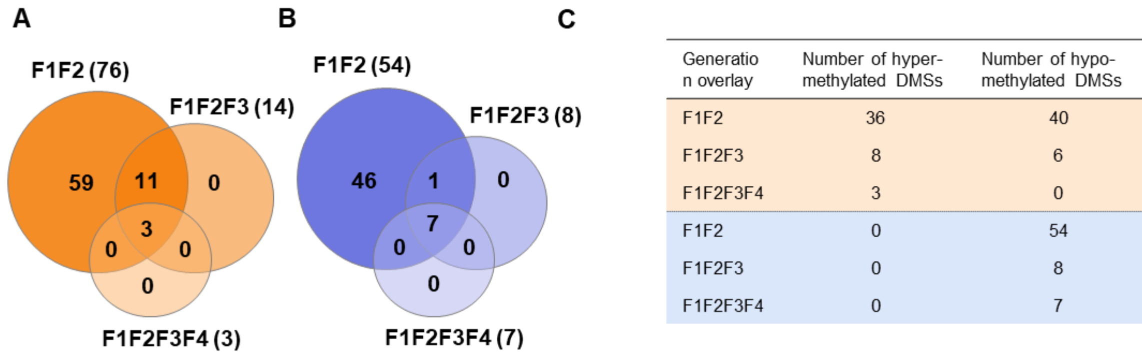


Figure 2.11.4. Dietary FA supplementation partially rescues or corrects dysregulated DNA methylation expression induced by *in utero* POPs exposure. Venn diagrams comparing overlapping genes associated with DMSs due to POPs, FA and POPs+FA in **(A)** F1, **(B)** F2, **(C)** F3, and **(D)** F4. Color-labeled tables illustrate the DMS' associated gene names and accompanying DNA methylation percentage between POPs and POPs+FA for the overlapping area sharing POPs, FA and POPs+FA (upper panel) and POPs and POPs+FA (lower panel).



Generation overlay	Number of hyper-methylated DMSs	Number of hypo-methylated DMSs
F1F2	36	40
F1F2F3	8	6
F1F2F3F4	3	0
F1F2	0	54
F1F2F3	0	8
F1F2F3F4	0	7

Figure 2.11. 5. Significantly conserved DMSs due to FA (orange) and POPs+FA (blue) across generations. **(A)** FA, **(B)** POPs+FA, and **(C)** distribution of hyper- versus hypo-methylated DMSs due to FA (orange) and POPs+FA (blue) across generations.

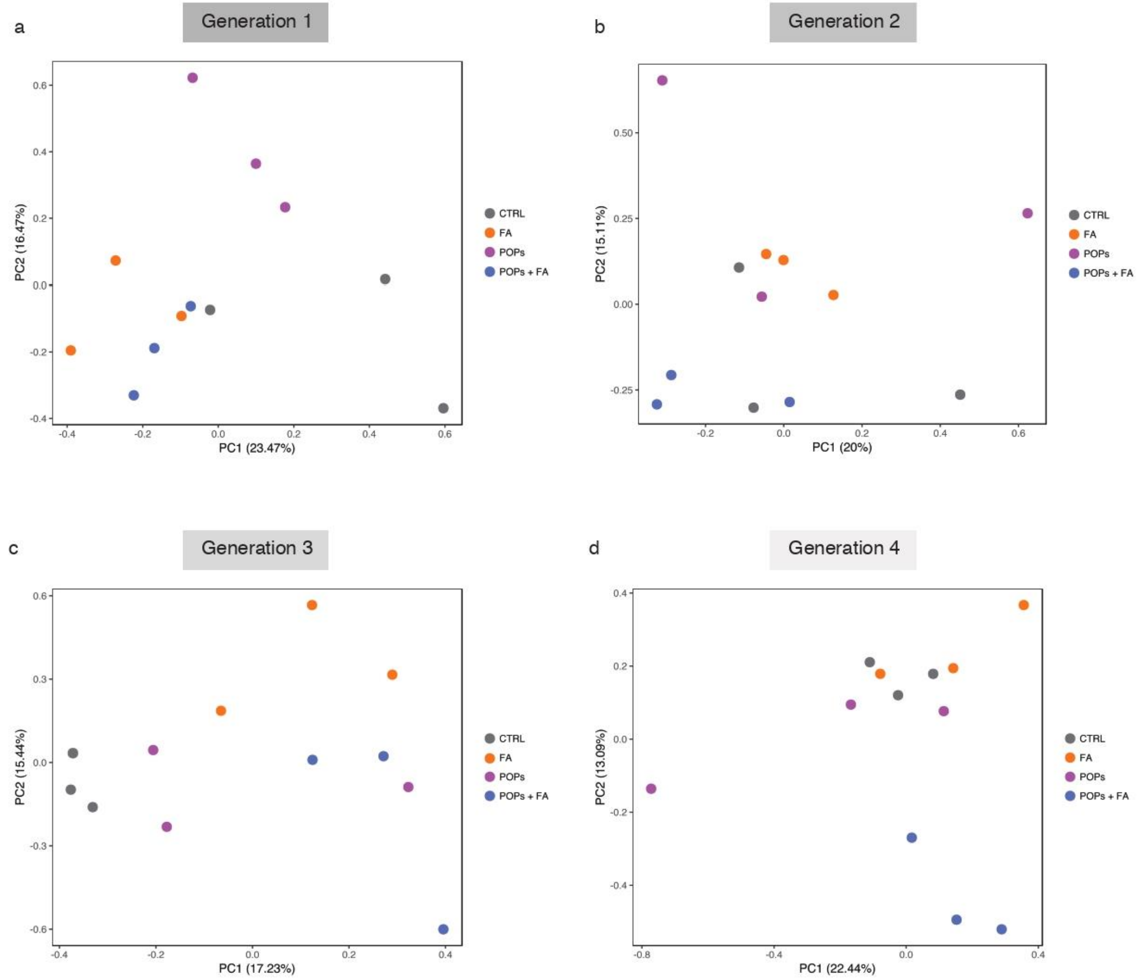


Figure 2.11.6. PCA plots depicting trends associated to sperm H3K4me3 following *in utero* POPs, FA and POPs+FA vs. CTRL per generation. PCA plots of filtered normalized H3K4me3 reads across all treatment groups for **(A)** F1, **(B)** F2, **(C)** F3, and **(D)** F4 sperm.

2.12 Tables

Table 2.12.1. Composition of environmentally relevant POPs mixture (Anas et al. 2005 Biol Reprod). [a]AccuStandard Inc. (New Haven, CT); Sigma-Aldrich Inc (St Louis, Missouri). [b]Aroclor and congener neat mix contains 2,4,4′-trichlorobiphenyl (PCB 28, 1%), 2,2′,4,4′-tetrachlorobiphenyl (PCB 47, 0.80%), 3,3,4,4′-tetrachlorobiphenyl (PCB 77, 0.0044%), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126, 0.02%), Aroclor 1254 (39.30%), and Aroclor 1260 (58.90%).

Compound	CAS no.	Origin[a]	% Weight
Aroclor and congener neat mix[b]		AccuStandard	32.40
Technical chlordane	57-74-9	AccuStandard	21.40
p,p′-Dichlorodiphenyldichloroethylene (DDE)	72-55-9	Sigma-Aldrich	19.30
p,p′-Dichlorodiphenyltrichloroethane (DDT)	50-29-3	Sigma-Aldrich	6.80
Technical toxaphene	8001-35-2	AccuStandard	6.50
α-Hexachlorocyclohexane (HCH)	319-84-6	Sigma-Aldrich	6.20
Aldrin	309-00-2	Sigma-Aldrich	2.50
Dieldrin	60-57-1	Sigma-Aldrich	2.10
1, 2, 4, 5-Tetrachlorobenzene	95-94-3	Sigma-Aldrich	0.90
p,p′-Dichlorodiphenyldichloroethane (DDD)	72-54-8	Sigma-Aldrich	0.50
β-Hexachlorocyclohexane (HCH)	319-85-7	Sigma-Aldrich	0.40
Hexachlorobenzene	118-74-1	AccuStandard	0.40
Mirex	2385-85-5	Sigma-Aldrich.	0.20
γ -hexachlorocyclohexane or Lindane (*γ*-HCH)	58-89-9	Sigma-Aldrich	0.20
Pentachlorobenzene	608-93-5	Sigma-Aldrich	0.20

Table 2.12.2. Distribution of DMSs, excluding distal intergenic regions, and average percentage difference of methylation changes compared to CTRL for POPS, FA, POPs+FA in F1-F4.

Treatment	Generation	Number DMSs	Avg % difference of methylation changes	Number unique genes	Genes with DMSs located on promoter region
POPs	F1	71 ↑	33.4 ± 12.1	62	*Zhx3, Kctd15, Grin2a, Kcnk5, Ppip5k2*
		63 ↓	-32.6 ± 10.9	49	*Wdr46, Rexo2, Tesk1, Pex10*
	F2	65 ↑	29.3 ± 8.9	55	*Atp5pd, Sgce, Crabp1, Zhx3, Srp9, Cyp2t1, Mapk4, Rpl10a, Olr143*
		50 ↓	-30.1 ± 7.8	45	*Cldn19, Rexo2, Vwa1, C1qtnf9*
	F3	49 ↑	28.1 ± 6.3	47	*LOC690478, Asb2, Mab21l3, Stk3, Tmprss3*
		0 ↓	0 ± 0	0	
	F4	93 ↑	31.8 ± 11.1	86	*Pkdrej, Mir3547, Spry2, Grk1, Zfp219, Atp5pd, Dapp1, Sh3rf2, LOC690478, Rpl10a, Ddx39b, C1qtnf9, Rexo2, Asb2, Slc25a54*
		0 ↓	0 ± 0	0	
FA	F1	113 ↑	32.2 ± 9.5	97	*Myo7a, Zfp24, Tex26, Kctd15, Chad, Pdgfrb, Plekhj1, Dmap1, Ppip5k2, Tigd5, Zfp879, Asb2, Kcng4*
		84 ↓	-34.1 ± 11.5	72	*Tesk1, Extl3, Mpped1, Iah1, Mab21l3, Wdr46, Rexo2, Mpp3*
	F2	80 ↑	32.7 ± 9.3	62	*Ddx39b, LOC690435, Elfn1, Wdr46, Asb2, RT1-DOa*
		101 ↓	-35.6 ± 9.9	81	*Wdr46, Extl3, Rexo2, Nphp4, Tmprss3, Ppip5k2, Mpp3, Kcnh6, Fkbpl, Mir678*
	F3	67 ↑	28.7 ± 8.9	53	*Ppil1, Mir3547, LOC690478*
		43 ↓	-30.2 ± 8.2	41	*Rexo2, Nphp4, LOC498122, Nacad, Zfp219, Btbd16*
	F4	123 ↑	31.3 ± 9.9	93	*Diras2, Grk1, Amz1, Shank2, Gldn, LOC690478, Sh3rf2, Mir3547, Atp5pd, Cyp2t1, Sardh, Tesk1*
		0 ↓	0 ± 0		
POPs+FA	F1	54 ↑	33.8 ± 9.2	47	*Aqp9, Grin2a, Mir3547, Sgce, Ppip5k2, Dmap1, Slc25a54*
		107 ↓	-29.0 ± 6.9	71	*Osbpl8, Ltbp4, Evpl, Taok3, Coch, Zbtb4, Prop1, Trir, Dok3, C1qtnf9*
	F2	40 ↑	31.6 ± 9.9	34	*Rn5-8s, Cyp2t1, Elfn1, Aqp9*
		144 ↓	-27.7 ± 6.9	102	*Matn1, Ppp1r9a, Kctd15, C1qtnf9, Tesk1, Cplx4, Mab21l3, LOC690478, Rexo2, Vwa1, RT1-DOa, Prop1, Map2k6*
	F3	48 ↑	27.1 ± 6.2	42	*LOC690478, Dmap1, Mir3547, Mir671, Tmprss3, Olr143*
		59 ↓	-30.1 ± 7.5	45	*Nacad, Dolpp1, RT1-DOa, Wdr46, C1qtnf9, Mpped1, Vwa1, Scarf1*
	F4	72 ↑	31.9 ± 9.3	63	*Trim40, Mir3547, Grin2a, Rn5-8s, LOC690478*

116 ↓	-30.9 ± 8.7	77	*Zfp24, Sgce, Dolpp1, Acap1, Bhlha15, Wdr3, Kcne2, Grin3b, Ppip5k2, Lnc081, Rpl10a, Kcnk5, Cd2*

2.13 Supplementary information

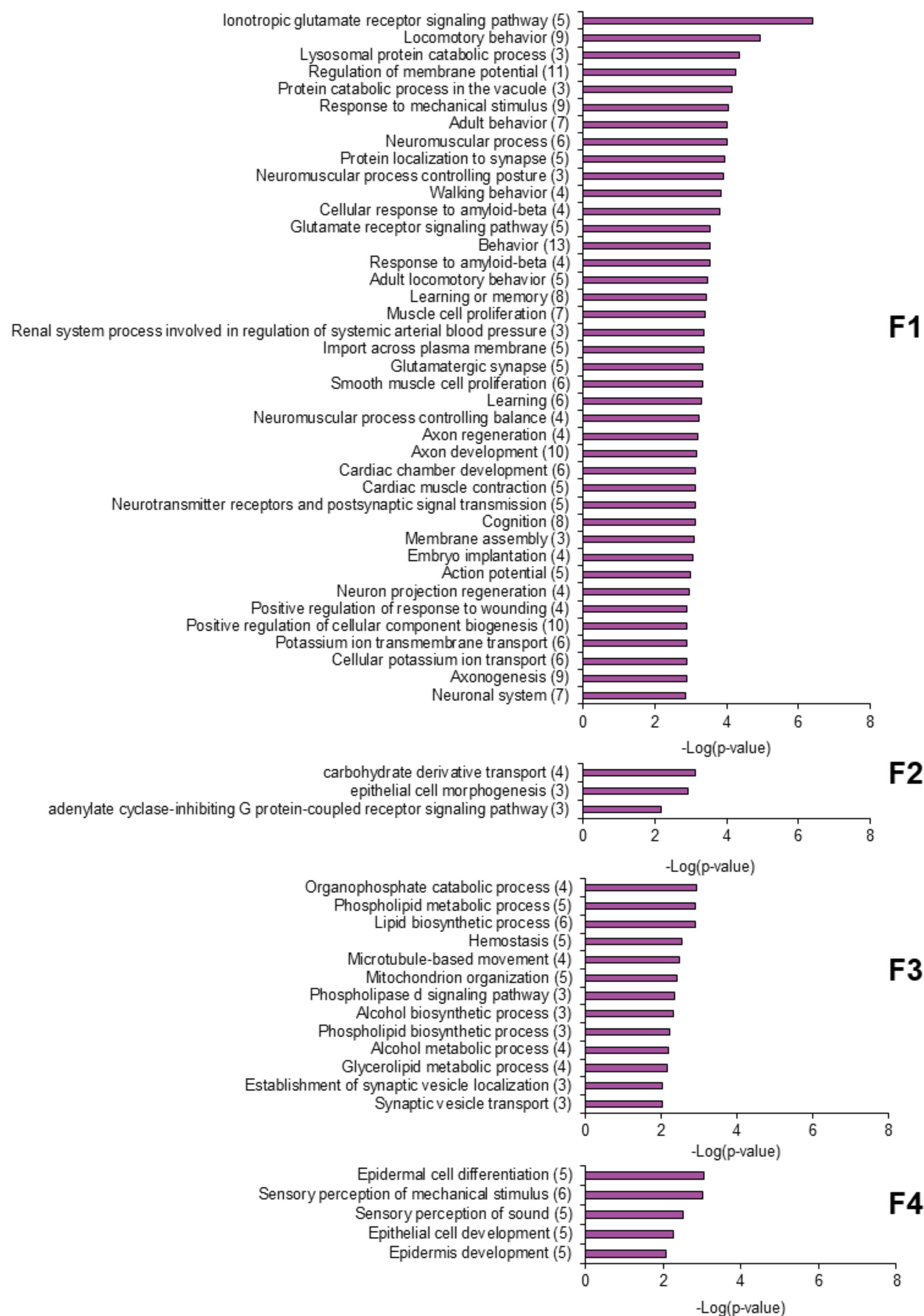
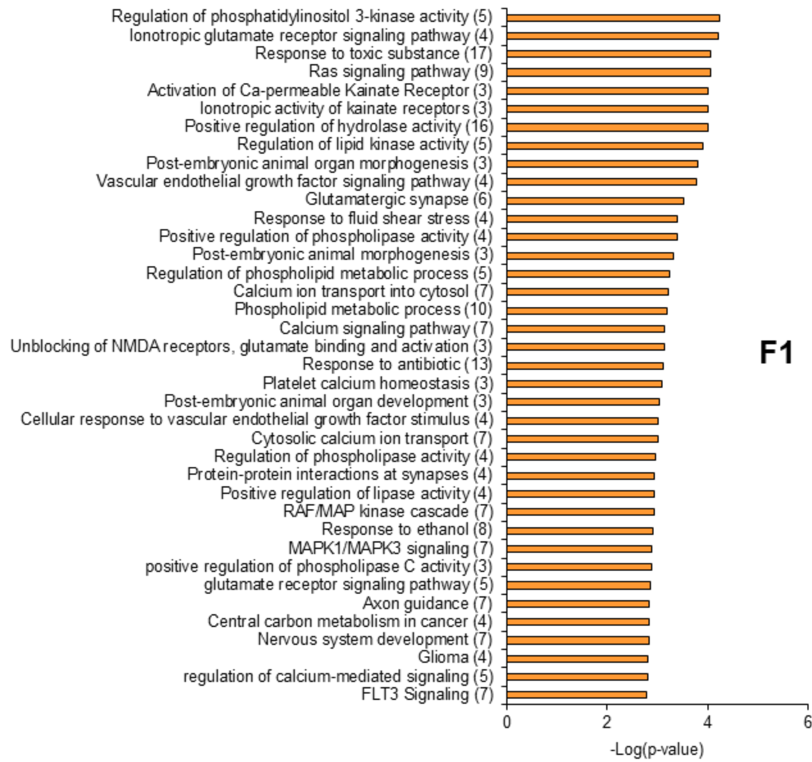
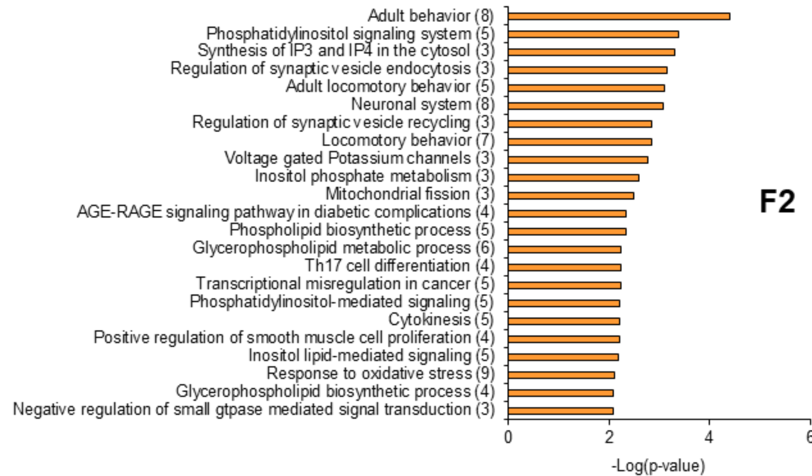


Figure S2.13.1. Gene ontology (GO) enrichment analysis of the genes associated to DMSs affected due to POPs regardless of methylation status in F1-F4. Up to 40 most significant biological pathways are presented. The number of genes associated to each pathway is in parenthesis.


Regulation of phosphatidylinositol 3-kinase activity (5)
Ionotropic glutamate receptor signaling pathway (4)
Response to toxic substance (17)
Ras signaling pathway (9)
Activation of Ca-permeable Kainate Receptor (3)
Ionotropic activity of kainate receptors (3)
Positive regulation of hydrolase activity (16)
Regulation of lipid kinase activity (5)
Post-embryonic animal organ morphogenesis (3)
Vascular endothelial growth factor signaling pathway (4)
Glutamatergic synapse (6)
Response to fluid shear stress (4)
Positive regulation of phospholipase activity (4)
Post-embryonic animal morphogenesis (3)
Regulation of phospholipid metabolic process (5)
Calcium ion transport into cytosol (7)
Phospholipid metabolic process (10)
Calcium signaling pathway (7)
Unblocking of NMDA receptors, glutamate binding and activation (3)
Response to antibiotic (13)
Platelet calcium homeostasis (3)
Post-embryonic animal organ development (3)
Cellular response to vascular endothelial growth factor stimulus (4)
Cytosolic calcium ion transport (7)
Regulation of phospholipase activity (4)
Protein-protein interactions at synapses (4)
Positive regulation of lipase activity (4)
RAF/MAP kinase cascade (7)
Response to ethanol (8)
MAPK1/MAPK3 signaling (7)
positive regulation of phospholipase C activity (3)
glutamate receptor signaling pathway (5)
Axon guidance (7)
Central carbon metabolism in cancer (4)
Nervous system development (7)
Glioma (4)
regulation of calcium-mediated signaling (5)
FLT3 Signaling (7)
0
2
4
6
-Log(p-value)
F1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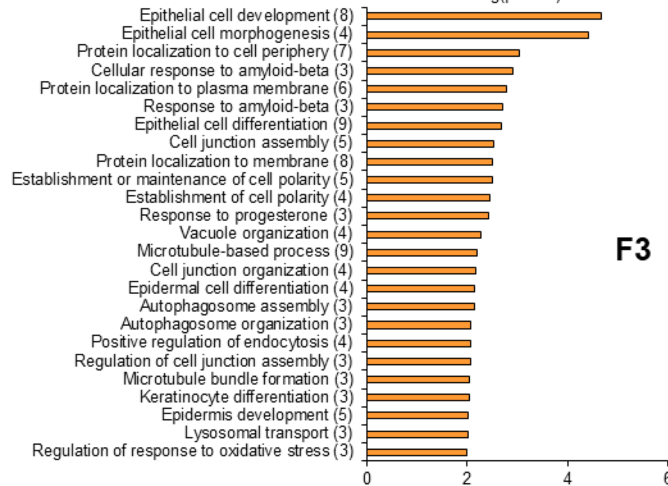
Phosphatidylinositol signaling system (5)
Synthesis of IP3 and IP4 in the cytosol (3)
Regulation of synaptic vesicle endocytosis (3)
Adult locomotory behavior (5)
Neuronal system (8)
Regulation of synaptic vesicle recycling (3)
Locomotory behavior (7)
Voltage gated Potassium channels (3)
Inositol phosphate metabolism (3)
Mitochondrial fission (3)
AGE-RAGE signaling pathway in diabetic complications (4)
Phospholipid biosynthetic process (5)
Glycerophospholipid metabolic process (6)
Th17 cell differentiation (4)
Transcriptional misregulation in cancer (5)
Phosphatidylinositol-mediated signaling (5)
Cytokinesis (5)
Positive regulation of smooth muscle cell proliferation (4)
Inositol lipid-mediated signaling (5)
Response to oxidative stress (9)
Glycerophospholipid biosynthetic process (4)
Negative regulation of small gtpase mediated signal transduction (3)
0
2
4
6
-Log(p-value)
F2


Epithelial cell development (8)
Epithelial cell morphogenesis (4)
Protein localization to cell periphery (7)
Cellular response to amyloid-beta (3)
Protein localization to plasma membrane (6)
Response to amyloid-beta (3)
Epithelial cell differentiation (9)
Cell junction assembly (5)
Protein localization to membrane (8)
Establishment or maintenance of cell polarity (5)
Establishment of cell polarity (4)
Response to progesterone (3)
Vacuole organization (4)
Microtubule-based process (9)
Cell junction organization (4)
Epidermal cell differentiation (4)
Autophagosome assembly (3)
Autophagosome organization (3)
Positive regulation of endocytosis (4)
Regulation of cell junction assembly (3)
Microtubule bundle formation (3)
Keratinocyte differentiation (3)
Epidermis development (5)
Lysosomal transport (3)
Regulation of response to oxidative stress (3)
0
2
4
6
-Log(p-value)
F3

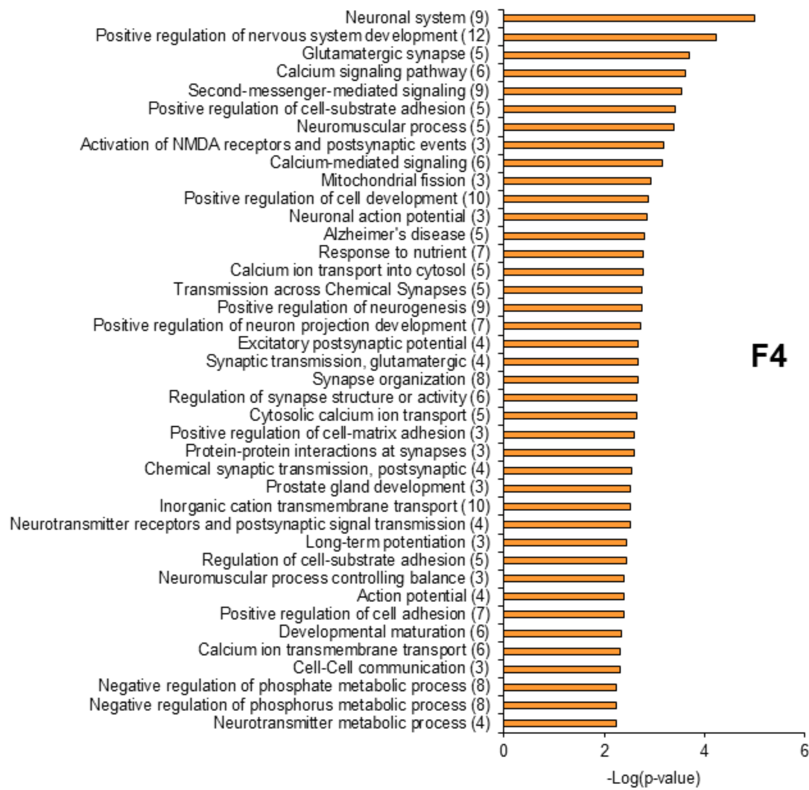
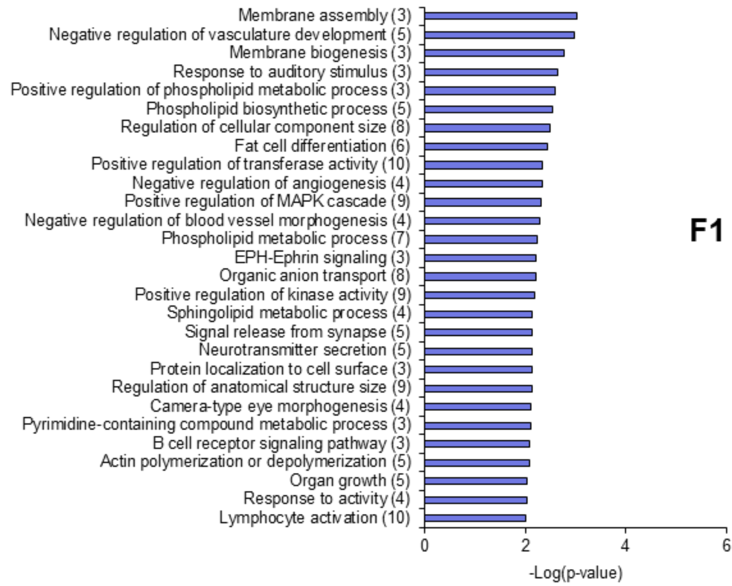
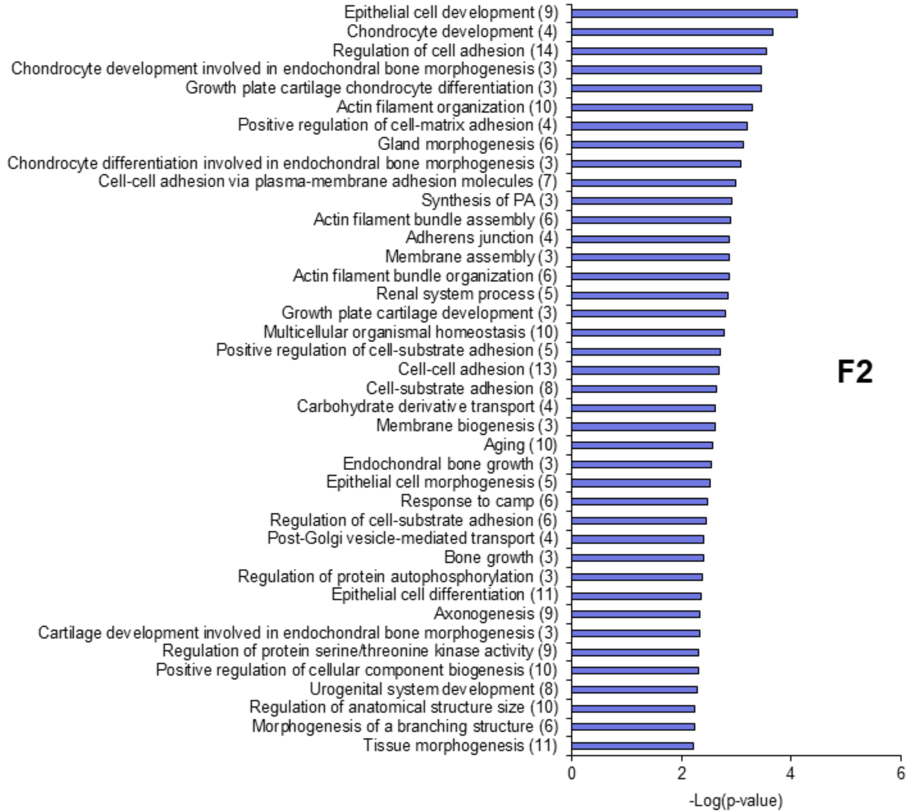
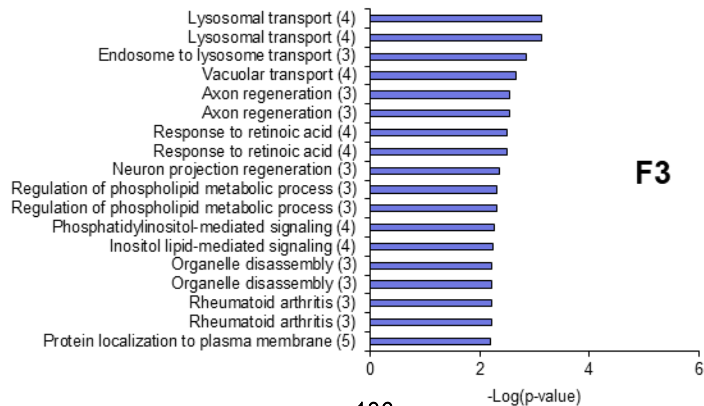


Figure S2.13.2. Gene ontology (GO) enrichment analysis of the genes associated to DMSs affected due to FA regardless of methylation status in F1-F4. Up to 40 most significant biological pathways are presented. The number of genes associated to each pathway is in parenthesis.


Membrane assembly (3)
Negative regulation of vasculature development (5)
Membrane biogenesis (3)
Response to auditory stimulus (3)
Positive regulation of phospholipid metabolic process (3)
Phospholipid biosynthetic process (5)
Regulation of cellular component size (8)
Fat cell differentiation (6)
Positive regulation of transferase activity (10)
Negative regulation of angiogenesis (4)
Positive regulation of MAPK cascade (9)
Negative regulation of blood vessel morphogenesis (4)
Phospholipid metabolic process (7)
EPH-Ephrin signaling (3)
Organic anion transport (8)
Positive regulation of kinase activity (9)
Sphingolipid metabolic process (4)
Signal release from synapse (5)
Neurotransmitter secretion (5)
Protein localization to cell surface (3)
Regulation of anatomical structure size (9)
Camera-type eye morphogenesis (4)
Pyrimidine-containing compound metabolic process (3)
B cell receptor signaling pathway (3)
Actin polymerization or depolymerization (5)
Organ growth (5)
Response to activity (4)
Lymphocyte activation (10)
0
2
4
6
-Log(p-value)
F1


Epithelial cell development (9)
Chondrocyte development (4)
Regulation of cell adhesion (14)
Chondrocyte development involved in endochondral bone morphogenesis (3)
Growth plate cartilage chondrocyte differentiation (3)
Actin filament organization (10)
Positive regulation of cell-matrix adhesion (4)
Gland morphogenesis (6)
Chondrocyte differentiation involved in endochondral bone morphogenesis (3)
Cell-cell adhesion via plasma-membrane adhesion molecules (7)
Synthesis of PA (3)
Actin filament bundle assembly (6)
Adherens junction (4)
Membrane assembly (3)
Actin filament bundle organization (6)
Renal system process (5)
Growth plate cartilage development (3)
Multicellular organismal homeostasis (10)
Positive regulation of cell-substrate adhesion (5)
Cell-cell adhesion (13)
Cell-substrate adhesion (8)
Carbohydrate derivative transport (4)
Membrane biogenesis (3)
Aging (10)
Endochondral bone growth (3)
Epithelial cell morphogenesis (5)
Response to camp (6)
Regulation of cell-substrate adhesion (6)
Post-Golgi vesicle-mediated transport (4)
Bone growth (3)
Regulation of protein autophosphorylation (3)
Epithelial cell differentiation (11)
Axonogenesis (9)
Cartilage development involved in endochondral bone morphogenesis (3)
Regulation of protein serine/threonine kinase activity (9)
Positive regulation of cellular component biogenesis (10)
Urogenital system development (8)
Regulation of anatomical structure size (10)
Morphogenesis of a branching structure (6)
Tissue morphogenesis (11)
0
2
4
6
-Log(p-value)
F2


Lysosomal transport (4)
Lysosomal transport (4)
Endosome to lysosome transport (3)
Vacuolar transport (4)
Axon regeneration (3)
Axon regeneration (3)
Response to retinoic acid (4)
Response to retinoic acid (4)
Neuron projection regeneration (3)
Regulation of phospholipid metabolic process (3)
Regulation of phospholipid metabolic process (3)
Phosphatidylinositol-mediated signaling (4)
Inositol lipid-mediated signaling (4)
Organelle disassembly (3)
Organelle disassembly (3)
Rheumatoid arthritis (3)
Rheumatoid arthritis (3)
Protein localization to plasma membrane (5)
0
2
4
6
-Log(p-value)
F3

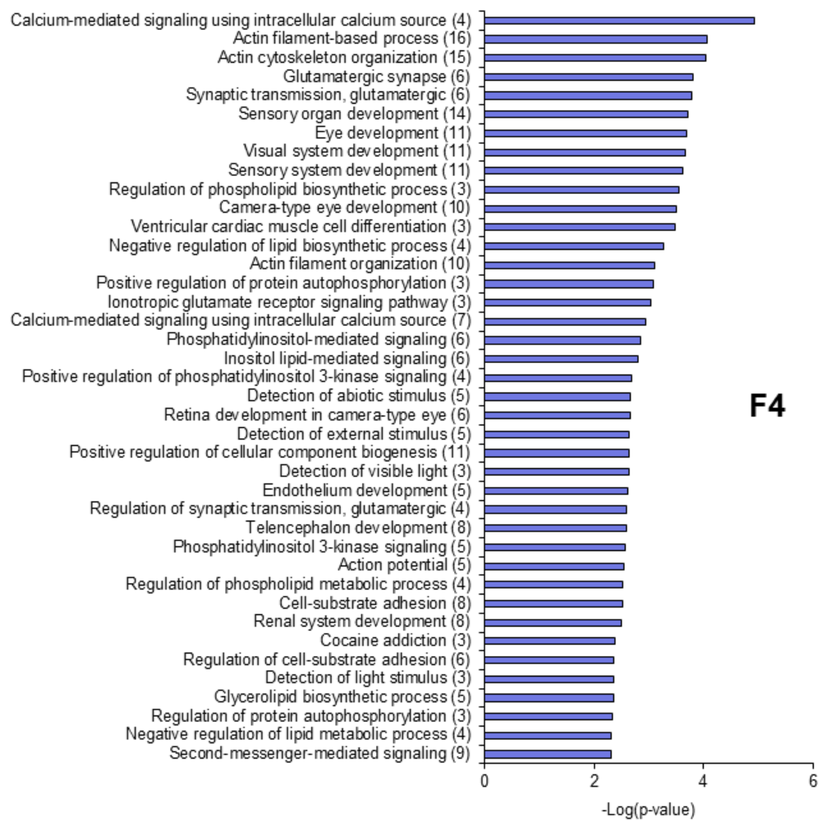
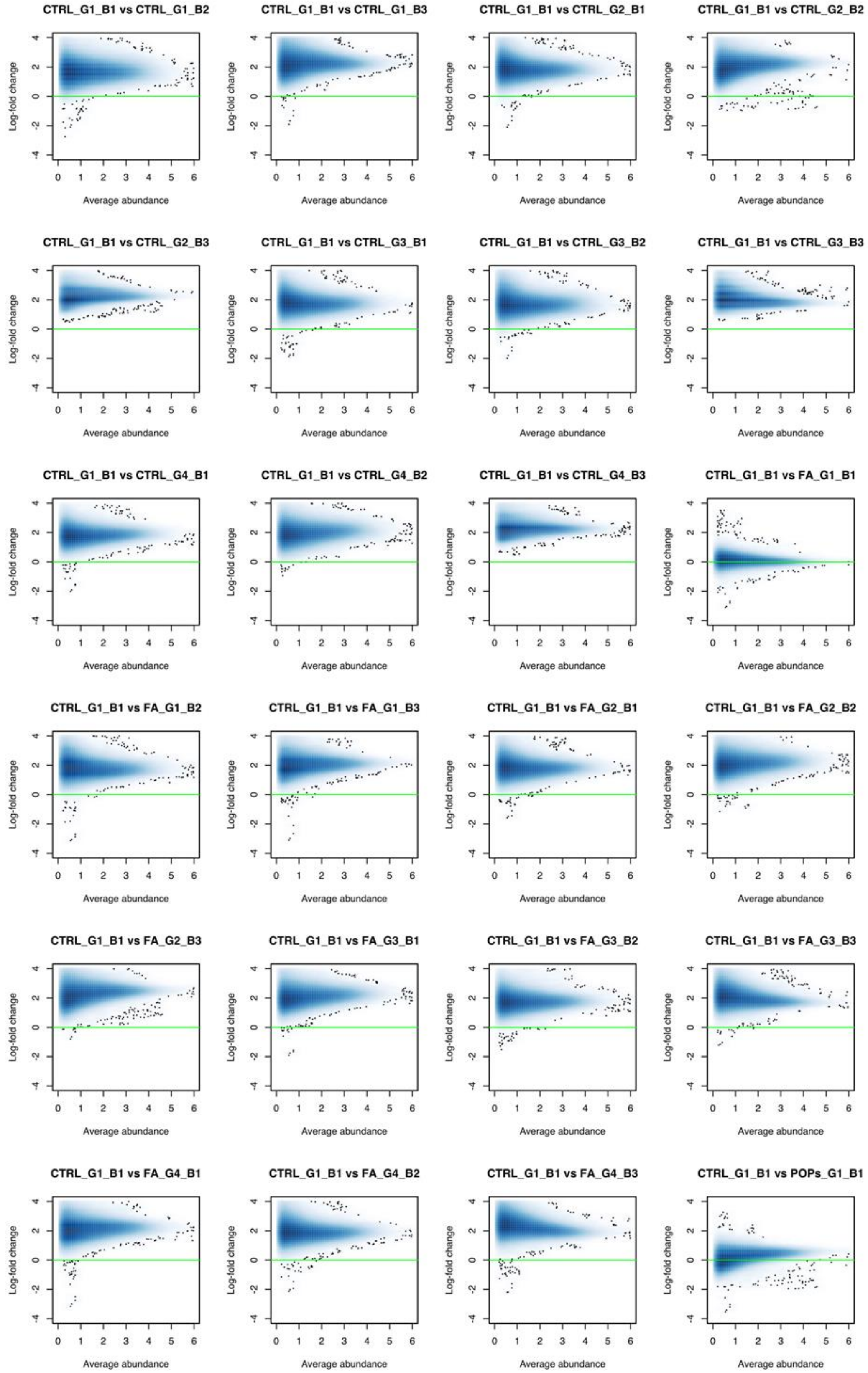


Figure S2.13.3. Gene ontology (GO) enrichment analysis of the genes associated to DMSs affected due to POPs+FA regardless of methylation status in F1-F4. Up to 40 most significant biological pathways are presented. The number of genes associated to each pathway is in parenthesis.



CTRL_G1_B1 vs CTRL_G1_B2
CTRL_G1_B1 vs CTRL_G1_B3
CTRL_G1_B1 vs CTRL_G2_B1
CTRL_G1_B1 vs CTRL_G2_B2
CTRL_G1_B1 vs CTRL_G2_B3
CTRL_G1_B1 vs CTRL_G3_B1
CTRL_G1_B1 vs CTRL_G3_B2
CTRL_G1_B1 vs CTRL_G3_B3
CTRL_G1_B1 vs CTRL_G4_B1
CTRL_G1_B1 vs CTRL_G4_B2
CTRL_G1_B1 vs CTRL_G4_B3
CTRL_G1_B1 vs FA_G1_B1
CTRL_G1_B1 vs FA_G1_B2
CTRL_G1_B1 vs FA_G1_B3
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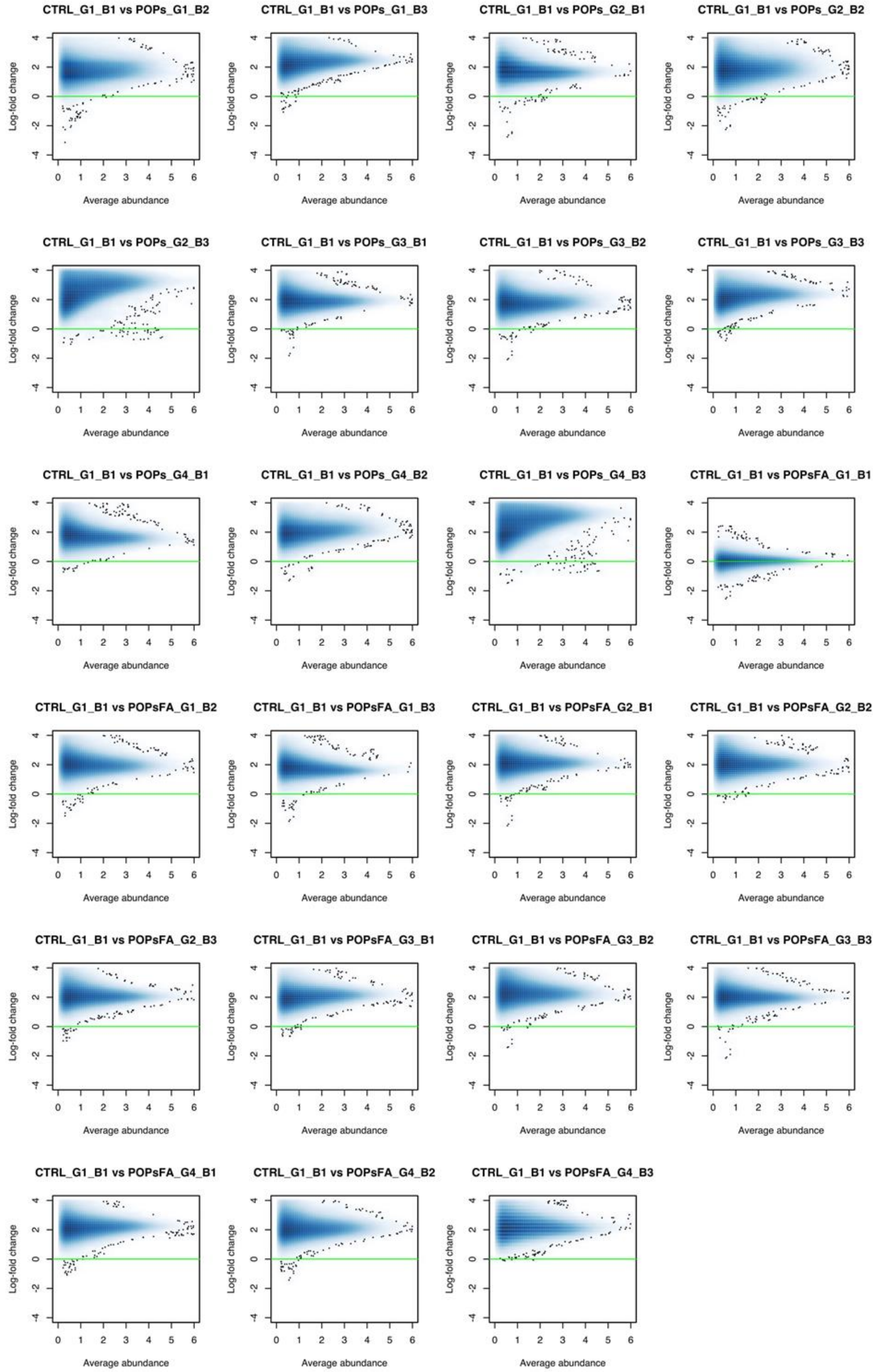
CTRL_G1_B1 vs FA_G2_B3
CTRL_G1_B1 vs FA_G3_B1
CTRL_G1_B1 vs FA_G3_B2
CTRL_G1_B1 vs FA_G3_B3
CTRL_G1_B1 vs FA_G4_B1
CTRL_G1_B1 vs FA_G4_B2
CTRL_G1_B1 vs FA_G4_B3
CTRL_G1_B1 vs POPs_G1_B1
Log-fold change
Average abundance


CTRL_G1_B1 vs POPs_G1_B2
CTRL_G1_B1 vs POPs_G1_B3
CTRL_G1_B1 vs POPs_G2_B1
CTRL_G1_B1 vs POPs_G2_B2
CTRL_G1_B1 vs POPs_G2_B3
CTRL_G1_B1 vs POPs_G3_B1
CTRL_G1_B1 vs POPs_G3_B2
CTRL_G1_B1 vs POPs_G3_B3
CTRL_G1_B1 vs POPs_G4_B1
CTRL_G1_B1 vs POPs_G4_B2
CTRL_G1_B1 vs POPs_G4_B3
CTRL_G1_B1 vs POPsFA_G1_B1
CTRL_G1_B1 vs POPsFA_G1_B2
CTRL_G1_B1 vs POPsFA_G1_B3
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CTRL_G1_B1 vs POPsFA_G2_B2
CTRL_G1_B1 vs POPsFA_G2_B3
CTRL_G1_B1 vs POPsFA_G3_B1
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CTRL_G1_B1 vs POPsFA_G3_B3
CTRL_G1_B1 vs POPsFA_G4_B1
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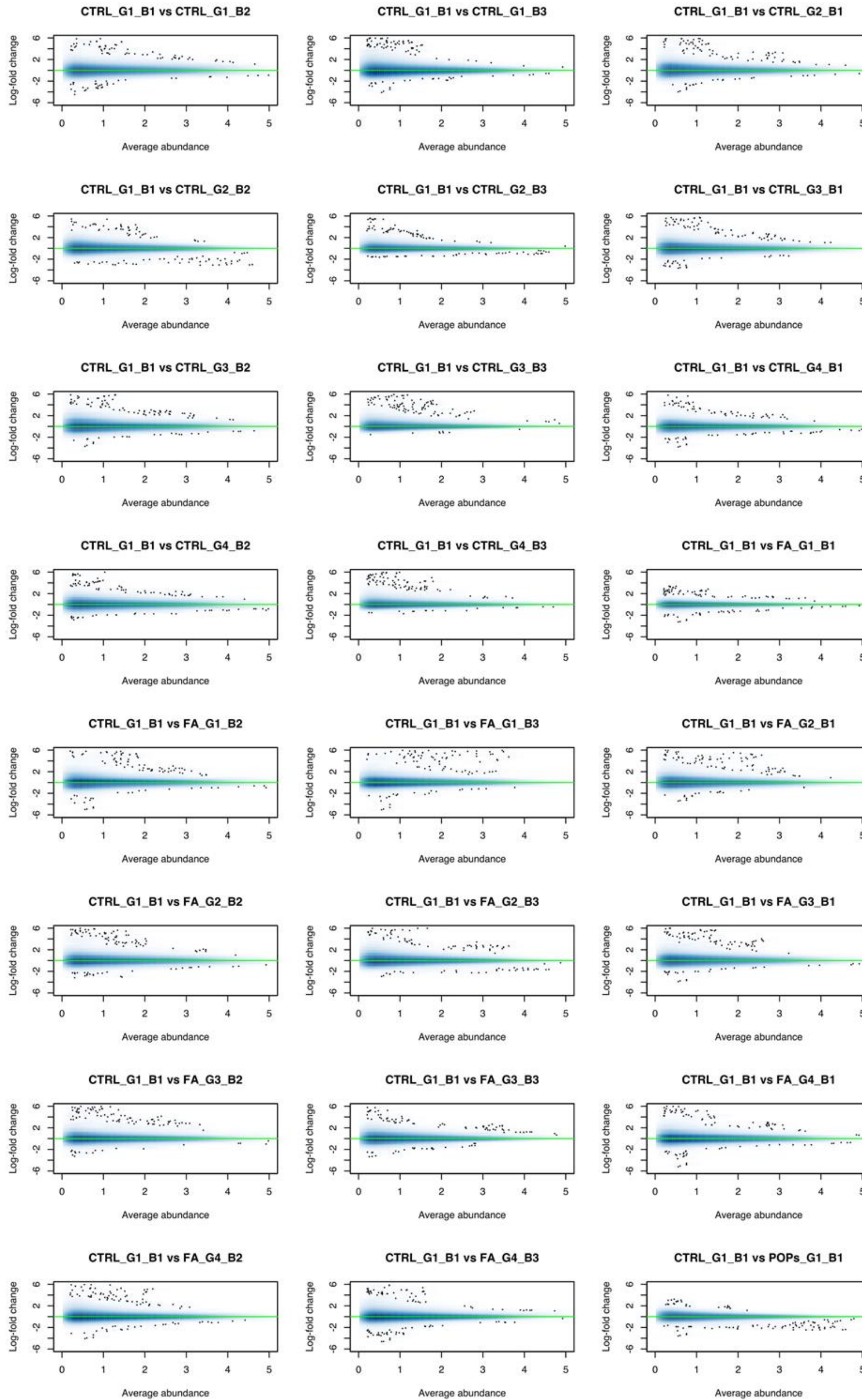
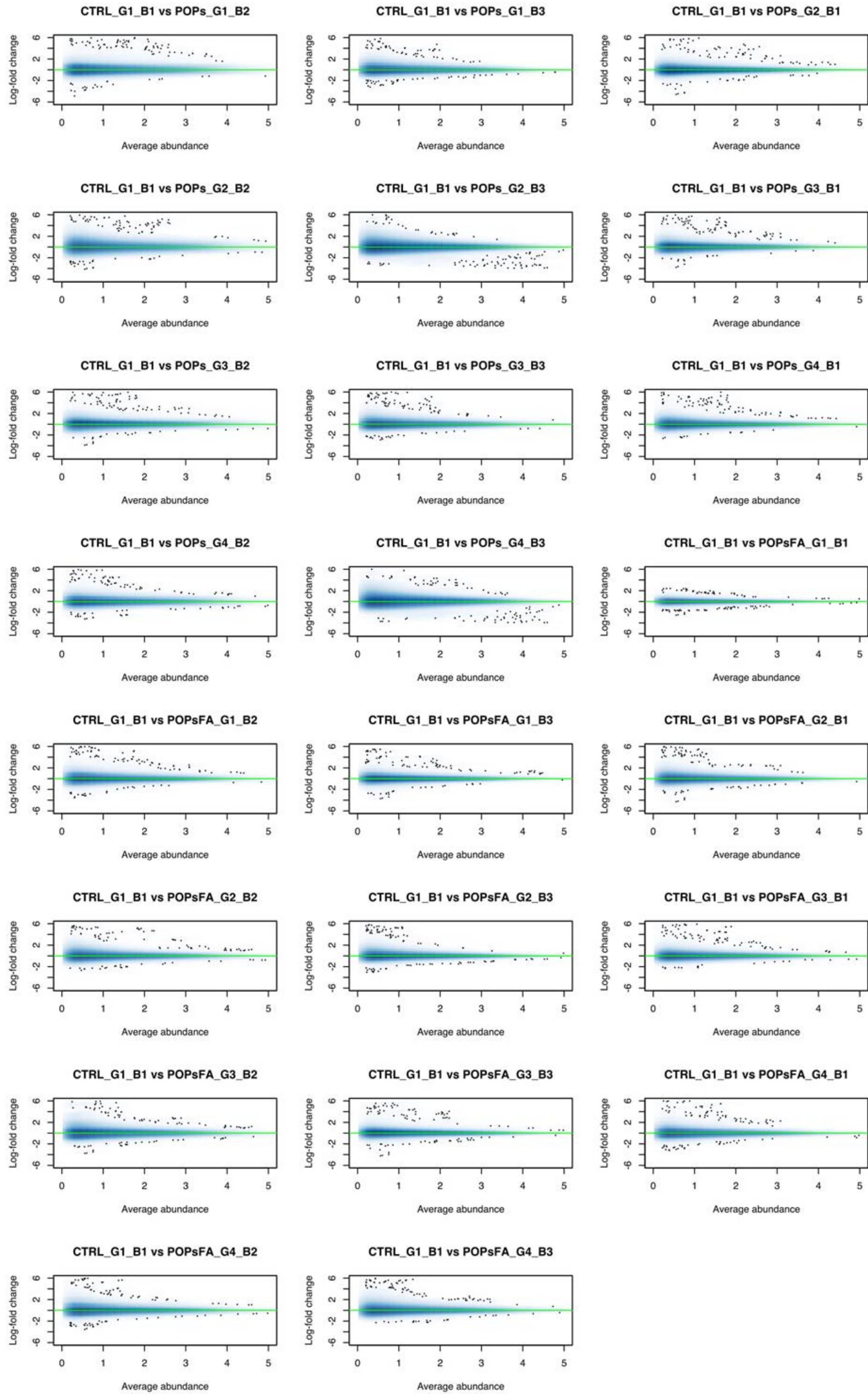
CTRL_G1_B1 vs POPsFA_G4_B3
Log-fold change
Average abundance


CTRL_G1_B1 vs CTRL_G1_B2
CTRL_G1_B1 vs CTRL_G1_B3
CTRL_G1_B1 vs CTRL_G2_B1
CTRL_G1_B1 vs CTRL_G2_B2
CTRL_G1_B1 vs CTRL_G2_B3
CTRL_G1_B1 vs CTRL_G3_B1
CTRL_G1_B1 vs CTRL_G3_B2
CTRL_G1_B1 vs CTRL_G3_B3
CTRL_G1_B1 vs CTRL_G4_B1
CTRL_G1_B1 vs CTRL_G4_B2
CTRL_G1_B1 vs CTRL_G4_B3
CTRL_G1_B1 vs FA_G1_B1
CTRL_G1_B1 vs FA_G1_B2
CTRL_G1_B1 vs FA_G1_B3
CTRL_G1_B1 vs FA_G2_B1
CTRL_G1_B1 vs FA_G2_B2
CTRL_G1_B1 vs FA_G2_B3
CTRL_G1_B1 vs FA_G3_B1
CTRL_G1_B1 vs FA_G3_B2
CTRL_G1_B1 vs FA_G3_B3
CTRL_G1_B1 vs FA_G4_B1
CTRL_G1_B1 vs FA_G4_B2
CTRL_G1_B1 vs FA_G4_B3
CTRL_G1_B1 vs POPs_G1_B1
Log-fold change
Average abundance



CTRL_G1_B1 vs POPs_G1_B2
CTRL_G1_B1 vs POPs_G1_B3
CTRL_G1_B1 vs POPs_G2_B1
CTRL_G1_B1 vs POPs_G2_B2
CTRL_G1_B1 vs POPs_G2_B3
CTRL_G1_B1 vs POPs_G3_B1
CTRL_G1_B1 vs POPs_G3_B2
CTRL_G1_B1 vs POPs_G3_B3
CTRL_G1_B1 vs POPs_G4_B1
CTRL_G1_B1 vs POPs_G4_B2
CTRL_G1_B1 vs POPs_G4_B3
CTRL_G1_B1 vs POPsFA_G1_B1
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CTRL_G1_B1 vs POPsFA_G1_B3
CTRL_G1_B1 vs POPsFA_G2_B1
CTRL_G1_B1 vs POPsFA_G2_B2
CTRL_G1_B1 vs POPsFA_G2_B3
CTRL_G1_B1 vs POPsFA_G3_B1
CTRL_G1_B1 vs POPsFA_G3_B2
CTRL_G1_B1 vs POPsFA_G3_B3
CTRL_G1_B1 vs POPsFA_G4_B1
CTRL_G1_B1 vs POPsFA_G4_B2
CTRL_G1_B1 vs POPsFA_G4_B3
Log-fold change
Average abundance

Figure S2.13.4. Normalization of ChIP-seq sperm H3K4me3 data. Two upper panels illustrate data before normalization was applied. Two lower panels show data after normalization. MA-plots were used to compare the log2 ratio of counts per filtered windows (M) against the average abundance (A) of the window across all libraries, before and after normalization. M value comparison at high abundance showed a non-linear distribution for samples, entailing a slight immunoprecipitation efficiency bias. Consequently, to correct for non-linear biases, loess normalization was used for all libraries, which assumes that most windows are not differentially enriched (Ballman *et al.*, 2004; Robinson *et al.*, 2010). The counts per million normalization was also used for all libraries to correct for differences in library size. “B” = batch and/or pool; “G” = generation.

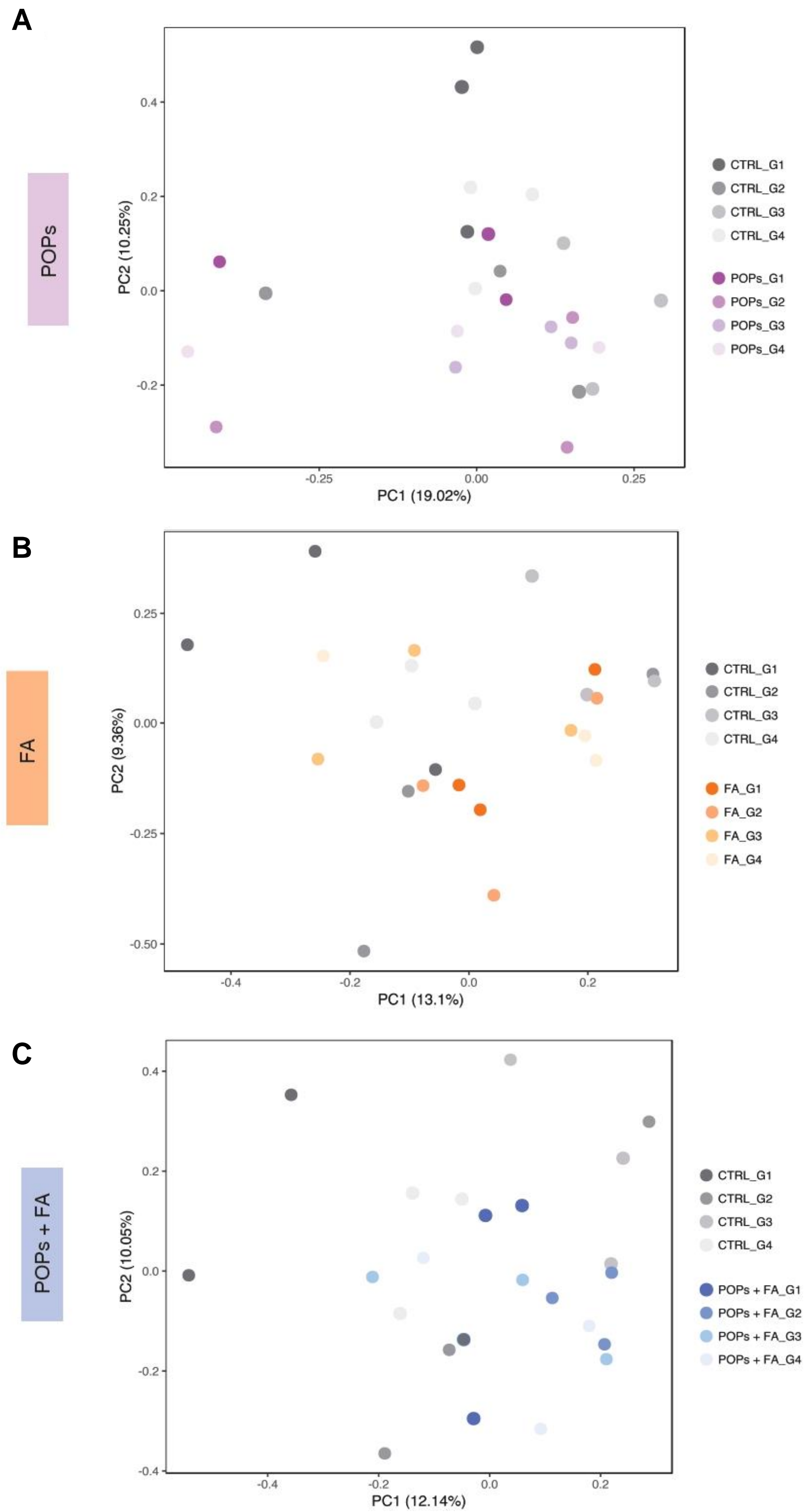

Figure S2.13.5. PCA plots depicting generational trends associated to sperm H3K4me3 following *in utero* POPs, FA and POPs+FA vs. CTRL. PCA plots of filtered normalized H3K4me3 reads across all generations for **(A)** CTRL vs. POPs, **(B)** CTRL vs. FA sperm, **(C)** CTRL vs POPs+FA ancestrally exposed sperm.

Table S2.13.1. Distribution of DMSs associated to distal intergenic regions, and average percentage difference of methylation changes compared to CTRL for POPS, FA, POPs+FA in F1-F4.

Treatment	Generation	Number DMSs	Avg % difference of methylation changes
POPs	F1	90↑	30.9 ± 9.9
		71↓	-31.3 ± 7.7
	F2	99↑	28.6 ± 6.3
		57↓	-32.5 ± 11.2
	F3	53↑	31.1 ± 8.2
	F4	137↑	33.9 ± 11.5
FA	F1	162↑	33.0 ± 10.8
		105↓	-34.0 ± 11.1
	F2	101↑	31.3 ± 9.9
		118↓	-29.7 ± 8.0
	F3	82↑	31.0 ± 10.9
		49↓	23.5 ± 9.0
	F4	176↑	29.4 ± 9.4
POPs+FA	F1	107↑	29.6 ± 8.5
		140↓	-31.3 ± 8.1
	F2	54↑	30.9 ± 8.7
		182↓	-27.2 ± 5.7
	F3	51↑	28.6 ± 6.8
		53↓	-30.7 ± 7.5
	F4	107↑	30.3 ± 8.4
		161↓	-30.4 ± 7.9

Table S2.13.2. Unique genes associated with significantly altered DMSs inter- and transgenerationally (F1F2 and F1F2F3, F1F2F3F4) due to *in utero* FA exposure. Methylation status depicted in ↑ for hyper-methylation and ↓ for hypo-methylation. Few genes (*LOC100360106, Zmat4, Mir678*) were associated to multiple DMSs with opposing methylation status and genomic annotation.

F1F2		
Gene symbol	**Genomic annotation**	**Methylation status**
Lpar2	3' UTR	↑
Ppil1	3' UTR	↓
Gbx1	3' UTR	↓
Zfp418	Distal Intergenic	↑
Vom2r30	Distal Intergenic	↑
Grk2	Distal Intergenic	↑
LOC100360106	Distal Intergenic	↑↓
Crybb3	Distal Intergenic	↑
Atp6v1g3	Distal Intergenic	↑
Prag1	Distal Intergenic	↑
Zmat4	Distal Intergenic + Intron (NM_001134747/684961, intron 1 of 7)	↑↓
Tubgcp3	Distal Intergenic	↑
Tex29	Distal Intergenic	↑
Zfp532	Distal Intergenic	↑
Smim15	Distal Intergenic	↑
Tuft1	Distal Intergenic	↑
Rhobtb1	Distal Intergenic	↑
Dab2ip	Distal Intergenic	↑
Ndufa8	Distal Intergenic	↑
LOC100909675	Distal Intergenic	↑
Grin3a	Distal Intergenic	↑
Slc44a1	Distal Intergenic	↑
Six3	Distal Intergenic	↑
Dusp6	Distal Intergenic	↑
Gsdmc	Distal Intergenic	↑
Srp9	Distal Intergenic	↓
Sertad4	Distal Intergenic	↓
Pspc1	Distal Intergenic	↓
Dusp26	Distal Intergenic	↓
Myom2	Distal Intergenic	↓
Cln8	Distal Intergenic	↓
Iars	Distal Intergenic	↓
Gins2	Distal Intergenic	↓
Banp	Distal Intergenic	↓
Dclk1	Distal Intergenic	↓
S1pr1	Distal Intergenic	↓
LOC499843	Distal Intergenic	↓

Rpl10a	Distal Intergenic	↓
Galntl5	Distal Intergenic	↓
Cdca4	Distal Intergenic	↓
Zfp386	Distal Intergenic	↓
Mageb5	Distal Intergenic	↓
Tnrc18	Exon (NM_001107123/304302, exon 4 of 35)	↓
RGD1562726	Exon (NM_001109052/498060, exon 3 of 3)	↑
Fcgbp	Exon (NM_001164657/100303643, exon 7 of 20)	↓
Fat1	Exon (NM_031819/83720, exon 2 of 27)	↑
Tmem218	Intron (NM_001008325/300516, intron 3 of 3)	↑
MGC94891	Intron (NM_001044277/681210, intron 1 of 9)	↓
Zfhx2	Intron (NM_001098803/305888, intron 1 of 9)	↓
Galnt2	Intron (NM_001106196/292090, intron 2 of 13)	↑
Syne3	Intron (NM_001106762/299356, intron 20 of 23)	↑
Calm1	Intron (NM_001108719/362768, intron 15 of 15)	↑
Grik3	Intron (NM_001112716/298521, intron 1 of 15)	↓
Zfpm2	Intron (NM_001130501/314930, intron 3 of 6)	↓
Gaa	Intron (NM_001134688/287867, intron 24 of 26)	↓
Mphosph9	Intron (NM_001170554/288654, intron 9 of 21)	↑
Rbpms	Intron (NM_001271244/498642, intron 1 of 6)	↓
Ppp1r21	Intron (NM_001271305/362697, intron 17 of 21)	↓
Fam210a	Intron (NM_001271365/679578, intron 6 of 7)	↓
Map7	Intron (NM_001277694/365057, intron 9 of 34)	↓
Actg2	Intron (NM_012893/25365, intron 2 of 7)	↓
Ell3	Intron (NM_017319/29468, intron 10 of 13)	↓
Fbln5	Intron (NM_019153/29158, intron 5 of 10)	↑
Nrxn2	Intron (NM_053846/116595, intron 8 of 21)	↑
Cabyr	Intron (NM_172023/259221, intron 24 of 27)	↓
Extl3	Promoter (<=1kb)	↓
Mir678	Intron (NM_001025144/499420, intron 6 of 10) + Promoter (<=1kb)	↑↓
Asb2	Promoter (2-3kb)	↑
Nphp4	Promoter (2-3kb)	↓
Rexo2	Promoter (2-3kb)	↓
	F1F2F3	
Gene symbol	**Genomic annotation**	**Methylation status**
Lpar2	3' UTR	↑
Atp6v1g3	Distal Intergenic	↑
Prag1	Distal Intergenic	↑
Smim15	Distal Intergenic	↑
Slc44a1	Distal Intergenic	↑
Gsdmc	Distal Intergenic	↑
Pspc1	Distal Intergenic	↑
Gins2	Distal Intergenic	↑
Fat1	Exon (NM_031819/83720, exon 2 of 27)	↑
Zfpm2	Intron (NM_001130501/314930, intron 3 of 6)	↑
Mphosph9	Intron (NM_001170554/288654, intron 9 of 21)	↑

Map7	Intron (NM_001277694/365057, intron 9 of 34)	↑
Nphp4	Promoter (2-3kb)	↑
Rexo2	Promoter (2-3kb)	↑
F1F2F3F4		
Gene symbol	**Genomic annotation**	**Methylation status**
Lpar2	3' UTR	↑
Prag1	Distal Intergenic	↑
Gsdmc	Distal Intergenic	↑

Table S2.13.3. Unique genes associated with significantly altered DMSs inter- and transgenerationally (F1F2 and F1F2F3, F1F2F3F4) due to *in utero* POPs+FA exposure. Methylation status depicted in ↑ for hyper-methylation and ↓ for hypo-methylation. All genes were associated with hypo-methylated DMSs.

Gene symbol	Genomic annotation	Methylation status
F1F2		
Bclaf1	Distal Intergenic	↓
Irx4	Distal Intergenic	↓
Kctd15	Distal Intergenic	↓
Cacng5	Distal Intergenic	↓
Cxcr4	Distal Intergenic	↓
LOC498308	Distal Intergenic	↓
Barhl2	Distal Intergenic	↓
Bmp4	Distal Intergenic	↓
Scel	Distal Intergenic	↓
Glg1	Distal Intergenic	↓
Ccnh	Distal Intergenic	↓
Tspan5	Distal Intergenic	↓
Cth	Distal Intergenic	↓
Fbxw2	Distal Intergenic	↓
Capzb	Distal Intergenic	↓
Exd2	Distal Intergenic	↓
Dusp6	Distal Intergenic	↓
Adpgk	Distal Intergenic	↓
Pygo1	Distal Intergenic	↓
Tgfbr2	Distal Intergenic	↓
MGC116197	Distal Intergenic	↓
Ilkap	Distal Intergenic	↓
Pja2	Distal Intergenic	↓
Evpl	Exon (NM_001107066/303687, exon 22 of 22)	↓
Tnrc18	Exon (NM_001107123/304302, exon 4 of 35)	↓
Ctsh	Exon (NM_001170531/192213, exon 23 of 35)	↓
Fam210a	Intron (NM_001007688/307343, intron 3 of 3)	↓
Btbd9	Intron (NM_001013073/294318, intron 7 of 10)	↓
Sulf2	Intron (NM_001034927/311642, intron 1 of 20)	↓
Tmem26	Intron (NM_001107624/309728, intron 4 of 11)	↓
Zmat4	Intron (NM_001134747/684961, intron 1 of 7)	↓
Add3	Intron (NM_001164103/25230, intron 1 of 14)	↓
Kcnk10	Intron (NM_023096/65272, intron 1 of 6)	↓
Ly6g5c	Intron (NM_198739/294245, intron 2 of 2)	↓
Prop1	Promoter (2-3kb)	↓
F1F2F3		
Gene symbol	**Genomic annotation**	**Methylation status**
Scel	Distal Intergenic	↓
Exd2	Distal Intergenic	↓
Evpl	Exon (NM_001107066/303687, exon 22 of 22)	↓

Ly6g5c	Intron (NM_198739/294245, intron 2 of 2)	↓
F1F2F3F4		
Gene symbol	**Genomic annotation**	**Methylation status**
Scel	Distal Intergenic	↓
Evpl	Exon (NM_001107066/303687, exon 22 of 22)	↓
Ly6g5c	Intron (NM_198739/294245, intron 2 of 2)	↓

Chapter 3

Prenatal Exposure to Environmentally-Relevant Contaminants Perturbs Male Reproductive Parameters Across Multiple Generations that are Partially Protected by Folic Acid Supplementation.

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3.1 Resumé

On pense que l'environnement paternel influence la qualité des spermatozoïdes et que la progéniture future pourrait également être affectée. Nous avons émis l'hypothèse que l'exposition prénatale à des contaminants environnementaux nuit à la reproduction masculine et altére l'expression des gènes embryonnaires sur plusieurs générations. L'acide folique (AF) peut améliorer la qualité des spermatozoïdes et les résultats de la grossesse, nous avons donc émis l'hypothèse que l'AF atténue les effets des contaminants. Des rates Sprague-Dawley F0 traitées avec des polluants organiques persistants (POPs) ou de l'huile de maïs et nourries avec un régime basal ou enrichi en AF, ont été utilisées pour initier quatre générations de portées. Seules les femelles F0 ont reçu des traitements POPs et / ou AF. L'exposition *in utero* aux POPs a modifié les paramètres spermatiques en F1, qui ont été en partie protégé par la supplémentation en AF. L'exposition paternelle aux POPs a réduit la qualité spermatique des mâles F2, et la fertilité des mâles F3 a été modifiée par les POPs et l'AF. La supplémentation ancestrale en AF a amélioré les paramètres spermatiques des mâles F4, tandis que l'effet des POPs les a diminué. Curieusement, les mâles F3 ont eu les résultats de grossesse les plus pauvres et ont généré les embryons avec les gènes les plus différentiellement exprimés. L'exposition précoce aux POPs nuit à la reproduction des mâles sur plusieurs générations. La supplémentation en AF a en partie atténué l'impact des POPs. Le transcriptome des embryons à deux cellules est sensible à l'environnement paternel et pourrait être le fondement de l'issue de la grossesse future.

3.2 Abstract

The paternal environment is thought to influence sperm quality and future progeny may also be impacted. We hypothesized that prenatal exposure to environmentally-relevant contaminants impairs male reproduction, altering embryo gene expression over multiple generations. Folic acid (FA) can improve sperm quality and pregnancy outcomes, thus we further hypothesized that FA mitigates the contaminants.

Sprague-Dawley F0 female rats treated with persistent organic pollutants (POPs) or corn oil and fed basal or supplemented FA diets, then used to yield four generations of litters. Only F0 females received POPs and/or FA treatments.

In utero POPs exposure altered sperm parameters in F1, which were partly rescued by FA supplementation. Paternal exposure to POPs reduced sperm quality in F2 males, and the fertility of F3 males was modified by both POPs and FA. Ancestral FA supplementation improved sperm parameters of F4 males, while the POPs effect diminished. Intriguingly, F3 males had the poorest pregnancy outcomes and generated the embryos with the most significantly differentially expressed genes. Early-life exposure to POPs harms male reproduction across multiple generations. FA supplementation partly mitigated the impact of POPs. The two-cell embryo transcriptome is susceptible to paternal environment and could be the foundation for later pregnancy outcomes.

3.3 Introduction

Levels of persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs) and legacy pesticides such as dichlorodiphenyltrichloroethane (DDT) have been declining since their use was restricted by the Stockholm Convention in 2001 (Wohrnschimmel *et al.*, 2016). Nonetheless, due to their long half-life and lipophilic nature, they remain present in the environment (NCP, 2013). Many POPs are known to be endocrine disruptors (Zoeller *et al.*, 2012) and may impair spermatogenesis and sperm function, thereby reducing male fertility and reproductive health (Chia, 2000; Hauser *et al.*, 2002). With the alarming decline in sperm counts since 1970, it is of interest to determine whether and to what extent POPs may play in male fertility (Latif *et al.*, 2017).

In men, semen quality can be considered a biomarker for overall health (Eisenberg *et al.*, 2016; Jensen *et al.*, 2009; Latif *et al.*, 2017). Men with reduced sperm quality have a shorter life expectancy (Jensen *et al.*, 2009) and have a higher risk of adverse health outcomes such as cardiovascular disease, hypertension and diabetes (Eisenberg *et al.*, 2016; Latif *et al.*, 2017). Exposure of men to POP contaminants may thus influence not just their fertility but also their overall health (Consales *et al.*, 2016; Vested *et al.*, 2014).

There is an increasing body of research demonstrating that the adverse effects of environmental contaminants can be transmitted to subsequent generations (Guerrero-Bosagna *et al.*, 2014; Skinner, Guerrero-Bosagna, *et al.*, 2013; Veenendaal *et al.*, 2013). Although historical presumption links the health of subsequent generations to the mother (Sharp *et al.*, 2018), paternal exposures can also affect the development of his descendants (J. M. Braun *et al.*, 2017; Soubry, 2015).

Maternal folic acid (FA) supplementation in the periconceptional period reduces neural tube defects (Czeizel *et al.*, 1992; MRC, 1991) and supplementation is recommended for women of childbearing age (Canada, 2001). In Canada, and many other countries, higher folic acid intake is ensured by mandatory fortification of white wheat flour and other enriched grain products (Canada, 1998) Folic acid deficiency in mouse models reduces sperm function and

sperm count (Lambrot *et al.*, 2013; B.G. Swayne *et al.*, 2012; Wallock *et al.*, 2001) and supplementation could protect the father's sperm from the adverse effects of environmental contaminants (Shorter *et al.*, 2015).

This study tested the hypothesis that prenatal exposure to environmentally-relevant POPs disrupts sperm quality, fertility and early embryo gene expression across multiple, unexposed generations in a rat model. The POPs composition and dosage has been previously confirmed to be comparable to lower concentrations measured in maternal and umbilical cord plasma in Arctic populations (Anas *et al.*, 2005). Further, dietary FA supplementation was assessed for whether it mitigated the effects of POPs to improve male reproductive parameters.

3.4 Materials and methods

3.4.1 Environmentally-relevant mixture

As described previously, the POPs mixture (Anas *et al.*, 2005; Maurice *et al.*, 2018) was designed to approximate the profile the Canadian northern food chain (D. Muir *et al.*, 1999). The experimental dose, which is considered to be environmentally-relevant (Anas *et al.*, 2005), is reported as 500 µg PCBs/kg body weight plus other POPs in lower proportions (Table 3.12.4).

3.4.2 Diets

Animals were fed one of two FA-defined diets based on the AIN-93G formula (Reeves *et al.*, 1993): control (2 mg FA/kg diet) or supplemented (6 mg FA/kg diet) (# 110700 and #117819 respectively by Dyets, Inc, Bethlehem, PA). The control diet approximates the FA intake of Canadian adults during the post-fortification era (0.4 mg/day), whereas the supplemented diet corresponds to the total FA intake from fortified foods plus a daily multivitamin containing 1 mg FA (B.G. Swayne *et al.*, 2012).

3.4.3 Study design

Animal care and all treatment procedures were in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Université Laval animal Research Ethics Committee (certificate # 2015010-2). Forty healthy five-week-old female Sprague-Dawley rats (Charles River Laboratories, Saint-Constant, Quebec, Canada) were used as founder dams (F0). Rats were housed in standard cages at 22°C (50% humidity) with 12-hour light-dark cycle. Food and water were provided *ad libitum*.

F0 females were housed two/cage for 10 days of acclimatization and then randomly assigned to four treatment groups (n = 6; Figure 3.12.2): (1) Control group ("CTRL") were fed the control diet (2 mg FA/kg diet described above); (2) POPs group ("POPs") received the POPs mixture (Table 3.12.4) by gavage and fed the control diet; (3) FA-supplemented group ("FA") were fed the FA-supplemented diet (6 mg FA/kg diet); (4) POPs and FA supplemented group ("POPsFA") were gavaged with the POPs mixture and fed the FA-supplemented diet. All groups were weighed and gavaged thrice weekly with corn oil (CTRL and FA groups) or the POPs mixture (POPs and POPsFA groups) for five weeks before

mating with untreated males (aged 90 days, Charles River). Mating was confirmed by the presence of sperm in vaginal smears and pregnant females were then housed individually. Gavage continued until the birth of F1 pups. After parturition, all F0 females received the control diet. Pups were weaned at Postnatal Day (PND) 21 and housed two/cage (n = 12). All subsequent generations received the control diet and no additional treatment.

At PND 90, randomly selected F1 males (two/F0 dam) were mated with untreated females (aged 63-74 days, Charles River) to generate F2 (Figure 3.12.2). Likewise, F3 and F4 lineages were generated. After 36 h of pregnancy, females were sacrificed to collect two cell embryos (n = 12). At Gestational Day (GD) 19.5, females were sacrificed to collect fetuses (n = 6) and the remaining females gave birth (n = 6). Rats were anesthetized with 3% isoflurane then euthanized by exsanguination via cardiac puncture and CO_2 asphyxiation. F4 adult males were not mated.

The body weights of the animals were closely monitored thrice weekly to ensure animal welfare. No apparent signs of general or systemic toxicity, such as behavioral changes or increased excitability, were observed. Weight gain increased over the nine weeks of treatment for all generations (*Supplementary Figure 3.13.1*).

3.4.4 Assessment of sperm concentration, morphology and viability

Adult males at PND 150 (n = 12 males) were euthanized to collect the *cauda* epididymides and sperm obtained by diffusion (Klinefelter *et al.*, 1991) into M199 medium (GIBCO®, ThermoFisher Scientific, Mississauga, ON, Canada) at 37°C for 30 min. Simultaneously, testes were collected, flash frozen and stored at -80°C for spermatid counts.

Cauda epididymal sperm concentrations were counted using a hemocytometer (Bright-Line™, Sigma-Aldrich, Oakville, ON, Canada) by diluting 1/50 in fixation medium (WHO, 2010). At least 200 spermatozoa/replicate were counted. Sperm counts were normalized by epididymal weight.

Sperm samples (50 µl) were further diluted in 450 µl warmed M199 medium. To assess morphology, sperm were smeared on a slide and dipped in SpermBlue fixative as previously

reported (van der Horst *et al.*, 2009) (Ref. SB-250-N, ©MICROPTIC 2018, Spain). To evaluate viability, sperm were mixed with equal parts eosin-nigrosin stain (WHO, 2010). Slides were prepared in duplicate. Morphology and viability were evaluated on 100 spermatozoa/slide using phase-contrast microscopy (400X).

Testicular spermatid counts were obtained using frozen testes (n = 4). The tunica albuginea was removed as described in Seung, Wolfe (2003). Weighted testes were homogenized in 10% DMSO / 0.9% NaCl using a Polytron TissueMiser (Fisher Scientific, Pittsburgh, PA) and sonicated (Labsonic M, Sartorius Stedim, Oakville, ON, Canada) for 1 min at 40%. Spermatids were visualized using 0.1% trypan blue staining and counted by hemacytometry. Spermatid production was calculated as the mean count of both hemacytometer chambers divided by $0.4 \cdot 10^{-5}$ ml X dilution volume ($0.4 \cdot 10^{-5}$ ml is the suspension volume used per chamber).

3.4.5 Sperm motility analysis

Sperm motility (n = 12) was assessed by computer-assisted sperm analysis (CASA) with a CEROS analyzer (Version. 12 CEROS, Hamilton Thorne Research, Beverly, MA, USA). After incubation at 37°C for 30 min, samples were loaded into 100 µm Rat Toxicology Slides (Leja Compagny, #SC 100-01-02-B, B.V., The Netherlands) and assessed for motility, progressive motility, average path velocity (VAP), linear velocity (VSL), curvilinear velocity (VCL), mean amplitude of lateral head displacement (ALH), frequency of head displacement (BCF), straightness coefficient (STR) and linearity coefficient (LIN). The following settings were used: frame rate, 60 Hz; frames acquired, 30; minimum contrast, 80 and minimum cell size, 7 (Zhou *et al.*, 2008). A minimum of 200 spermatozoa from five different fields was assessed.

3.4.6 RNA analysis from two-cell embryos

Two-cell embryos were collected from multiple pregnancies sired by PND 90 males (n = 12) to examine the transcriptomes of F2 to F4 two-cell embryos. The untreated females were super-ovulated by intraperitoneal injection with 150 IU/kg PMSG (CDMV, Québec, Canada) followed 48 h later by 300 IU/kg hCG (CDMV, Québec, Canada). After hCG, super-ovulated females were combined with PND 90 F1, F2 or F3 males. Females were sacrificed 36 h after

copulation and ovaries plus oviducts were collected in 37°C M2 medium (Sigma, M7167). Using a pre-heated IVF Work Station (Origio Midatlantic Devices, NJ, USA), ovaries were separated from oviducts and small incisions were made to release the two-cell embryos. With a 100 µM Cook® Flexipet® Pipette (Cook Medical LLC, Bloomington, IN, USA), two-cell embryos were collected and stored at -80°C until all embryos were obtained. To ensure sufficient material, 15 embryos from three sires (descended from different F0 dams) within each treatment were pooled for total RNA extraction. For each treatment, three replicates were assessed. From each pool, total RNA was extracted using the PicoPure™ RNA Isolation Kit (ThermoFisher Scientific, Mississauga, ON, Canada).

3.4.7 Two-cell embryo RNA sequencing.

cDNA libraries were constructed using SMART-Seq v4 Ultra low input RNA Kit (Clontech Laboratories, Takara Bio Company; CA, USA). First-strand cDNA was synthesized using total RNA from 15 two-cell embryos by the 3' SMART-Seq CDS Primer II A and template switching was performed by SMART-Seq v4 Oligonucleotide at the 5' end of the transcript. cDNA from SMART sequences was amplified by PCR Primer II A. After 14 cycles of long-distance PCR, amplified cDNA was purified using the Agencourt AMPure XP Kit (Beckman Coulter, Cat.No. A63882). The quality of cDNA construction was validated using Agilent Tapestation 2200 system. Final libraries for Illumina Next Generation sequencing were prepared with 150 pg of cDNA using Nextera XT DNA Library Preparation Kits (Illumina Inc., San Diego, CA, USA). Twenty-four libraries with unique indices were pooled in equimolar ratio and sequenced for paired-end 125 pb sequencing using three lanes of a high output flow cell on an Illumina HiSeq 2500 V4 System. The average insert size for the paired-end libraries was 225 bp. Reads were trimmed using Trimmomatic v0.36 with the following options: TRAILING:30, SLIDINGWINDOW:4:20 and MINLEN:30. All other options used the default values. Quality checks were performed on raw and trimmed data using FastQC v0.11.5 and MultiQC v1.5. Quantification was performed with Kallisto v0.44 and differential expression analysis using R v3.5.0 using the DESeq2 v1.20.0. For subsequent analysis, only genes with a normalized count of >10, p value ≤ 0.05, Discovery Rate (FDR) ≤ 5% and -2 ≥ |Log2 Fold change| ≥ 2 (CTRL vs. other treatments) were included. Ingenuity® Pathway Analysis (IPA®, Ingenuity Systems Inc., Redwood City, CA) was used to identify gene ontology pathways altered by the significantly DE genes.

3.4.8 Assessment of pregnancy outcomes (F1-F3 males)

Fertility parameters were assessed as a function of the father's treatment lineage. Fetal analysis at GD 19.5, sex ratio and number of fetuses, *corpora lutea* and implantation sites were assessed to calculate the fertility rate (# fetuses / # *corpora lutea*)*100, pre-implantation losses ((# *corpora lutea* – # implantation sites) / # *corpora lutea*)*100 and post-implantation losses ((# implantation sites - # fetuses) / # implantation sites)*100. Litter size and number of dead and live pups were noted on PND 0, PND 6 and PND 11. Sex was confirmed by genital observation at PND 21.

3.4.9 Statistical analysis

Data were analyzed using SAS University (Copyright © SAS Institute Inc., SAS Campus Drive, Cary, North Carolina 27513, USA) using *Mixed* Procedure with a one-way analysis of variance (ANOVA) in 2 X 2 factorial design. Main effects of POPs, FA and interaction (POPs*FA) were considered. The number of pups/litter and the F0 females were also included in the expanded model and excluded when not significant. To increase diversity and minimize the effect of the F0 dams, male lineages were derived from an equivalent number of the F0 founder females throughout the four generations. Differences were considered significant at $p \leq 0.05$.

3.5 Results

3.5.1 Sperm characteristics following exposure to POPs and/or FA

The impact of POPs and/or FA supplementation exposure on sperm characteristics (counts, morphology and viability) are presented in Table 3.12.1. Testicular spermatid count is lower in the F2 generation for rats sired by F1 fathers whose germ cells were directly exposed to POPs *in utero* through F0 females' exposure ($p = 0.01$). The effect of FA supplementation on sperm morphology varied due to POPs in the F2 generation. When the F2 rats were not exposed to POPs, FA supplementation decreased normal sperm morphology. When combined with POPs, FA supplementation brings sperm morphology to levels comparable to control group (CTRL; $p = 0.01$). Sperm viability decreased due to POPs exposure in both F1 and F2 generations ($p = 0.02$; $p = 0.003$). FA-supplementation partially restored sperm viability in the F1 rats, but not in the F2 generation. ($p = 0.23$).

Prenatal POPs exposure reduced sperm motility and progressive motility in the F1 males ($p = 0.04$; Table 3.12.2). Similarly, prenatal POPs exposure and FA supplementation separately decreased both the straightness coefficient (STR; $p = 0.02$) and the linearity coefficient (LIN; $p = 0.01$). When combined POPs and FA, the coefficient returned to a level similar to CTRL in the same generation. Interestingly, POPs did not alter sperm motility in the F2 generation, although FA supplementation decreased both STR ($p = 0.02$) and LIN ($p = 0.03$; Table 3.12.2).

POPs exposure and/or FA supplementation also affected sperm in paternal lineages not directly exposed (F3 and F4 generations). Ancestral POPs treatment decreased STR ($p = 0.02$) in F3 males, while, the linear velocity (VSL; $p = 0.03$) and the curvilinear velocity (VCL; $p = 0.05$) parameters increased with ancestral FA supplementation in F4 males (Table 3.12.2). FA supplementation increased sperm viability in the F3 lineage ($p = 0.03$; Table 3.12.1).

3.5.2 POPs and/or FA supplementation alter gene expression in two-cell embryos

To understand whether the sperm from exposed males induced a phenotype in subsequent generations, we examined the transcriptome of F2 two-cell embryos (Figure 3.12.1). The

two-cell embryo stage was specifically chosen, because the gene expression patterns are more related to the paternal genome (Oswald *et al.*, 2000; Santos *et al.*, 2002).

First, we analyzed the impact of each treatment on gene expression and overlap of significantly differentially expressed (DE) genes (p value ≤ 0.05, FDR ≤ 5% and -2 ≥ |Log2 Fold change| ≥ 2) between treatments compared to CTRL in F2-F4 (Figure 3.12.1A). In F2, 29 significantly DE genes were common among POPs, FA and POPsFA treatments (*Supplementary Table 3.13.1*). POPs and POPsFA shared only one common gene in F2; though, FA and POPsFA, and POPs and FA share 239 and 26 significantly DE genes, respectively (Figure 3.12.1A). The number of significantly DE genes decreased for all treatments in F3 (POPs 429 to 10; FA 543 to 50; POPsFA 305 to 10); this dilution effect supports the rationale that reprogramming will minimize generational transmission. In comparison to the F2, F3 gene expression more closely resembles that of the unexposed CTRL. In F4, however, the number of significantly DE genes increased substantially POPs 10 to 445; FA 50 to 322 and POPsFA 10 to 465.

Next, we examined the distribution of up- and down-regulated genes due to POPs and/or FA supplementation compared to CTRL in F2-F4 (Figure 3.12.1B). In F2, of the 429 POPs-induced significantly DE genes, 422 were down-regulated (|Log2 Fold change| ≤ -2) and 7 were up-regulated (|Log2 Fold change| ≥ 2). In contrast, the majority of all significantly DE genes in FA and POPsFA embryos were upregulated, FA 43↓; 500↑ and POPsFA 1↓; 304↑, respectively. This expression pattern changed in F3 and F4 such that all treatments decreased gene expression (Figure 3.12.1B).

We performed gene-ontology analysis (GO) to identify pathways altered by the significantly DE genes due to direct and ancestral exposure to POPs and/or FA supplementation in F2-F4 (Fig. 1C). In F2, exposure to POPs, FA and POPsFA affected multiple similar pathways ($p \leq 0.05$) notably cell movement, apoptosis and cell homeostasis. Interestingly, pathways involved in male germ cell development were only affected by POPsFA in F2 ($p \leq 0.05$). In contrast, in F3, those male germ cell development pathways were affected in FA embryos.

In F4, multiple different pathways were altered by all treatment groups, except apoptosis, which was altered in POPs embryos.

To determine whether dietary FA supplementation rescued the impact of POPs on two-cell embryo transcriptomes, we compared the gene expression of shared genes among all treatments in F2-F4 (Figure 3.12.1D). In F2, 29 significantly DE genes (p value ≤ 0.05, FDR $\leq 5\%$ and $-2 \geq$ |Log2 Fold change| ≥ 2) were shared among POPs, FA and POPsFA, of which 27 genes were down-regulated in POPs embryos and up-regulated in FA embryos. The same 27 genes were up-regulated in POPsFA embryos but to a lesser extent. Only two genes, RragB and LOC108348078 (encodes ATP-dependent zinc metalloprotease YME1L1), were affected (up or down-regulated) by all treatments. This consistent effect was only observed in F2 and disappeared in subsequent generations (Figure 3.12.1D).

Lastly, we identified genes that were significantly DE (p value ≤ 0.05, FDR $\leq 5\%$ and $-2 \geq$ |Log2 Fold change| ≥ 2) across generations. Prenatal paternal POPs exposure led to one conserved significantly DE gene between F2 and F3. FA and POPsFA, however, altered 41 and 8 significantly DE genes from F2 to F3, respectively. None of these genes were significantly altered from F2-F4 in all treatments.

3.3 Ancestral POPs and/or FA supplementation were associated with negative pregnancy outcomes

Direct exposure of POPs and/or FA supplementation had no apparent effect on fertility parameters of the F1 and F2 males or pregnancy outcomes in their litters. We observed, however, transgenerational effects on pregnancy outcomes (Table 3.12.3), as the number of fetuses sired by F3 fathers decreased with ancestral POPs exposure ($p = 0.04$). The harmful effect of ancestral POPs exposure was also apparent as the number of implantation sites tended to be lower than CTRL ($p = 0.06$).

FA supplementation did not protect against POPs in our study, since the fertility rate was lower ($p = 0.03$) and preimplantation losses are higher ($p = 0.04$) in litters sired by the F3

fathers who were ancestrally exposed to both POPs and FA supplementation (Table 3.12.3). Moreover, there were more postimplantation losses due to ancestral FA supplementation in the absence of POPs ($p = 0.03$). Fertility parameters were not studied in the fourth generation as the F4 males were not mated.

3.6 Discussion

An increasing body of evidence suggests that, in men, poor sperm parameters are predictive of other pathologies later in life (Eisenberg *et al.*, 2016; Jensen *et al.*, 2009; Latif *et al.*, 2017). Therefore, our observations that direct and ancestral exposure to environmental contaminants decreases sperm function is of concern. We report here that prenatal exposure of the paternal lineage to a POPs mixture induces subtle reductions in sperm quality (F1-F4) and male fertility (F1-F3) in a Sprague-Dawley rat model, thereby affecting males that were not directly exposed to the contaminants. Concomitant supplementation with FA was hypothesized to counteract harmful effects of POPs, however, relatively moderate effects were observed, albeit over multiple generations.

These modest phenotypes likely reflect the physiologically appropriate treatments tested. Indeed, the present study used oral administration of environmentally relevant POPs concentrations intended to approximate body burdens in Inuit people (Anas *et al.*, 2005) as a pertinent model of contemporary pollutant exposure. In addition, we used a mixture of contaminants and the cumulative effect may differ from effects observed using a single contaminant (Mumford *et al.*, 2015). Moreover, the dietary FA levels used are physiological, not pharmacological. The control diet represents recommended adult intake (0.4 mg/d) and the supplemented diet reflects the FA intake levels of women in Canada, consuming FA-fortified food and a daily periconceptional supplements, corresponding to 3-fold the recommended dietary allowance (B. G. Swayne *et al.*, 2012).

3.6.1 Generational adverse effects due to prenatal paternal POPs exposure

F1 generation – POPs particularly affected sperm viability and motility

Prenatal paternal POPs exposure caused moderate, but adverse effects on sperm parameters (F1-F4) and male fertility (F1-F3). Sperm viability and motility appeared to be the most sensitive to POPs exposure, impacting predominantly sperm from F1 and F2 males (Tables 3.12.1 and 2). Similar effects were observed in men from the far North as they were exposed to POP contaminants, through their diet (Bonde *et al.*, 2016; Spano *et al.*, 2005; Toft, 2014). Other regions of concern include malaria-endemic regions, as previous studies indicate that

men have lower sperm quality associated with DDT exposure (Aneck-Hahn *et al.*, 2007; De Jager *et al.*, 2006).

Several POP chemicals have structural similarities with naturally occurring steroid and thyroid hormones, which may induce pseudo-hormonal or endocrine-disrupting behaviors causing imbalances in normal physiological processes (Skakkebaek *et al.*, 2016). Multiple studies demonstrated a link between prenatal exposure to endocrine disruptors and altered male reproductive health in adulthood (Hass *et al.*, 2012; Sanabria *et al.*, 2016; Vidaeff *et al.*, 2005). Endocrine disruption during a critical fetal developmental window may affect endocrine homeostatic mechanisms, thereby compromising adult reproductive function (Vested *et al.*, 2013) and explaining the reduced sperm quality observed in F1 rats following prenatal POPs exposure.

F2 generation – Prenatal POPs exposure altered F2 two-cell embryo gene expression and sperm phenotype in adult F2 males

Emerging evidence supports the concept that prenatal exposure to environmental pollutants can alter the sperm phenotype of subsequent generations (Vieira *et al.*, 2017). To determine whether the altered sperm parameters in POPs-exposed F1 males compromised the F2 generation, we examined early-embryo gene expression and later, sperm parameters in the adult F2 males. The two-cell embryo stage was selected because paternal epigenetic reprogramming is not yet complete and allows observation of heritable paternal traits (Santos *et al.*, 2002).

Gene expression was dramatically down-regulated due to prenatal paternal POPs exposure in F2 two-cell embryos (Figure 3.12.1B). Interestingly, gene ontology analysis revealed that similar pathways including cell movement, apoptosis and cellular homeostasis were affected by POPs (Figure 3.12.1D). Since the F2 two-cell embryos were not directly exposed to the treatments, it can be assumed that differences in their gene expression profiles are due to *in utero* POPs exposure in F1 male germ cells. Furthermore, these early embryo changes could explain the observed differences in sperm quality and overall phenotypic outcomes later in

adult F2 males. The testicular spermatid counts (Table 3.12.1) and sperm viability (Table 3.12.1) were decreased due to POPs in F2 adults.

Ancestral POPs exposure affects particularly F4 two-cell embryo gene expression

The F3 generation is the first unexposed and any phenotypes observed considered to be transgenerational. Therefore, appearance of any phenotypic traits due to F1 exposure is solely dependent on transmission via the male germ cell lineage.

Ancestral exposure to POPs still perturbed gene expression in the F3 two-cell embryos, albeit less than in the F2 embryos (10 versus 429, respectively). The affected genes in the F3 embryos are implicated in apoptosis (Figure 3.12.1C, F3 graph). Furthermore, only one gene, V-set immunoregulatory receptor (*Vsir*; ENSRNOG00000000569), was significantly DE expressed in both F2 and F3 two-cell embryos.

Perturbed gene expression in the embryos must be due to paternal factors in our model. Male germ cells undergo extensive epigenomic reprogramming during development from primordial germ cells in the fetal gonad through postnatal spermatogenesis and after fertilisation, and are thus vulnerable to environmental stressors during these reprogramming windows (Ly *et al.*, 2015). Prenatal paternal POPs exposure may have altered epigenetic marks in the F1 sperm that are transmitted to the F2 two-cell embryos, thereby changing their gene expression (Alegria-Torres *et al.*, 2011) and even postnatal development and health (Grova *et al.*, 2019). Indeed, in F3 adult males, ancestral POPs exposure altered sperm function (Table 3.12.2), in concordance with a previous rat study where ancestral DDE diminished the percentage of motile sperm until F3 (Song *et al.*, 2014).

In the F4 two-cell embryos, 448 genes were significantly DE as a result of ancestral POPs exposure of the F3 males. The majority of these genes were down-regulated and again implicated in apoptosis, but also cell movement, cellular homeostasis, viability of gonadal cells, proliferation of endocrine cells and germ cell viability. There were no significantly DE genes common to all generations of embryos. It is tempting to speculate that POPs exposure of F1 germ cells induced some gene modifications that escape reprogramming and are

responsible for the embryo and adult phenotypes observed in subsequent generations. Nonetheless, ancestral POPs exposure did not affect F4 sperm quality parameters.

3.6.2 Protective effect of FA supplementation

To the best of our knowledge, this is the first report that attempts to counter the harmful effects of POPs. We selected FA supplementation as a nutritional intervention, since it is well known to reduce the incidence of congenital birth anomalies (De Wals *et al.*, 2007; R. D. Wilson *et al.*, 2015) and to improve sperm quality in men (Salarkia *et al.*, 2017). Furthermore, dietary FA has been shown to counteract the effects of bisphenol A following maternal exposure (Dolinoy *et al.*, 2007) and protected against chromatin damage and mutation in the male germline (B. G. Swayne *et al.*, 2012).

In support of our hypothesis, FA supplementation protected F1 sperm viability (Table 3.12.1) and motility parameters (Table 3.12.2) against prenatal POPs exposure. Recent studies have also shown FA to protect sperm quality against lead (Gomaa *et al.*, 2017) and the carbamate insecticide, methyomyl (Sakr *et al.*, 2018). Furthermore, a high dose of FA (20 mg/kg/day) protected male germ cells, including sperm viability against the harmful effects of bisphenol A in a rat model (Gules *et al.*, 2019). It should be noted, however, that similar levels of FA are reported to induce adverse health outcomes (Aarabi *et al.*, 2015; Y. Deng *et al.*, 2017).

Partially supporting our hypothesis, in the F2 two-cell embryos, prenatal paternal exposure to FA supplementation with POPs counteracted (upregulated) the POPs-induced downregulation of shared genes (Fig. 1D). Interestingly, pathways implicated in sperm development were among those that were only affected by prenatal paternal FA supplementation with POPs, although whether these are beneficial is unknown according to our gene ontology analysis (Fig. 1C). In F2 adults, POPs and FA interact, suggesting that FA supplementation is beneficial for sperm morphology in the presence of these contaminants (Table 3.12.1). In contrast, when F2 males were only exposed to FA supplementation, the percentage of morphologically normal sperm (Table 3.12.1) and motility parameters dropped (Table 3.12.2). Relevant to this F2 sperm phenotype, prenatal paternal exposure to FA supplementation alone markedly altered gene pathways related to cell movement, gonadal

cell morphology and apoptosis in the F2 embryos (Fig. 1C). Corresponding to these pathways, previous studies have shown that moderate to high (2 – 10X) gestational FA supplementation is linked to altered DNA methylation patterns in offspring somatic tissues, including the sperm and brain (Aarabi *et al.*, 2015; Barua *et al.*, 2016; Schaible *et al.*, 2011).

Similar to what was observed in the F3 POPs two-cell embryos, a small number of genes were significantly DE in POPsFA embryos, and the two shared genes were downregulated as a result of both treatments, which does not support our hypothesis (Fig. 1A and B). Unlike the F2 embryos, therefore, FA supplementation did not counteract the POPs effects in the F3 embryos (Fig. 1D). With respect to sperm, no phenotypic alterations were observed in F3 POPsFA males, although ancestral FA supplementation in the absence of POPs increased sperm viability (Table 3.12.1).

In the F4 embryos, the number of significantly DE genes increased substantially compared to the F3 embryos due to all lineage treatments. In contrast to our hypothesis, genes were expressed always in the same manner across treatments, thus ancestral FA supplementation did not counter the POPs. Even several generations after initial exposure, FA supplementation increased sperm motility parameters, VSL and VCL, in the F4 male lines.

3.6.3 Pregnancy outcomes are affected in F3

To our knowledge, we are the first to show the transgenerational effect of an environmentally relevant pollutant mixture at a physiological dose that extends to the F4 generation. Previous studies have demonstrated that exposure to POPs has little to no immediate effect on pregnancy outcomes (Savitz *et al.*, 2014; Toft *et al.*, 2004). In contrast, our study demonstrates that the F3 paternal lineage showed the worst pregnancy outcomes amongst all generations (Table 3.12.3). These results are unexpected, especially since the F3 generation did not display any altered sperm parameters, however, transcriptomic analysis of the F4 two-cell embryos revealed a surprisingly high number of significantly DE genes with all treatments (Fig. 1A). These early developmental alterations could be the foundation for later adverse pregnancy phenotypes (Table 3.12.3).

Although we have no mechanistic evidence for these observations, we postulate that the sperm produced by the F3 paternal lineage contained accumulated upstream epimutations such as perturbed DNA methylation, histone modification, or noncoding RNAs, that escaped remodeling during development and spermatogenesis. These epimutations in the F3 sperm were unable to be corrected, resulting in substantial alternations in the F4 two-cell embryo transcriptome and associated pregnancy outcomes. Thus, the F3 males did not appear to have sperm parameter abnormalities, yet their reproductive competence was compromised. Also, although not assessed in this study, DNA sequence changes cannot be not ruled out (Heard *et al.*, 2014).

Various indicators of pregnancy outcome from both the F3 POPs and POPsFA lineages indicate compromised paternal competence to produce healthy litters (Table 3.12.3). Therefore, no counteracting effect of FA supplementation against POPs was observed in F4 pregnancy outcomes; in fact, the FA supplemented lineage also showed increased postimplantation fetal losses (Table 3.12.3).

3.7 Conclusion

Semen quality should be now considered as a biomarker of overall health (Jensen *et al.*, 2009; Latif *et al.*, 2017), since men with reduced sperm quality also have a shorter life expectancy and a higher risk of adverse health outcomes such as cardiovascular disease, hypertension and diabetes (Eisenberg *et al.*, 2016). The present study confirms our hypothesis that prenatal exposure to environmentally-relevant POPs subtly but significantly reduces male reproductive function for at least three generations. These results with a rodent model support speculation that declining sperm quality in men may be due to environmental factors (J. M. Braun *et al.*, 2017; Latif *et al.*, 2018; Levine *et al.*, 2017; Skakkebaek *et al.*, 2016). In contrast, our results do not fully support the hypothesis that prenatal FA supplementation offsets the effects of POPs. Compared to other animal studies using non-physiological FA doses, our FA supplementation was modest, which may explain the lack of convincing results in its ability to protect paternal lineages from prenatal exposure to POPs. This finding, however, does not negate the public health benefit of periconceptional FA supplementation in reducing neural tube defects (Y. M. Chan *et al.*, 2015; MacFarlane *et al.*, 2011; R. D. Wilson *et al.*, 2015).

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3.9 Author Contributions

Authors contributed to initial conception or design (JLB, JMT, SK, AJM); final design (ML, PMH, JLB); data acquisition, analysis, or interpretation (ML, PMH, PLC, MD, JLB), drafting the manuscript (ML, PMH); and critically revising the manuscript (ML, PMH, PLC, PN, JMT, SK, AJM, MOBB, CJB, AD, MD, JLB). All authors gave final approval and agreed to be accountable for all aspects of work in ensuring that questions relating the accuracy or integrity of any part of the work are appropriately investigated and resolved.

3.10 Competing Interests Statement

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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3.12 Figures and tables

Table 3.12.1. Sperm characteristics. Testicular spermatid counts in F1-F4 generation. F1 to F4 rat spermatozoa concentration (cauda epididymal sperm count normalized by epididymal weight). F1-F4 rat spermatozoa morphology. F1 to F4 rat spermatozoa viability. SEM= standard error of the mean.

(n = 12)	Testicular spermatid count ($x10^6$ ± SEM)	*Cauda* epididymal sperm count ($x10^6$ ± SEM)	Sperm morphology (% ± SEM)	Sperm viability (% ± SEM)
F1				
CTRL	74.32 ± 6.5	73.85 ± 3.8	92 ± 2	48[a] ± 3
POPs	70.68 ± 6.1	70.44 ± 4.0	90 ± 2	34[b] ± 3
FA	71.83 ± 6.1	74.91 ± 3.8	91 ± 2	45[a] ± 3
POPsFA	69.50 ± 6.5	71.71 ± 4.0	88 ± 2	44[a] ± 3
p value	POPs (*p*=0.63) FA (*p*=0.80) POPs*FA (*p*=0.91)	POPs (*p*=0.40) FA (*p*=0.77) POPs*FA (*p*=0.97)	POPs (*p*=0.26) FA (*p*=0.60) POPs*FA (*p*=0.88)	POPs (*p*=0.009) FA (*p*=0.17) **POPs*FA (*p*=0.02)**
F2				
CTRL	80.04[a] ± 6.2	82.42 ± 5.8	96[a] ± 1	39[a] ± 2
POPs	63.60[b] ± 5.6	65.14 ± 6.7	95[a] ± 1	30[b] ± 2
FA	78.40[a] ± 6.2	76.43 ± 5.8	93[b] ± 1	39[a] ± 2
POPsFA	57.24[b] ± 7.7	76.98 ± 6.0	96[a] ± 1	35[b] ± 2
p value	**POPs (*p*=0.01)** FA (*p*=0.64) POPs*FA (*p*=0.72)	POPs (*p*=0.17) FA (*p*=0.63) POPs*FA (*p*=0.14)	POPs (*p*=0.18) FA (*p*=0.09) **POPs*FA (*p*=0.01)**	**POPs (*p*=0.003)** FA (*p*=0.32) POPs*FA (*p*=0.23)
F3				
CTRL	66.39 ± 12.1	110.41 ± 10.9	92 ± 2	37[a] ± 3
POPs	47.75 ± 12.6	103.77 ± 10.9	93 ± 2	29[a] ± 3
FA	46.16 ± 10.6	109.48 ± 10.9	91 ± 2	39[b] ± 3
POPsFA	57.11 ± 10.7	105.03 ± 10.9	91 ± 2	39[b] ± 3
p value	POPs (*p*=0.75) FA (*p*=0.62) POPs*FA (*p*=0.26)	POPs (*p*=0.92) FA (*p*=0.98) POPs*FA (*p*=0.61)	POPs (*p*=0.74) FA (*p*=0.53) POPs*FA (*p*=0.55)	POPs (*p*=0.09) **FA (*p*=0.03)** POPs*FA (*p*=0.14)
F4				
CTRL	71.85 ± 11.0	111.72 ± 9.0	94 ± 1	57 ± 3
POPs	62.67 ± 11.0	120.37 ± 9.0	92 ± 1	54 ± 3
FA	90.39 ± 11.0	120.29 ± 9.0	94 ± 1	53 ± 3
POPsFA	77.39 ± 10.9	135.26 ± 9.0	94 ± 1	54 ± 3
p value	POPs (*p*=0.23) FA (*p*=0.37) POPs*FA (*p*=0.88)	POPs (*p*=0.19) FA (*p*=0.19) POPs*FA (*p*=0.72)	POPs (*p*=0.35) FA (*p*=0.34) POPs*FA (*p*=0.38)	POPs (*p*=0.71) FA (*p*=0.42) POPs*FA (*p*=0.51)

Table 3.12.2. Sperm motility parameters in F1 - F4 generations. (VAP: Average path velocity; VSL: Linear velocity; VCL: Curvilinear velocity; ALH: mean amplitude of lateral head displacement; BCF: frequency of head displacement; STR: straightness coefficient; LIN: linearity coefficient). SEM= standard error of the mean.

(n = 12)	Total motility (%) ± SEM	Progressive motility (%) ± SEM	**VAP** (μm/s) ± SEM	**VSL** (μm/s) ± SEM	**VCL** (μm/s) ± SEM	**ALH** (μm/s) ± SEM	**BCF** (Hz) ± SEM	**STR** (%) ± SEM	**LIN** (%) ± SEM
F1									
CTRL	78[a] ± 6	67[a] ± 5	326.7 ± 18	243.6 ± 16	504.9 ± 23	20.1 ± 0.5	15.9 ± 0.9	67[a] ± 1	46[a] ± 1
POPs	64[b] ± 6	53[b] ± 5	282.9 ± 18	204.6 ± 16	454.6 ± 23	19.5 ± 0.5	17.7 ± 0.9	65[b] ± 1	42[b] ± 1
FA	82[a] ± 6	70[a] ± 5	316.0 ± 18	228.8 ± 16	503.3 ± 23	20.8 ± 0.5	17.2 ± 0.9	65[b] ± 1	42[b] ± 1
POPsFA	72[b] ± 6	61[b] ± 5	308.4 ± 18	227.4 ± 16	485.0 ± 23	20.0 ± 0.5	16.9 ± 0.9	66[a] ± 1	45[a] ± 1
p value	**POPs (*p*=0.04)** FA (*p*=0.32) POPs*FA	**POPs (*p*=0.04)** FA (*p*=0.31) POPs*FA	POPs (*p*=0.17) FA (*p*=0.69) POPs*FA	POPs (*p*=0.20) FA (*p*=0.79) POPs*FA	POPs (*p*=0.14) FA (*p*=0.53) POPs*FA	POPs (*p*=0.14) FA (*p*=0.20) POPs*FA	POPs (*p*=0.39) FA (*p*=0.77) POPs*FA	POPs (*p*=0.69) FA (*p*=0.69) **POPs*FA**	POPs (*p*=0.44) FA (*p*=0.87) **POPs*FA**
F2									
CTRL	52 ± 7	43 ± 6	212.5 ± 9	159.3 ± 8	319.6 ± 12	13.7 ± 0	14.9 ± 0.9	68[a] ± 1	47[a] ± 2
POPs	42 ± 8	32 ± 7	207.7 ± 11	164.4 ± 10	299.1 ± 13	12.5 ± 1	14.8 ± 1.0	70[a] ± 1	50[a] ± 2
FA	56 ± 7	42 ± 6	207.7 ± 9	151.0 ± 8	307.0 ± 12	13.3 ± 0	14.6 ± 0.9	66[b] ± 1	44[b] ± 2
POPsFA	53 ± 8	41 ± 6	189.1 ± 10	139.9 ± 9	286.7 ± 12	12.6 ± 1	15.7 ± 0.9	66[b] ± 1	45[b] ± 2
p value	POPs (*p*=0.40) FA (*p*=0.34) POPs*FA	POPs (*p*=0.37) FA (*p*=0.50) POPs*FA	POPs (*p*=0.24) FA (*p*=0.24) POPs*FA	POPs (*p*=0.72) FA (*p*=0.06) POPs*FA	POPs (*p*=0.10) FA (*p*=0.31) POPs*FA	POPs (*p*=0.09) FA (*p*=0.80) POPs*FA	POPs (*p*=0.60) FA (*p*=0.77) POPs*FA	POPs (*p*=0.36) **FA (*p*=0.02)** POPs*FA	POPs (*p*=0.26) **FA (*p*=0.03)** POPs*FA
F3									
CTRL	48 ± 7	37 ± 5	241.2 ± 10	173.7 ± 8	398.2 ± 17	18.6 ± 7	19.4 ± 0.7	64[a] ± 1	41 ± 1
POPs	46 ± 7	34 ± 6	227.5 ± 10	151.0 ± 8	370.3 ± 17	18.0 ± 7	20.3 ± 0.7	61[b] ± 1	40 ± 1
FA	44 ± 7	34 ± 6	237.7 ± 10	161.6 ± 8	387.8 ± 17	32.4 ± 7	20.1 ± 0.7	62[a] ± 1	41 ± 1
POPsFA	52 ± 7	39 ± 6	242.2 ± 10	163.7 ± 8	397.5 ± 17	18.1 ± 7	20.4 ± 0.7	61[b] ± 1	40 ± 1
p value	POPs (*p*=0.66) FA (*p*=0.87) POPs*FA	POPs (*p*=0.79) FA (*p*=0.88) POPs*FA	POPs (*p*=0.65) FA (*p*=0.59) POPs*FA	POPs (*p*=0.21) FA (*p*=0.96) POPs*FA	POPs (*p*=0.58) FA (*p*=0.62) POPs*FA	POPs (*p*=0.29) FA (*p*=0.33) POPs*FA	POPs (*p*=0.41) FA (*p*=0.56) POPs*FA	**POPs (*p*=0.02)** FA (*p*=0.80) POPs*FA	POPs (*p*=0.21) FA (*p*=0.87) POPs*FA
F4									
CTRL	56 ± 6	43 ± 5	221.0 ± 21	146.6[a] ± 20	349.0[a] ± 23	16.8 ± 1	18.9 ± 0.9	64 ± 1	43 ± 1
POPs	57 ± 6	45 ± 5	231.6 ± 21	167.1[a] ± 20	350.2[a] ± 23	15.7 ± 1	18.5 ± 0.9	66 ± 1	46 ± 1
FA	65 ± 6	52 ± 5	250.9 ± 21	184.7[b] ± 20	385.6[b] ± 23	16.9 ± 1	17.4 ± 0.9	66 ± 1	45 ± 1
POPsFA	58 ± 6	45 ± 5	279.9 ± 21	219.3[b] ± 21	409.3[b] ± 23	16.1 ± 1	17.5 ± 0.9	68 ± 1	47 ± 1
p value	POPs (*p*=0.56) FA (*p*=0.44) POPs*FA	POPs (*p*=0.62) FA (*p*=0.38) POPs*FA	POPs (*p*=0.34) FA (*p*=0.07) POPs*FA	POPs (*p*=0.17) **FA (*p*=0.03)** POPs*FA	POPs (*p*=0.58) **FA (*p*=0.05)** POPs*FA	POPs (*p*=0.10) FA (*p*=0.62) POPs*FA	POPs (*p*=0.87) FA (*p*=0.16) POPs*FA	POPs (*p*=0.14) FA (*p*=0.08) POPs*FA	POPs (*p*=0.14) FA (*p*=0.31) POPs*FA

Table 3.12.3. Pregnancy outcomes for F1-F3 fathers. Fertility rate corresponds to (# fetuses / # corpus lutea)*100, pre-implantation losses corresponds to ((# corpus lutea – # implantation sites) / # corpus lutea)*100 and post-implantation losses corresponds to ((# implantation sites - # fetuses) / # implantation sites)*100. Neonatal deaths correspond to pups dead after birth during the first week of life (PND 1 to PND 6).

	Nb fetuses	Implantation sites	Fertility rate (% ± SEM)	Preimplantation losses (% ± SEM)	Postimplantation losses (% ± SEM)	Neonatal deaths (% ± SEM)
F1						
CTRL	12 ± 1	13 ± 1	85 ± 7	11 ± 4	4 ± 8	18 ± 12
POPs	13 ± 1	14 ± 1	85 ± 7	10 ± 4	6 ± 7	20 ± 13
FA	14 ± 1	14 ± 1	90 ± 7	9 ± 4	1 ± 7	1 ± 13
POPsFA	11 ± 1	13 ± 1	82 ± 7	6 ± 4	16 ± 7	2 ± 12
p value	POPs (*p*=0.38) FA (*p*=0.88) POPs*FA (*p*=0.15)	POPs (*p*=0.66) FA (*p*=1.00) POPs*FA (*p*=0.20)	POPs (*p*=0.62) FA (*p*=0.90) POPs*FA (*p*=0.58)	POPs (*p*=0.64) FA (*p*=0.52) POPs*FA (*p*=0.77)	POPs (*p*=0.31) FA (*p*=0.67) POPs*FA (*p*=0.40)	POPs (*p*=0.92) FA (*p*=0.19) POPs*FA (*p*=0.95)
F2						
CTRL	12 ± 2	12 ± 2	85 ± 13	8 ± 13	7 ± 3	10 ± 5
POPs	14 ± 2	15 ± 2	89 ± 15	6 ± 15	4 ± 3	6 ± 5
FA	10 ± 2	10 ± 2	68 ± 13	30 ± 13	3 ± 3	2 ± 5
POPsFA	12 ± 2	12 ± 2	96 ± 15	1 ± 15	5 ± 3	1 ± 5
p value	POPs (*p*=0.27) FA (*p*=0.37) POPs*FA (*p*=0.99)	POPs (*p*=0.30) FA (*p*=0.30) POPs*FA (*p*=0.97)	POPs (*p*=0.28) FA (*p*=0.73) POPs*FA (*p*=0.41)	POPs (*p*=0.33) FA (*p*=0.58) POPs*FA (*p*=0.37)	POPs (*p*=0.77) FA (*p*=0.64) POPs*FA (*p*=0.44)	POPs (*p*=0.60) FA (*p*=0.19) POPs*FA (*p*=0.64)
F3						
CTRL	14^{a} ± 1	14^{a} ± 1	84^{a} ± 8	14^{a} ± 7	2^{a} ± 6	11.3 ± 9
POPs	12^{b} ± 1	13^{b} ± 1	88^{a} ± 8	11^{a} ± 7	1^{a} ± 6	1.3 ± 8
FA	13^{a} ± 1	14^{a} ± 1	89^{a} ± 8	5^{a} ± 7	6^{b} ± 6	3.6 ± 9
POPsFA	9^{b} ± 1	11^{b} ± 1	54^{b} ± 8	33^{b} ± 7	24^{b} ± 6	15.6 ± 8
p value	**POPs (*p*=0.04)** FA (*p*=0.11) POPs*FA (*p*=0.32)	**POPs (*p*=0.06)** FA (*p*=0.43) POPs*FA (*p*=0.61)	POPs (*p*=0.08) FA (*p*=0.10) **POPs*FA (*p*=0.03)**	POPs (*p*=0.09) FA (*p*=0.35) **POPs*FA (*p*=0.04)**	POPs (*p*=0.16) **FA (*p*=0.03)** POPs*FA (*p*=0.12)	POPs (*p*=0.90) FA (*p*=0.71) POPs*FA (*p*=0.23)

Table 3.12.4. Composition of POPs mixture used (Anas et al., 2005; Maurice et al., 2018).

Products	CAS no.	Origin[a]	% in mixture	Dose (µg/kg body weight)
Aroclor and congener neat mix[b]		AccuStandard	32.4	500
Technical chlordane	57-74-9	AccuStandard	21.4	330.3
Dichlorodiphenyldichloroethylene (*p,p'*-DDE)	72-55-9	Sigma-Aldrich	19.3	297.8
Dichlorodiphenyltrichloroethane (*p,p'*-DDT)	50-29-3	SigmaAldrich	6.8	104.9
Technical toxaphene	8001-35-2	AccuStandard	6.5	100.0
α-hexachlorocyclohexane (α-HCH)	319-84-6	Sigma-Aldrich	6.2	95.7
Aldrin	309-00-2	Sigma-Aldrich	2.5	38.6
Dieldrin	60-57-1	Sigma-Aldrich	2.1	32.4
1, 2, 4, 5-tetrachlorobenzene	95-94-3	Sigma-Aldrich	0.9	13.9
Dichlorodiphenyldichloroethane (*p, p'*-DDD)	72-54-8	Sigma-Aldrich	0.5	7.7
β-hexachlorocyclohexane (β-HCH)	319-85-7	Sigma-Aldrich	0.4	6.2
Hexachlorobenzene	118-74-1	AccuStandard	0.4	6.2
Mirex	2385-85-5	Sigma-Aldrich	0.2	3.1
Lindane	58-89-9	Sigma-Aldrich	0.2	3.1
Pentachlorobenzene	608-93-5	Sigma-Aldrich	0.2	3.1

[a] AccuStandard Inc (New Haven, Connecticut); Sigma-Aldrich Inc (St Louis, Missouri).
[b] Mix containing PCBs : Aroclor 1260 (58.9%); Aroclor 1254 (39.3%); 2,4,4'-trichlorobiphenyl (PCB 28; 1%); 2,2',4,4'-tetrachlorobiphenyl (PCB 47; 0.8%); 3,3',4,4',5-pentachlorobiphenyl (PCB 126; 0.02%), and 3,3',4,4'-tetrachlorobiphenyl (PCB 77; 0.004%).

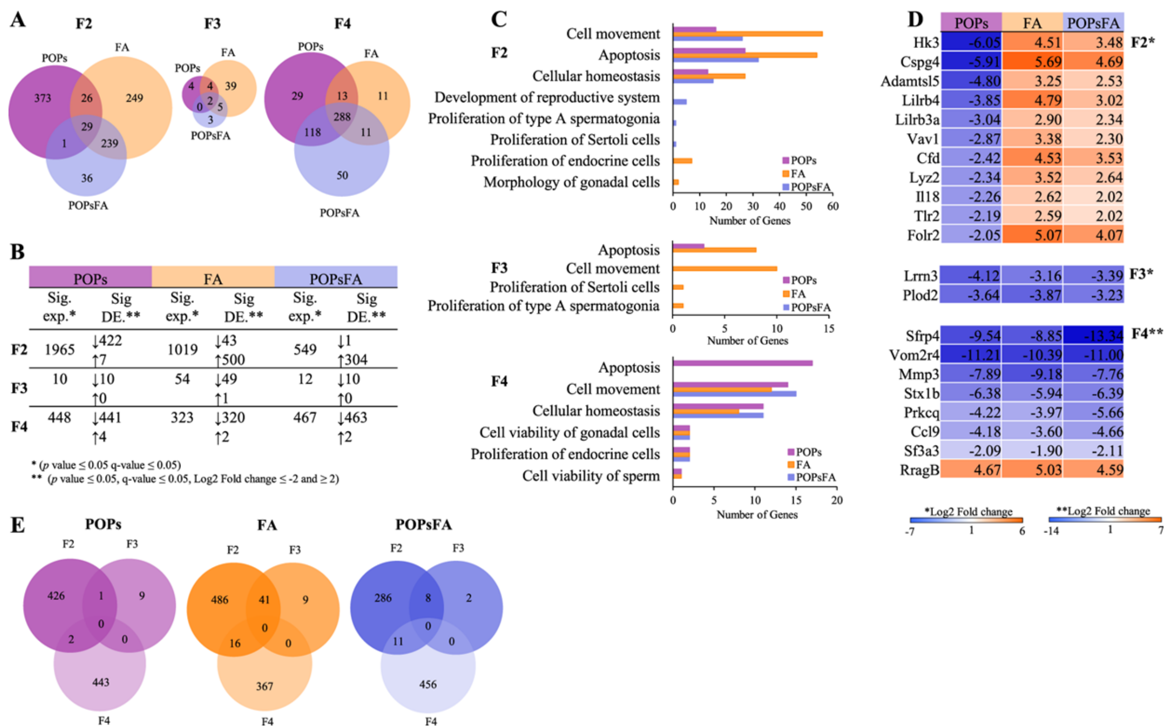

Figure 3.12.1. Differential gene expression in two-cell embryos due to prenatal paternal POPs, FA and POPsFA exposure. (A) Venn diagrams comparing the number of significantly differentially expressed genes due to POPs, FA and POPsFA in F2-F4 two-cell embryos. (B) Table including total number of significantly expressed (Sig. exp.) genes (p value ≤ 0.05, FDR ≤ 5%) and the number of significantly differentially expressed (Sig. DE) genes (p value ≤ 0.05, FDR ≤ 5% and -2 ≥ |Log2 Fold change| ≥ 2) that are up- or downregulated indicated by ↑ and ↓ respectively due to POPs, FA and POPsFA in F2-F4. (C) Gene ontology (GO) and pathway analysis showing only significantly affected pathways (p value ≤ 0.05). (D) Heatmaps representing common genes shared between all treatments per generation. (E) Candidate genes that are altered across multiple generations due to POPs, FA and POPsFA across F2-F4.

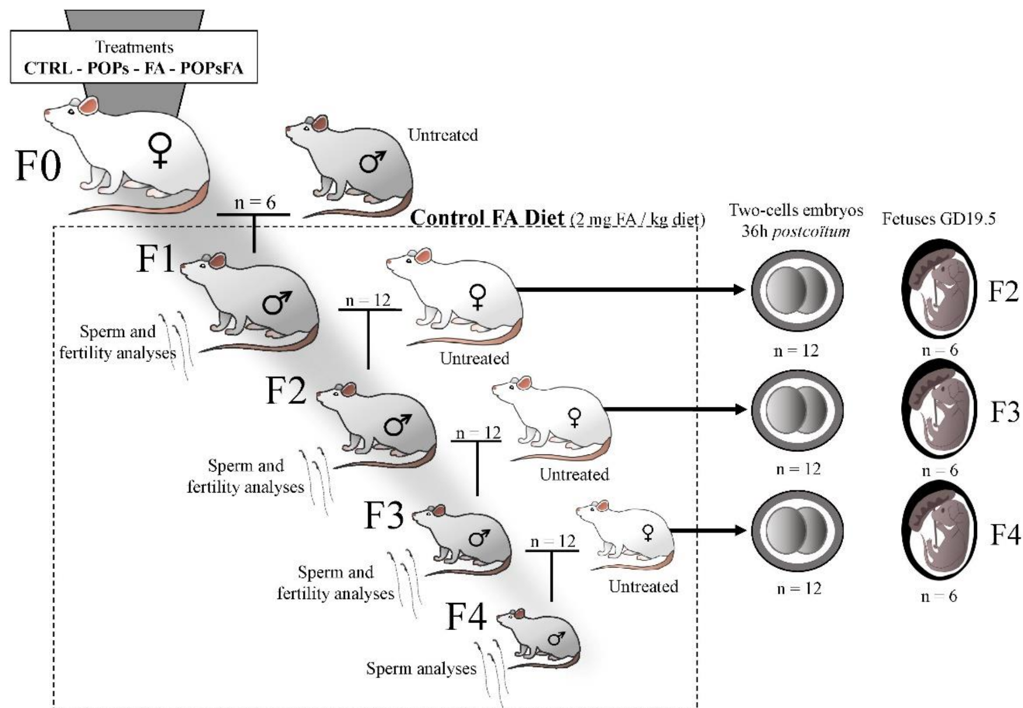

Figure 3.12.2. Study design. Four treatment groups of Sprague-Dawley F0 founder females (n = 6) were gavaged with an environmentally-relevant POPs mixture or corn oil and received either a control diet (2 mg FA/kg diet) or supplemented diet (6 mg FA/kg diet). F0 females were treated for 5 weeks and then mated to untreated males; POPs and FA supplementation treatments continued until the birth of the F1 litters. Only F0 females received POPs or supplemented FA diet.

3.13 Supplementary data

Table S3.13.1 Fold change of all shared significantly differentially expressed (DE) genes between POPs, FA and POPsFA in F2 (29 genes total), F3 (2 genes total) and F4 (288 genes total).

F2

Ensembl ID	Official gene symbol	POPs	FA	POPsFA
ENSRNOG00000003160	RragB	7.48	8.01	7.01
ENSRNOG00000017100	LOC108348078	9.81	6.46	7.21
ENSRNOG00000026235	Hk3	-6.05	4.51	3.48
ENSRNOG00000017208	Cspg4	-5.91	5.69	4.69
ENSRNOG00000010253	Cd163	-5.76	4.22	3.47
ENSRNOG00000021424	Cd300lf	-5.00	2.63	2.29
ENSRNOG00000033787	Adamtsl5	-4.80	3.25	2.53
ENSRNOG00000027811	Lilrb4	-3.85	4.79	3.02
ENSRNOG00000010183	Fam198b	-3.58	3.65	2.91
ENSRNOG00000021161	Fermt3	-3.42	3.97	2.84
ENSRNOG00000016294	Cd4	-3.32	3.13	2.27
ENSRNOG00000053260	Lilrb3a	-3.04	2.90	2.34
ENSRNOG00000050430	Vav1	-2.87	3.38	2.30
ENSRNOG00000016512	Sema3b	-2.77	2.14	2.23
ENSRNOG00000046663	LOC100911825	-2.64	4.37	3.05
ENSRNOG00000039390	Slc37a2	-2.63	3.17	2.08
ENSRNOG00000012749	C1qb	-2.56	3.55	2.77
ENSRNOG00000012807	C1qa	-2.55	3.51	2.55
ENSRNOG00000028668	Slc28a2	-2.47	3.54	2.78
ENSRNOG00000003622	Cybb	-2.45	3.76	2.73
ENSRNOG00000006094	Cd44	-2.44	3.78	2.87
ENSRNOG00000033564	Cfd	-2.42	4.53	3.53
ENSRNOG00000025001	Pcolce	-2.39	2.28	2.79
ENSRNOG00000005825	Lyz2	-2.34	3.52	2.64
ENSRNOG00000009848	Il18	-2.26	2.62	2.02
ENSRNOG00000009822	Tlr2	-2.19	2.59	2.02
ENSRNOG00000054251	Clec7a	-2.12	3.48	2.60
ENSRNOG00000052219	Gm2a	-2.07	2.98	2.72
ENSRNOG00000019890	Folr2	-2.05	5.07	4.07

F3

Ensembl ID	Official gene symbol	POPs	FA	POPsFA
ENSRNOG00000006368	Lrrn3	-4.12	-3.16	-3.39
ENSRNOG00000030183	Plod2	-3.64	-3.87	-3.23

F4

Ensembl ID	Official gene symbol	POPs	FA	POPsFA
ENSRNOG00000047388	Vom2r4	-11.21	-10.39	-11.00
ENSRNOG00000050370	Vom2r6	-9.22	-10.22	-10.27
ENSRNOG00000012057	Olig3	-9.40	-9.75	-8.73
ENSRNOG00000016421	Tyr	-10.53	-9.72	-10.55
ENSRNOG00000060573	AABR07004437.1	-7.29	-9.28	-7.48
ENSRNOG00000002167	Dmp1	-8.28	-9.25	-10.31
ENSRNOG00000032626	Mmp3	-7.89	-9.18	-7.76
ENSRNOG00000051929	Phox2b	-6.92	-9.17	-9.54
ENSRNOG00000054957	Sfrp4	-9.54	-8.85	-13.34
ENSRNOG00000019377	Amh	-7.75	-8.84	-8.44
ENSRNOG00000021108	Slc22a12	-9.13	-8.79	-9.11
ENSRNOG00000058285	Nme8	-8.47	-8.44	-8.62
ENSRNOG00000012671	Gan	-8.03	-8.23	-9.21
ENSRNOG00000048390	AABR07006536.1	-8.25	-7.86	-8.31
ENSRNOG00000058560	Col2a1	-7.66	-7.82	-7.47
ENSRNOG00000054716	AABR07015907.1	-7.44	-7.70	-8.40
ENSRNOG00000051678	AABR07012475.1	-8.95	-7.70	-7.93
ENSRNOG00000053805	Akain1	-8.18	-7.70	-8.50
ENSRNOG00000011892	Slc36a2	-7.78	-7.49	-6.17
ENSRNOG00000047891	Foxg1	-6.62	-7.40	-7.28
ENSRNOG00000024904	Pla2g4e	-6.79	-7.34	-9.46
ENSRNOG00000032745	Slc17a3	-6.62	-7.22	-7.61
ENSRNOG00000049019	Tmem170a	-7.04	-7.19	-7.08
ENSRNOG00000016362	Gpr4	-8.13	-7.14	-8.13
ENSRNOG00000057392	AABR07042859.1	-9.31	-7.06	-9.80
ENSRNOG00000022044	Cabp4	-7.25	-6.92	-6.03
ENSRNOG00000046611	Dzip1-ps1	-7.97	-6.87	-6.77
ENSRNOG00000045689	AABR07037645.1	-8.15	-6.87	-7.28
ENSRNOG00000057463	AABR07008293.4	-7.96	-6.85	-7.95
ENSRNOG00000000196	Cyp19a1	-7.21	-6.82	-7.57
ENSRNOG00000006741	Podnl1	-7.37	-6.79	-7.27
ENSRNOG00000029876	Gucy1a2	-7.33	-6.74	-6.39
ENSRNOG00000042897	Nmrk2	-6.84	-6.63	-7.06
ENSRNOG00000042222	RGD1563562	-7.68	-6.63	-8.01
ENSRNOG00000012428	Maf	-6.41	-6.61	-7.18
ENSRNOG00000033883	Stard8	-6.62	-6.58	-6.43
ENSRNOG00000038068	Pcdh9	-8.36	-6.58	-7.43
ENSRNOG00000006776	Smyd1	-7.31	-6.54	-7.59
ENSRNOG00000059091	Potem	-8.65	-6.51	-6.77
ENSRNOG00000052093	Smok2a	-7.48	-6.38	-6.96
ENSRNOG00000049504	AABR07009221.1	-7.04	-6.35	-7.32

ENSRNOG00000017333	Syt4	-9.97	-6.35	-6.06
ENSRNOG00000020097	Inha	-6.73	-6.33	-6.65
ENSRNOG00000018286	Chrna1	-6.24	-6.30	-6.78
ENSRNOG00000008121	Slc13a1	-5.73	-6.28	-5.58
ENSRNOG00000015002	Abhd15	-5.69	-6.26	-6.98
ENSRNOG00000045797	Lep	-6.50	-6.24	-6.25
ENSRNOG00000051598	AABR07013583.1	-7.11	-6.19	-7.28
ENSRNOG00000004679	Fign	-7.41	-6.17	-4.89
ENSRNOG00000032706	Kcnk18	-6.61	-6.16	-6.19
ENSRNOG00000055570	AABR07034940.2	-5.86	-6.15	-6.23
ENSRNOG00000030156	LOC102547344	-8.42	-6.12	-7.41
ENSRNOG00000049016	AABR07048012.1	-7.43	-6.11	-7.02
ENSRNOG00000049451	AABR07048013.1	-7.43	-6.11	-7.02
ENSRNOG00000010348	Cacna1f	-6.64	-6.10	-5.26
ENSRNOG00000046711	AC128353.1	-5.89	-6.05	-6.99
ENSRNOG00000057039	AC124874.1	-7.41	-6.04	-7.39
ENSRNOG00000003997	Pld5	-5.72	-6.02	-5.32
ENSRNOG00000037435	Tmem196	-5.28	-6.02	-5.49
ENSRNOG00000032558	AABR07030263.1	-5.89	-6.01	-6.96
ENSRNOG00000060832	AC240408.2	-7.20	-5.98	-7.23
ENSRNOG00000032002	Hapln1	-6.22	-5.97	-6.58
ENSRNOG00000042535	AABR07010907.1	-7.34	-5.97	-6.66
ENSRNOG00000054519	AABR07053179.1	-7.32	-5.97	-7.27
ENSRNOG00000014253	Pax2	-6.58	-5.95	-6.30
ENSRNOG00000019193	Stx1b	-6.38	-5.94	-6.39
ENSRNOG00000050847	AABR07027902.1	-7.00	-5.94	-6.99
ENSRNOG00000051648	AABR07016566.1	-5.98	-5.90	-6.39
ENSRNOG00000059400	AABR07070796.1	-7.53	-5.85	-7.20
ENSRNOG00000058491	AABR07017104.1	-7.09	-5.83	-7.07
ENSRNOG00000057038	AABR07029803.1	-7.78	-5.82	-6.94
ENSRNOG00000016679	Tmc7	-6.71	-5.74	-6.11
ENSRNOG00000017686	Pi15	-7.39	-5.73	-6.56
ENSRNOG00000014871	Zic4	-5.29	-5.73	-5.95
ENSRNOG00000008431	Gabbr2	-6.36	-5.73	-6.38
ENSRNOG00000010106	Faxc	-6.31	-5.70	-6.39
ENSRNOG00000017197	Pdgfb	-5.74	-5.69	-4.91
ENSRNOG00000003899	Krt14	-7.03	-5.69	-5.42
ENSRNOG00000045560	Gvin1	-4.90	-5.67	-6.26
ENSRNOG00000007942	Fscn3	-6.26	-5.67	-5.70
ENSRNOG00000031879	AABR07026048.1	-5.86	-5.67	-5.27
ENSRNOG00000000871	Cd40lg	-5.83	-5.67	-5.88
ENSRNOG00000052460	AABR07004812.2	-6.58	-5.63	-6.82
ENSRNOG00000058101	AABR07067387.1	-6.72	-5.62	-6.73
ENSRNOG00000024789	LOC499219	-6.59	-5.48	-6.49

ENSRNOG00000008053	Atp8a2	-5.35	-5.46	-5.18
ENSRNOG00000053122	Scn1a	-5.74	-5.43	-6.83
ENSRNOG00000053229	AABR07007905.2	-6.64	-5.38	-6.41
ENSRNOG00000013312	Kcnt2	-4.65	-5.38	-4.57
ENSRNOG00000005749	Foxred2	-5.85	-5.37	-6.10
ENSRNOG00000010834	Mpped1	-5.73	-5.32	-6.30
ENSRNOG00000030266	Plekhg2	-4.98	-5.28	-6.12
ENSRNOG00000004623	Rag2	-5.81	-5.27	-6.51
ENSRNOG00000002624	Edaradd	-6.06	-5.24	-5.14
ENSRNOG00000010478	LOC299282	-6.47	-5.23	-6.18
ENSRNOG00000026302	Lrrn4cl	-5.86	-5.22	-5.99
ENSRNOG00000047671	AABR07060588.2	-5.79	-5.21	-6.22
ENSRNOG00000042860	Pappa2	-6.91	-5.20	-4.73
ENSRNOG00000038369	AABR07038477.1	-5.02	-5.19	-5.65
ENSRNOG00000002265	Casr	-6.21	-5.19	-6.06
ENSRNOG00000055846	AABR07013065.1	-6.23	-5.12	-6.11
ENSRNOG00000060553	AC240408.1	-5.67	-5.11	-6.33
ENSRNOG00000012165	Ccdc129	-5.45	-5.10	-6.52
ENSRNOG00000008644	Nkx2-1	-6.20	-5.09	-6.17
ENSRNOG00000057092	Slfn4	-4.61	-5.06	-6.71
ENSRNOG00000049140	AABR07060678.1	-6.14	-5.06	-6.13
ENSRNOG00000056033	AC111632.2	-6.02	-5.03	-6.10
ENSRNOG00000011258	Masp2	-4.80	-5.03	-4.91
ENSRNOG00000058277	AC080157.1	-5.55	-4.98	-5.53
ENSRNOG00000028112	LOC499796	-6.01	-4.97	-6.84
ENSRNOG00000060618	AABR07027267.1	-5.72	-4.95	-5.89
ENSRNOG00000059691	AABR07033318.1	-5.77	-4.95	-5.73
ENSRNOG00000002723	Sele	-5.58	-4.93	-6.53
ENSRNOG00000010798	Blk	-5.42	-4.93	-5.79
ENSRNOG00000016760	Kctd19	-4.81	-4.90	-4.69
ENSRNOG00000047765	AABR07010682.1	-5.93	-4.89	-5.95
ENSRNOG00000036661	Rab40b	-5.54	-4.89	-4.43
ENSRNOG00000012920	Col9a1	-5.36	-4.88	-5.27
ENSRNOG00000061900	AABR07047771.1	-5.62	-4.88	-5.86
ENSRNOG00000011177	Sftpc	-5.54	-4.86	-5.49
ENSRNOG00000051345	AABR07048469.2	-5.20	-4.86	-4.91
ENSRNOG00000040314	AABR07034637.1	-5.88	-4.85	-5.88
ENSRNOG00000008050	Stac3	-4.16	-4.85	-5.25
ENSRNOG00000008367	Krt86	-5.60	-4.84	-5.90
ENSRNOG00000034226	LOC100362981	-6.80	-4.84	-6.42
ENSRNOG00000057744	AC112355.1	-5.37	-4.83	-5.60
ENSRNOG00000000618	Mdga2	-5.52	-4.83	-4.63
ENSRNOG00000022565	Lrrc25	-5.45	-4.80	-5.33
ENSRNOG00000053741	AABR07052664.1	-5.73	-4.78	-5.57

ENSRNOG00000010128	Slc27a2	-4.35	-4.76	-4.70
ENSRNOG00000061132	LOC103694210	-5.44	-4.75	-5.74
ENSRNOG00000011068	Papss2	-5.58	-4.75	-5.78
ENSRNOG00000050885	Sult1c2	-4.73	-4.72	-3.18
ENSRNOG00000032995	AABR07025089.1	-5.83	-4.68	-5.51
ENSRNOG00000052774	AC123253.2	-5.38	-4.65	-5.60
ENSRNOG00000031202	Trim30c	-5.58	-4.62	-5.67
ENSRNOG00000029588	LOC100364769	-5.51	-4.62	-5.62
ENSRNOG00000017890	Crhbp	-4.65	-4.61	-5.24
ENSRNOG00000030538	Slco1b2	-5.21	-4.61	-5.54
ENSRNOG00000055015	AABR07015559.1	-5.86	-4.59	-5.31
ENSRNOG00000043357	Zfp407	-4.47	-4.58	-4.91
ENSRNOG00000024390	Osm	-5.84	-4.58	-6.30
ENSRNOG00000024569	Gimap9	-4.99	-4.57	-5.17
ENSRNOG00000030689	Ms4a6e	-5.28	-4.57	-5.55
ENSRNOG00000046547	Rbm24	-3.73	-4.55	-3.87
ENSRNOG00000049791	AABR07059552.1	-5.99	-4.55	-5.62
ENSRNOG00000025235	Tmem130	-5.09	-4.54	-4.31
ENSRNOG00000028908	Eppin	-4.38	-4.54	-4.62
ENSRNOG00000059027	AC098008.5	-5.73	-4.53	-5.37
ENSRNOG00000026238	RGD1562618	-4.43	-4.51	-4.66
ENSRNOG00000007307	Syde1	-4.86	-4.51	-5.54
ENSRNOG00000048305	AABR07013922.1	-6.16	-4.50	-5.44
ENSRNOG00000058003	Spon1	-5.00	-4.50	-5.51
ENSRNOG00000000378	AABR07044914.1	-5.38	-4.50	-5.50
ENSRNOG00000062191	AABR07036016.1	-4.98	-4.49	-4.45
ENSRNOG00000008587	Tek	-6.50	-4.49	-6.12
ENSRNOG00000004459	Sdr9c7	-5.24	-4.48	-5.21
ENSRNOG00000047234	AABR07057530.1	-4.87	-4.48	-4.73
ENSRNOG00000048917	Hoxa6	-3.84	-4.46	-4.24
ENSRNOG00000058209	AABR07063893.1	-5.80	-4.46	-5.61
ENSRNOG00000059506	AC124926.2	-5.67	-4.45	-5.55
ENSRNOG00000052264	AABR07034393.1	-6.17	-4.44	-5.02
ENSRNOG00000059362	Has3	-4.66	-4.43	-4.35
ENSRNOG00000010997	Ednrb	-4.79	-4.43	-5.29
ENSRNOG00000058984	AC115181.1	-5.29	-4.42	-5.15
ENSRNOG00000046774	AABR07045307.1	-5.55	-4.41	-5.73
ENSRNOG00000061874	AABR07047036.1	-5.32	-4.39	-5.31
ENSRNOG00000013373	Rmt1	-4.81	-4.38	-4.35
ENSRNOG00000015691	Fam212b	-4.64	-4.38	-4.64
ENSRNOG00000015538	Abcd2	-5.29	-4.33	-5.08
ENSRNOG00000061625	AABR07048698.1	-5.46	-4.33	-5.34
ENSRNOG00000053337	Ly49s6	-5.48	-4.31	-5.47
ENSRNOG00000028072	Chit1	-4.88	-4.30	-5.25

ENSRNOG00000042471	RGD1560324	-5.63	-4.28	-5.34
ENSRNOG00000017833	Actn2	-3.86	-4.27	-4.72
ENSRNOG00000007059	Atp1b4	-4.74	-4.24	-4.90
ENSRNOG00000007664	Tnfrsf13c	-4.33	-4.21	-4.64
ENSRNOG00000060489	AABR07015639.1	-5.16	-4.21	-5.28
ENSRNOG00000001189	Sik1	-4.07	-4.20	-4.72
ENSRNOG00000037684	RGD1566159	-4.27	-4.19	-4.66
ENSRNOG00000039593	Ecscr	-4.02	-4.18	-4.00
ENSRNOG00000050966	AABR07024757.1	-5.38	-4.18	-5.22
ENSRNOG00000059468	AABR07034573.3	-4.87	-4.17	-4.96
ENSRNOG00000008074	Cyp11a1	-3.94	-4.17	-3.63
ENSRNOG00000054385	Rhebl1	-3.92	-4.16	-3.71
ENSRNOG00000049314	AABR07069524.1	-4.91	-4.15	-4.97
ENSRNOG00000059786	AC107531.3	-4.39	-4.12	-4.48
ENSRNOG00000056490	AABR07013566.1	-5.31	-4.11	-4.99
ENSRNOG00000010853	Chrna7	-4.49	-4.11	-4.45
ENSRNOG00000061264	AABR07058091.2	-4.35	-4.09	-4.69
ENSRNOG00000008842	Cyp4a8	-4.63	-4.07	-4.69
ENSRNOG00000024482	Tnrc18	-4.36	-4.04	-4.08
ENSRNOG00000009043	Piwil4	-4.10	-4.04	-4.06
ENSRNOG00000022022	AC128967.1	-4.71	-4.04	-4.71
ENSRNOG00000053367	AABR07064257.1	-4.71	-4.03	-4.94
ENSRNOG00000047635	Tmem178b	-4.01	-4.03	-5.38
ENSRNOG00000060246	Klrd1	-4.77	-4.02	-4.67
ENSRNOG00000003669	Myocd	-4.64	-3.99	-4.82
ENSRNOG00000037452	AABR07042542.1	-5.11	-3.99	-5.02
ENSRNOG00000046505	Bend3	-3.72	-3.99	-4.19
ENSRNOG00000043188	AABR07000261.1	-4.12	-3.99	-4.50
ENSRNOG00000019057	Prkcq	-4.22	-3.97	-5.66
ENSRNOG00000037449	Pole	-3.67	-3.97	-3.92
ENSRNOG00000037997	Tssk2	-4.32	-3.97	-4.33
ENSRNOG00000058653	Tmem52b	-3.89	-3.97	-4.35
ENSRNOG00000050509	Ebi3	-3.36	-3.95	-4.01
ENSRNOG00000060946	LOC100910506	-4.39	-3.89	-4.09
ENSRNOG00000032361	AABR07038798.1	-4.47	-3.88	-4.48
ENSRNOG00000009832	Slc39a14	-3.74	-3.88	-3.80
ENSRNOG00000056187	AC132752.1	-4.44	-3.88	-4.47
ENSRNOG00000021663	RGD1561849	-4.01	-3.87	-3.56
ENSRNOG00000000010	Cbln1	-3.95	-3.85	-4.47
ENSRNOG00000059705	Elmo1	-4.09	-3.84	-4.06
ENSRNOG00000006509	Srgap3	-4.22	-3.81	-4.69
ENSRNOG00000038835	Cd86	-4.29	-3.80	-4.31
ENSRNOG00000029244	Pcdhb10	-5.13	-3.79	-4.65
ENSRNOG00000037275	Tlcd2	-4.12	-3.79	-4.58

ENSRNOG00000051802	AABR07010609.1	-4.83	-3.77	-4.88
ENSRNOG00000006783	Neb	-4.90	-3.73	-4.15
ENSRNOG00000026582	RGD1310212	-3.64	-3.71	-3.99
ENSRNOG00000051664	AABR07010878.1	-4.54	-3.70	-4.63
ENSRNOG00000058766	AABR07019341.2	-4.22	-3.69	-4.29
ENSRNOG00000061317	AABR07011733.1	-4.73	-3.69	-4.83
ENSRNOG00000052304	AC096201.1	-4.66	-3.67	-4.92
ENSRNOG00000046332	AABR07004812.1	-4.82	-3.62	-4.55
ENSRNOG00000028548	Ccl9	-4.18	-3.60	-4.66
ENSRNOG00000060998	AABR07029467.2	-4.31	-3.60	-4.34
ENSRNOG00000010975	Adnp	-3.38	-3.58	-3.42
ENSRNOG00000059588	AC113785.2	-4.33	-3.55	-4.32
ENSRNOG00000014137	Fbln1	-3.20	-3.54	-4.02
ENSRNOG00000015133	Kmt2a	-3.88	-3.48	-4.03
ENSRNOG00000031951	LOC100361645	-4.38	-3.48	-4.41
ENSRNOG00000016848	Fzd4	-3.97	-3.48	-3.99
ENSRNOG00000031579	LOC100363469	-4.22	-3.47	-4.43
ENSRNOG00000025648	Dhrs7l1	-4.13	-3.43	-4.40
ENSRNOG00000051932	AABR07016635.1	-4.89	-3.41	-4.46
ENSRNOG00000010889	Fbxw7	-3.73	-3.39	-3.09
ENSRNOG00000009826	Bche	-3.59	-3.29	-3.62
ENSRNOG00000000113	Elac1	-3.27	-3.28	-3.45
ENSRNOG00000034134	Cpm	-3.23	-3.27	-3.55
ENSRNOG00000013004	Akr1d1	-4.61	-3.27	-3.60
ENSRNOG00000059145	AABR07026137.2	-4.24	-3.26	-4.33
ENSRNOG00000001271	Card6	-3.50	-3.24	-3.76
ENSRNOG00000059322	N4bp2l1	-3.51	-3.23	-3.84
ENSRNOG00000036726	Cd300e	-4.11	-3.22	-3.54
ENSRNOG00000004697	Baalc	-3.49	-3.22	-3.72
ENSRNOG00000019590	Smg5	-3.77	-3.22	-4.09
ENSRNOG00000046353	LOC501406	-3.07	-3.20	-2.81
ENSRNOG00000005871	Il1rn	-3.44	-3.19	-3.09
ENSRNOG00000027468	Slc6a15	-3.25	-3.19	-3.90
ENSRNOG00000042741	Adgb	-3.55	-3.19	-3.32
ENSRNOG00000036604	Ifit2	-2.76	-3.17	-3.13
ENSRNOG00000020444	Hcn3	-3.60	-3.16	-3.96
ENSRNOG00000048904	AABR07004232.1	-3.82	-3.15	-3.92
ENSRNOG00000018839	Ntrk2	-3.41	-3.12	-3.45
ENSRNOG00000059252	AABR07057108.1	-4.13	-3.08	-3.95
ENSRNOG00000052847	AABR07025673.1	-3.70	-3.04	-4.03
ENSRNOG00000014452	Zfhx3	-2.70	-3.02	-3.12
ENSRNOG00000006649	Thrb	-3.58	-3.00	-3.11
ENSRNOG00000011991	Slc10a7	-2.76	-2.98	-2.99
ENSRNOG00000060742	AABR07029809.1	-4.03	-2.97	-4.00

ENSRNOG00000007548	Polr3f	-3.09	-2.95	-3.16
ENSRNOG00000050205	Afmid	-3.28	-2.94	-3.54
ENSRNOG00000030870	LOC102550396	-3.93	-2.94	-3.78
ENSRNOG00000048762	AABR07000534.1	-4.20	-2.90	-3.90
ENSRNOG00000051390	AABR07035064.1	-3.63	-2.89	-3.48
ENSRNOG00000057986	AABR07034980.2	-3.63	-2.89	-3.48
ENSRNOG00000046647	Impad1	-2.78	-2.86	-3.06
ENSRNOG00000000456	Psmb8	-3.21	-2.84	-3.05
ENSRNOG00000012439	Bid	-3.05	-2.81	-3.36
ENSRNOG00000036842	Smug1	-2.84	-2.68	-2.82
ENSRNOG00000005904	Cdc27	-2.48	-2.67	-2.84
ENSRNOG00000058408	AC108588.1	-3.75	-2.66	-3.52
ENSRNOG00000015517	Zfp444	-2.96	-2.66	-2.66
ENSRNOG00000017066	Zfp384	-2.62	-2.65	-2.63
ENSRNOG00000014288	Fn1	-2.70	-2.65	-3.06
ENSRNOG00000002866	Rassf6	-2.90	-2.63	-2.51
ENSRNOG00000011829	Rpgrip1l	-2.55	-2.59	-2.53
ENSRNOG00000037251	Zfp248	-2.55	-2.57	-2.69
ENSRNOG00000024120	Rxfp1	-2.79	-2.55	-3.09
ENSRNOG00000001548	Nfe2l2	-2.37	-2.50	-2.52
ENSRNOG00000013521	Dhfr	-2.41	-2.47	-2.74
ENSRNOG00000003132	Mip	-2.36	-2.40	-2.97
ENSRNOG00000008908	Slc35a1	-2.70	-2.32	-2.93
ENSRNOG00000027434	Fitm2	-2.46	-2.29	-2.49
ENSRNOG00000013624	Uevld	-2.70	-2.28	-2.83
ENSRNOG00000017672	Akr1c14	-2.49	-1.93	-2.51
ENSRNOG00000003160	RragB	4.67	5.03	4.59

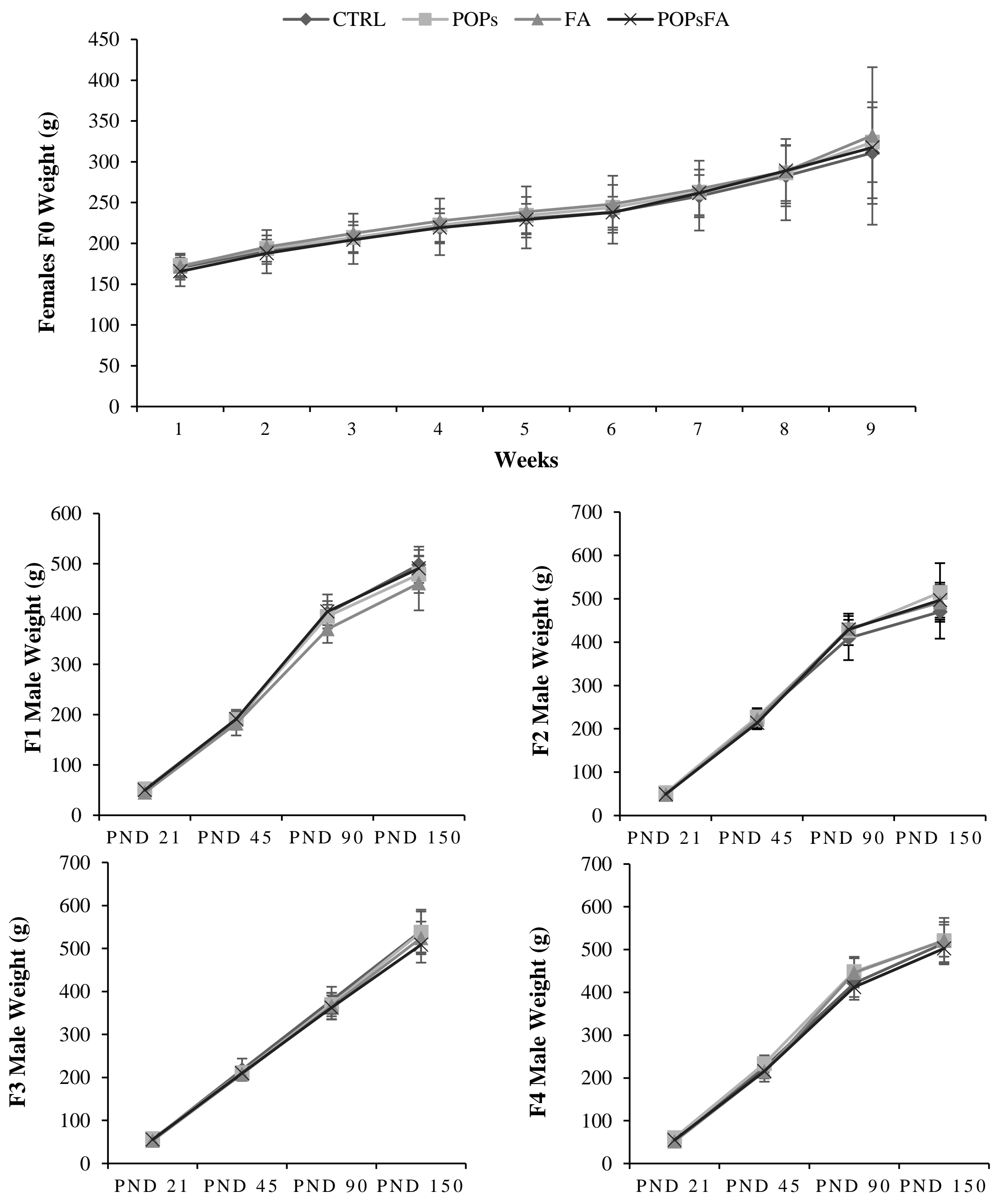

Figure S3.13.1. Weight monitoring of F0 female rats and F1 - F4 male offspring. The weight of the animals was closely monitored trice a week to ensure animal welfare. Mean weight is shown over time.

Chapter 4

Adipose tissue transcriptome is related to pollutant exposure in polar bear mother-cub pairs from Svalbard, Norway.

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4.1 Resumé

Étant au sommet de la chaîne alimentaire, les ours polaires (Ursus maritimus) sont fortement contaminés par des polluants organiques persistants (POPs). Les femelles transfèrent les POPs à leur progéniture pendant la gestation et la lactation, par conséquent, les jeunes oursons présentent des concentrations de POPs plus élevées que leur mère. Des études récentes suggèrent que les POPs affectent le métabolisme des lipides chez les ourses polaires, cependant, les mécanismes et l'impact sur leur progéniture restent inconnus. Ici, nous avons émis l'hypothèse que l'exposition aux POPs modifie différemment la transcription génique à l'échelle du génome dans le tissu adipeux des ourses polaires et de leurs oursons, mettant en évidence les différences physiologiques de réponse entre les adultes et les jeunes. Des biopsies de tissus adipeux ont été recueillies auprès de 13 ourses polaires adultes et de leurs petits jumeaux à Svalbard, en Norvège, en avril 2011, 2012 et 2013. L'ARN total extrait des biopsies a été soumis à un séquençage d'ARN à haut débit. Les concentrations plasmatiques de ΣPOPs chez les mères variaient de 897 à 13,620 ng/g de poids humide et étaient associées à une altération de l'expression des gènes du tissu adipeux chez les mères et les petits. Chez les mères, 2 502 et 2 586 gènes au total étaient respectivement positivement et négativement corrélés à l'exposition aux POPs, tandis que chez les petits, 2 585 gènes positifs et 1 690 gènes négatifs. Au total, 743 gènes positifs et négatifs se chevauchaient entre les mères et les petits suggérant des réponses moléculaires partiellement partagées aux ΣPOPs. Les gènes associés aux POPs sont impliqués dans de nombreuses voies métaboliques chez les mères et les petits, ce qui indique que l'exposition aux POPs modifie le métabolisme énergétique, qui, à son tour, peut être lié à un dysfonctionnement métabolique.

4.2 Abstract

Being at the food chain apex, polar bears (Ursus maritimus) are highly contaminated with persistent organic pollutants (POPs). Females transfer POPs to their offspring through gestation and lactation, therefore, young cubs present higher POPs concentrations than their mothers. Recent studies suggest that POPs affect lipid metabolism in female polar bears, however, the mechanisms and impact on their offspring remain unknown. Here, we hypothesized that exposure to POPs differentially alters genome-wide gene transcription in adipose tissue from mother polar bears and their cubs, highlighting physiological differences in response between adults and young. Adipose tissue biopsies were collected from 13 adult female polar bears and their twin cubs in Svalbard, Norway, in April 2011, 2012 and 2013. Total RNA extracted from biopsies was subjected to next-generation RNA sequencing. ΣPOPs plasma concentrations of summed PCBs, organochlorine pesticides and polybrominated diphenyl ethers in mothers ranged from 897 to 13,620 ng/g wet weight and were associated with altered adipose tissue gene expression in both mothers and cubs. In mothers, 2,502 and 2,586 genes in total were respectively positively and negatively correlated to POP exposure, whereas in cubs, 2,585 positively and 1,690 negatively genes. Between mothers and cubs, 743 positively and negatively genes overlapped between mothers and cubs suggesting partially shared molecular responses to ΣPOPs. ΣPOPs associated genes were involved in numerous metabolic pathways in mothers and cubs, indicating that POP exposure alters energy metabolism, which, in turn, may be linked to metabolic dysfunction.

4.3 Introduction

Environmental pollution poses an ongoing threat to Arctic wildlife. Although concentrations of many persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs) and organochlorinated pesticides (OCPs), have decreased in Arctic biota over the past few decades (Riget *et al.*, 2019), recent trends show that plasma concentrations of dichlorodiphenyldichloroethylene (*p,p'*-DDE), and hexachlorobenzene (HCB) in Barents Sea polar bears (*Ursus maritimus*) and adipose tissue concentrations of PCBs, chlordanes and hexachlorocyclohexanes in East Greenland polar bears have actually increased (Dietz, Desforges, Gustavson, *et al.*, 2018; Lippold *et al.*, 2019). Polar bears are at the top of the Arctic food web and, consequently, display some of the highest POPs concentrations among Arctic wildlife (Letcher *et al.*, 2010), particularly in subpopulations from the European Arctic (Andersen *et al.*, 2001; M. A. McKinney *et al.*, 2011; D. C. Muir *et al.*, 2006; Routti *et al.*, 2019; Verreault *et al.*, 2005). Their lipid-rich diet, consisting principally of ice-obligate seals, exposes them to a wide range of lipophilic POPs (Derocher *et al.*, 2002; M.A. McKinney *et al.*, 2017; Thiemann *et al.*, 2008), largely dominated by PCBs (Tartu, Bourgeon, *et al.*, 2017). Various POPs have endocrine disrupting properties (Gore *et al.*, 2015). Multiple studies on polar bears have associated POPs with endocrine perturbation, including altered lipid metabolism and thyroid hormone concentrations and reduced sexual organ size (Routti *et al.*, 2019; Sonne *et al.*, 2006; Tartu, Lille-Langoy, *et al.*, 2017). The effects of contaminants on energy homeostasis are of particular concern, as polar bears go through seasonal fasting and feeding cycles related to reproduction and food availability. In the absence of sea ice, polar bears may need to fast for several months. Subsequent maternal denning, when pregnant females retreat to their dens for four to five months, can lengthen the period of fasting up to eight months (Atkinson *et al.*, 1996; Ramsay *et al.*, 1988). During fasting, polar bears depend almost entirely on energy stored in white adipose tissue from which lipids are mobilized. Concentrations of lipophilic pollutants may increase several fold in adipose tissue and blood circulation due to reduced body fatness (Tartu, Bourgeon, *et al.*, 2017). Due to declining Arctic sea ice and habitat loss, polar bears will progressively have to endure longer periods of fasting (Stirling *et al.*, 2012) resulting in increased tissue pollutant concentrations.

White adipose tissue stores over 90% of the body burden of POPs in polar bears (Gebbink *et al.*, 2008). It plays a role in multiple biological functions ranging from lipid metabolism and appetite regulation to reproduction and immunity (Coelho *et al.*, 2013; Costa *et al.*, 2006; Laclaustra *et al.*, 2007). Previous *in vitro* research suggested that liver and fat extracts of POP contaminants from polar bears affect adipogenesis, i.e. differentiation of pre-adipocytes into mature fat cells, in cells originating from both polar bears and mouse (Routti *et al.*, 2016). Furthermore, individual PCBs and OCPs as well as POP mixtures, reflecting concentrations in polar bear adipose tissue from Svalbard (2010), modulated *in vitro* polar bear peroxisome proliferator-activated receptor gamma (*PPARG*), which is a key regulator of adipogenesis (Routti *et al.*, 2016). Transcript levels of several key regulators of energy metabolism including *PPARG*, PPARG coactivator 1 alpha (*PGC-1*), fatty acid synthase (*FASN*) and adiponectin (*ADIPOQ*) were altered according to adipose tissue concentrations of POPs in female polar bears (Tartu, Lille-Langoy*, et al.*, 2017). Moreover, these correlations were intensified when combined with reduced sea ice conditions (Tartu, Lille-Langoy*, et al.*, 2017). Analyses of gene transcript levels, however, focused on several target genes central to lipid metabolism, whereas analyses of other parameters related to lipid metabolism, such as ratios among fatty acids and several plasma parameters, suggested that multiple processes in energy metabolism could be targeted by pollutants (Tartu, Lille-Langoy*, et al.*, 2017).

Polar bear cubs are exposed to even higher levels of POPs than their mothers during lactation (Bernhoft *et al.*, 1997; Bytingsvik, Lie*, et al.*, 2012). Plasma and adipose tissue concentrations of POPs in cubs are tightly related to those in mothers, yet they can become over two times higher during their first year of life (Bytingsvik, Lie*, et al.*, 2012; Polischuk *et al.*, 1995). Besides having a direct effect on the adipose tissue itself, POP exposure occurring during sensitive developmental windows (e.g. pre- and postnatal development) could redirect gene expression inducing long-lasting effects that may not become evident until later in life (J. R. Barrett, 2013). To date, impacts of pollutants on polar bear offspring are unknown.

In the present study, we hypothesize that exposure to POPs differentially alters genome-wide gene transcription in adipose tissue from mother polar bears and their cubs, highlighting

physiological differences in response between adults and their young. To test our hypothesis, we utilized RNA sequencing to study the impact of POPs on both mother and cub adipose tissue transcriptomes.

4.4 Materials and Methods

4.4.1 Field sampling

Thirteen adult female polar bears with three to four months old twin cubs were captured opportunistically throughout Svalbard, Norway, in April, 2011-2013 as approved by the National Animal Research Authority of Norway (NARA). The field sampling was conducted within a few weeks after mothers with cubs emerge from maternal dens (Andersen et al. 2012). The adult females were immobilized by remote injection of tiletamine and zolazepam hydrochloride, as previously described (Zoletil Forte Vet; Virbac, France), from a helicopter, and the cubs were immobilized with injection from ground. Adipose tissue samples from adult females were collected using an 8 mm biopsy punch and taken approximately 15 cm lateral to the base of the tail. Biopsies from cubs were taken with a 2 mm biopsy punch 5-10 cm lateral to the base of the tail. Blood samples were collected only from adult females. They were taken from the femoral vein using heparinized tubes and kept cool and out of light until centrifuged within 10 h (3500 rpm, 9.65 cm rotor radius, 10 minutes). Biopsies were frozen in liquid nitrogen in the field and stored at -80°C. Plasma samples for contaminant analyses were frozen in the field and stored at -20ºC.

4.4.2 Analyses of pollutants

Plasma lipid weight concentrations of POPs in polar bear mothers were used as a proxy for contaminant exposure for both mothers and cubs. Preliminary assessment by the Norwegian Polar Institute indicates that lipid weight concentrations of POPs in plasma and wet weight concentrations in adipose tissue, which are likely the most relevant to study toxicological effect in adipose tissue, were highly correlated in female polar bears ($r=0.65$, $p<0.0001$, $n=90$). Furthermore, plasma ΣPCB concentrations correlate tightly between adult females and their 3-4 months old cubs (Bytingsvik, Lie*, et al.*, 2012). PCBs (-118, -138, -153 and -180), organochlorine pesticides (OCPs) including *p,p'*-DDE, hexachlorobenzene (HCB), oxychlordane, and polybrominated diphenyl ethers (PBDE-47 and -153), were determined in plasma sampled from adult female polar bears (n=13). The analytical procedures, QA/QC and the findings were published elsewhere (Lippold *et al.*, 2019; Lippold *et al.*, 2020; Tartu, Bourgeon*, et al.*, 2017) but a subset of the results are used herein to study associations between POP concentrations and adipose tissue transcriptome. The analytical procedures

including extraction, partitioning and clean-up, quantification and QA/QC are described in the supporting information. All contaminant analyses were conducted at the Norwegian University of Life Science (NMBU, Oslo, Norway), which is accredited for analysis of PCBs, OCPs and PBDEs in biological materials of animal origin according to the requirements of NS-EN ISO/IEC 17025 (Test 137)(ISO, 2005) . To assure quality control, blank, blind and spiked samples and in-house reference material were run for each series and certified reference materials were routinely analyzed. Limit of detection defined as three times noise level was ≤0.14 ng/g wet weight. Recovery rate of POPs in spiked samples varied between 82-118%.

4.4.3. RNA extraction

Prior to RNA extraction, adipose tissue from the 13 females and 26 cubs was separated from skin tissue using tweezers and a scalpel and kept on dry ice throughout the procedure. Two cub biopsies provided insufficient amounts of adipose tissue for RNA extraction and subsequent analyses, and thus were excluded from the study. Homogenous adipose tissue was then saturated in 500 µl QIAzol Lysis Reagent (Qiagen, Montreal, QC, Canada) (Tartu, Lille-Langoy, *et al.*, 2017) and disrupted using a tissue homogenizer until completely dissolved, followed by 5 min incubation at RT. For RNA extraction, the RNeasy® Lipid Tissue Mini Kit (Qiagen) was used according to manufacture's instructions. All samples were stored at -80ºC until sequencing. Adipose tissue biopsy quantities were insufficient to perform a parallel proteomic analysis.

4.4.4 RNA sequencing

The NEBNext Ultra II directional RNA library prep kit for Illumina (New England Biolabs Inc., Ipswich, MA, USA) was used to prepare mRNA sequencing libraries, according to manufacturer's instructions. Briefly 200 ng of total RNA were purified using the NEBNext poly(A) (New England Biolabs) and used as a template for cDNA synthesis by reverse transcriptase with random primers. The specificity of the strand was obtained by replacing the dTTP with the dUTP. This cDNA was subsequently converted to double-stranded DNA that was end-repaired. Ligation of adaptors was followed by a purification step with AxyPrep Mag PCR Clean-up kit (Axygen, Big Flats, NY, USA) by excising the strands containing the dUTPs and finally, by a PCR enrichment step of 12 cycles to incorporate specific indexed

adapters for the multiplexing. The quality of final amplified libraries was examined with a DNA screentape D1000 on a TapeStation 2200 and the quantification was done on the QuBit 3.0 fluorometer (Thermo Fisher Scientific, Canada). Subsequently, mRNA-seq libraries with unique indices were pooled together in equimolar ratio and subjected to paired-end 125 pb sequencing using two lanes of a high output flow cell on an Illumina HiSeq 2500 V4 system at the Next-Generation Sequencing Platform of the Genomics Center, CHU de Québec-Université Laval Research Center (Québec City, Canada). The average insert size for the paired-end libraries was 260 bp.

4.4.5 Reverse transcriptase PCR

To validate adipose tissue transcriptomic sequencing results, a reverse transcriptase PCR approach was chosen. Several key regulators of energy metabolism, presented in Figure 4.10.3, with a correlation value of preferably -0.5 ≥ |correlation value (rho)| ≥ 0.5 were selected for validation. Extracted RNA was reverse transcribed with provided random hexamer primers using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR kit (QIAGEN,) according to manufacturer's instructions. cDNAs were subjected to Real Time PCR using the LightCycler® 480 SYBR Green I Master (Roche, Canada) using primers described in supporting information (Table S4.11.1).

4.4.6 Bioinformatic analysis

Reads were trimmed using Trimmomatic v0.36 with the following options: TRAILING:30, SLIDINGWINDOW:4:20 and MINLEN:30 (Bolger *et al.*, 2014). All other options used the default values. Quality check was performed on raw and trimmed data to ensure the quality of the reads using FastQC v0.11.5 and MultiQC v1.5 (Andrews, 2010; Ewels *et al.*, 2016). The quantification was performed with Kallisto v0.44 (Bray *et al.*, 2016). Differential expression analysis (DESeq2 v1.18.1) and subsequent statistical analyses were performed in R v3.4.2 (Love *et al.*, 2014; R.Team, 2013). Heatmaps of gene expression were produced using the gplots v 3.0.1.1 using genes positively and negatively correlated to ΣPOP exposure according to Spearman correlation score for cubs and mothers separately (Warnes *et al.*, 2005). Regression analysis was conducted on the 10 genes with the highest absolute correlation scores, for mothers and cubs. We used linear modeling to investigate gene expression as a function of the ΣPOP exposure. Gene ontology analysis was performed using

the DAVID Gene Functional Classification Tool 6.8 to identify significantly enriched pathways ($p \leq 0.05$) that were related to ΣPOP exposure in mothers and cubs using polar bear as a reference species (Huang da *et al.*, 2009). Due to the small number of individuals, additional potential confounding factors such as mothers' age, parity, diet and body condition (Letcher *et al.*, 2010; Routti *et al.*, 2018) and cubs' weight and sex could not be included with validity in statistical analyses.

4.5 Results

The plasma ΣPOP concentrations measured in adult female polar bears ranged 15-fold, from 897 to 13,620 ng/g lipid weight with lipid percentage ranging from 0.78 to 1.97% (Figure 4.10.1). Structurally similar PCB-138, PCB-153 and PCB-180 with Cl-atoms positioned at 2,2′,3,4,4′,5′, 2,2',4,4',5,5' and 2,2',3,4,4',5,5' of the phenyl rings, respectively, were the dominant PCBs, accounting for 65-90% of the analyzed POPs. Oxychlordane accounted for 8-28% of the analyzed POPs, whilst PCB-118, p,p'-DDE, HCB, PBDE-47 and -153 comprised 1-17% of ΣPOPs.

The adipose tissue transcriptome of both adult females and their cubs was related to concentrations of ΣPOPs in mothers (Figure 4.10.2). In mother bears, transcript levels of 2502 and 2586 genes showed positive and negative correlations, respectively, with ΣPOP concentrations ($-0.5 \geq |rho| \geq 0.5$). In cubs, we observed 2025 and 1690 positively and negatively correlated genes, respectively. Overall, a clear distinction was observed across overall gene expression correlation patterns due to increasing ΣPOP concentration when comparing the lowest vs. the highest exposed adult female bears (Figure 4.10.2A, F) as well as their cubs (Figure 4.10.2B, G).

Shared and unique genes positively correlated to POP exposure in mothers and cubs

Between mothers and cubs, 341 positively correlated genes ($rho \geq 0.5$) were shared (Figure 4.10.2C). These genes were particularly involved in fundamental molecular pathways including ribosome (p = 3.30E-29), spliceosome (p = 4.70E-07), RNA transport (p = 1.90E-04), and ribosome biogenesis in eukaryotes (p = 1.10E-03) (Figure 4.10.2C).

Specific to only mothers, 2161 genes transcripts were positively related to ΣPOP concentrations (Figure 4.10.2C) of which 166 gene transcripts were involved in general metabolic pathways (p = 1.10E-02). In addition, transcript levels of genes involved in several neurodegenerative human diseases including Huntington's, Parkinson's and Alzheimer's disease ($p < 0.05$), non-alcoholic fatty liver disease (p = 1.10E-09), oxidative phosphorylation (p = 4.90E-13), purine and pyrimidine metabolism (p = 1.10E-04; p = 1.80E-03) pathways correlated positively with ΣPOP exposure in mothers (Figure 4.10.2D).

Exclusive to cubs, transcript levels of 123 genes related to metabolic pathways (p = 4.50E-02) also increased with increasing ΣPOP concentrations. Furthermore, we observed that transcript levels of genes involved in multiple pathways in cancer (including transcriptional mis regulation, miRNAs in cancer, basal cell carcinoma) ($p < 0.05$), arachidonic acid metabolism (p = 4.40E-04), sphingolipid metabolism (p = 2.10E-02) and linoleic acid metabolism (p = 5.00E-03) in cubs correlated positively with ΣPOP exposure.

Shared and unique genes negatively correlated to POP exposure in mothers and cubs

In contrast to the positively correlated genes, unique negatively correlated genes were involved in several distinctive metabolic pathways for mothers and cubs. Negatively correlated genes (rho ≤ -0.5), 402 in total, were shared between mothers and cubs (Figure 4.10.2H). These genes were particularly involved in P13K-Akt signaling (p = 1.40E-07), extracellular matrix (ECM)-receptor interaction (p = 5.10E-14), insulin signaling (p = 1.50E-03) and oxytocin signaling (p = 9.30E-03) (Figure 4.10.2H).

In mothers only, transcript levels of 2184 genes related to insulin signaling (p = 2.60E-04), insulin resistance (p = 1.20E-03), adipocytokine signaling (p = 4.10E-04), glucagon signaling (p = 8.80E-03) and type II diabetes mellitus (p = 4.10E-03), and thyroid hormone signaling (p = 2.30E-02), pathways were correlated with ΣPOP concentrations. Moreover, oxytocin- (p = 1.80E-05) and PI3K-Akt signaling (p = 3.70E-10) pathways were targeted in mothers once more. Solely in cubs, 1288 transcript levels of genes that correlated negatively with ΣPOP exposure were involved in other essential metabolic pathways, such as carbon metabolism (p = 4.20E-06), regulation of lipolysis in adipocytes (p = 4.00E-04), fatty acid metabolism (p = 1.00E-04) and degradation (p = 5.10E-05) and the citrate cycle (p = 8.10E-05) pathways as well as the PI3K-Akt signaling pathway (p = 1.40E-07).

Association between POP exposure and key regulators of energy metabolism in mothers and cubs

Regression analyses, confirmed by real time quantitative PCR, indicated that key regulators of energy metabolism in adipose tissue from mothers and cubs generally were or tended to

negatively correlate with ΣPOP exposure (Figure 4.10.3, S4.11.1). In mothers, only *ADIPOQ_5* was negatively correlated with ΣPOP exposure (rho $\leq$ -0.5; $p < 0.05$), whereas *ADIPOQ_2* and *3* (rho = -0.53; $p = 0.06$ and rho = -0.47; $p = 0.10$) as well as *SREBF1* (rho = -0.45; $p = 0.13$) and *LIPE* (rho = -0.41; $p = 0.16$) tended to correlate negatively with ΣPOP exposure. The other key regulators, including *FABP4*, *PNPLA2*, *PPARGC1A*, *CD36* and *SREBF2* were not correlated to POP exposure. In cubs however, *FABP4, LIPE, PNPLA2*, *PPARGC1A,* and *CD36_1* were negatively correlated to POP exposure (rho $\leq$ -0.5; $p < 0.05$) (Figure 4.10.3). Similar to mothers, *SREBF1* (rho = -0.40; $p = 0.05$) tended to negatively correlate to POP exposure; in contrast to *SREBF2* (rho = 0.46; $p = 0.02$) which tended to correlate positively with ΣPOPs.

4.6 Discussion

Results from the present study supports the hypothesis that exposure to POPs differentially alters genome-wide gene transcription in adipose tissue from mother polar bears and cubs. The study is the first to assess the impact of POP exposure on polar bear cubs using RNA sequencing and thereby highlights the physiological differences in response between adults and young. A limitation, however, is that the adipose tissue biopsies were insufficient to obtain proteomic information to validate the association between contaminant exposure and gene expression. Nonetheless, these preliminary data provide justification for further investigation.

Gene transcripts in adipose tissue from polar bear mother-cub pairs that correlated with circulating ΣPOP concentrations were predominantly involved in general metabolic pathways or specific signaling pathways related to energy metabolism. These associations suggest that POP exposure may alter energy metabolism in polar bears, which is in general agreement previous *in vitro* and correlative studies on adult polar bears (Routti *et al.*, 2019; Routti *et al.*, 2016; Tartu, Lille-Langoy, *et al.*, 2017), yet we expand to apply genome-wide next-generation sequencing and investigate the impact on the offspring as well.

Studies on ringed seals from the Baltic Sea, Svalbard and Canadian Arctic, also indicate that blubber transcript levels of genes involved in energy metabolism are related to POP exposure (Brown *et al.*, 2017; Castelli *et al.*, 2014). Overall, 749 genes, related to POP exposure, were shared between mothers and cubs, indicating that ΣPOPs partly alter the transcriptome via similar molecular responses which are likely not developmentally-influenced.

The increased intensity range of gene correlations for cubs compared to mothers (as shown on the color key of histogram -4 to 4, Figure 4.10.2B) may be related to higher tissue concentrations of POPs in cubs compared to their mothers. Cubs are directly exposed to POPs during gestation and mainly through lactation. Due to its richness in lipids, polar bear milk has higher levels of POPs than the adult diet resulting in weaning cubs with exceeding POP levels (Bernhoft *et al.*, 1997; Polischuk *et al.*, 1995).

Associations between POPs and genes related to energy metabolism in mothers and cubs

Genes that negatively correlated with ΣPOPs in mothers were involved in insulin, PI3K-Akt, thyroid, oxytocin, adipocytokine and glucagon signaling pathways as well as insulin resistance. Through the same genes, insulin and PI3K-Akt signaling pathways correlated negatively with ΣPOPs in cubs. Insulin stimulates glucose and free fatty acid uptake, inhibits lipolysis, and stimulates *de novo* fatty acid synthesis in adipocytes (Cignarelli *et al.*, 2019). Furthermore, insulin regulates adipose tissue growth and differentiation. Previous rodent studies demonstrated that POP exposure induces insulin resistance (Ibrahim *et al.*, 2011; Navarro *et al.*, 2019; Ruzzin *et al.*, 2010), whereas human studies investigating both insulin resistance and insulin secretion reported that serum POP concentrations were more strongly associated with decreased insulin secretion than insulin resistance (Jensen *et al.*, 2014; Y. M. Lee *et al.*, 2017; Park *et al.*, 2016). In polar bears, such relationships have not yet been established, however, POPs have been associated with increased expression of genes implicated in insulin use (Tartu, Lille-Langoy*, et al.*, 2017). Insulin is activated by an intracellular signaling cascade involving PI3K-Akt aside others. Since P13K-Akt signaling pathway plays an essential role in lipid biosynthesis and inhibits lipolysis (X. Huang *et al.*, 2018), the negative correlations between genes involved in PI3K-Akt signaling pathway and ΣPOPs implies elevated lipolysis in polar bear mothers. Furthermore, the negative correlations between POPs and adipocytokine and glucagon signaling pathways, and fatty acid metabolism and regulation of lipolysis in adipocytes signaling pathways, suggest that POPs may impair the release of stored energy from adipose tissue in polar bear mothers and cubs, respectively. Increased lipolysis in mothers may also enhance the release of POPs from adipose tissue into the circulation and, consequently, its bioavailability to other organs (Y. M. Lee *et al.*, 2018).

Negative relationships between POPs and genes involved in thyroid hormone signaling in mothers suggest decreased rate of lipolysis, since thyroid hormones (TH) regulate adipogenesis and related processes, including lipolysis and lipogenesis in white adipose tissue (Mullur *et al.*, 2014). Negative associations among genes involved thyroid hormone signaling pathways and POPs may be related to lowered circulating levels of the physiologically active TH, triiodothyronine, in female polar bears with high pollutant levels

(Bourgeon *et al.*, 2017; Braathen *et al.*, 2004; Bytingsvik *et al.*, 2013; Knott *et al.*, 2011). In addition, common POPs in polar bears, such as PCB153, PCB138, *p,p'*-DDE and oxychlordane, are antagonists of whale thyroid hormone receptor beta (THRB), which is 100% identical to polar bear THRB (Luhmann et al., in press).

With respect to genes that were positively correlated to POPs, processes such as fatty acid (including unsaturated) biosynthesis, degradation, and elongation, as well as glycolysis/gluconeogenesis and oxidative phosphorylation were observed in both mothers and cubs. The effect of POPs on energy metabolism has been previously investigated using *in vitro* and *in silico* approaches, and correlative field studies in adult female polar bears. A luciferase reporter assay for peroxisome proliferator-activated receptor gamma (PPARG), which is a major regulator of adipogenesis and promotor of lipid stores, revealed that an environmentally relevant POPs mixture antagonized polar bear PPARG (Routti *et al.*, 2016). Although we did not observe a strong correlation between POPs exposure and PPARG gene expression in the present study, several downstream targets of PPARG, including variants of ADIPOQ, were negatively correlated to ΣPOPs (Figure 4.10.3). Why PPARG was not directly related to POPs might be a result of differences in recent feeding history or body composition among mother bears.

Cubs exhibited negative correlations (rho $\leq$ -0.5; $p < 0.05$) of key regulators of energy metabolism including *FABP4, LIPE, PNPLA2*, *PPARGC1A,* and *CD36* with ΣPOP exposure, which further suggests a decrease in adipogenesis and lipid accumulation. In contrast, Tartu, Lille-Langoy*, et al.* (2017) reported that transcript levels of the same genes increased with increasing POP concentrations in adult female polar bears, some of which were also identified in the present study (Figure 4.10.3). This suggests that the energy metabolism of both polar bear mothers and cubs may partially respond differently to POP exposure.

Association between POPs and genes related to neurodegenerative diseases in mothers

Several genes related to neurogenerative diseases were associated with POPs in mothers. POP exposure is associated with neurological pathologies in humans (Grova *et al.*, 2019; Steenland *et al.*, 2014; Yan *et al.*, 2016), monkeys (Schantz *et al.*, 1989), rats (Pantaleoni *et*

al., 1988) and mice (Eriksson *et al.*, 1996). Although neurogenerative diseases have not been investigated in polar bears, *in vitro* and correlative field studies suggest that neurochemistry may be compromised by Hg and PFAS exposure in polar bears (Eggers Pedersen *et al.*, 2015; Krey *et al.*, 2014; Krey *et al.*, 2015). Adipose tissue regulates glucose and lipid homeostasis, which are both linked to neurodegenerative diseases (Parimisetty *et al.*, 2016; Rosen *et al.*, 2006). In addition, adipokines, which are cytokines secreted by adipose tissue, influence neuroinflammation and oxidative stress, which are associated with many neurodegenerative diseases (Parimisetty *et al.*, 2016). Indeed, adipokines Interleukin 6 (*IL-6*), *LEP* and *ADIPOQ* were negatively correlated to ΣPOP exposure in mother bears (Table S4.11.2). It should also be noted that a large number of the targeted genes associated with multiple neurodegenerative disease pathways overlapped with those of metabolic pathways (Figure S4.11.3). These common genes included 23 subunits of the NADH:ubiquinone oxidoreductase, also known as respiratory complex I, a key player in ATP synthesis in mammalian mitochondria (Zhu *et al.*, 2016). Defects within this complex have been associated with neurodegenerative pathologies, including Parkinson's disease (Giachin *et al.*, 2016; L. K. Sharma *et al.*, 2009). Five other genes in common coded for subunits in mitochondrial ATP synthase (Figure S4.11.3).

Associations between genes implicated in non-alcoholic fatty liver disease and POPs in mothers

Various positively correlated genes involved in non-alcoholic fatty liver disease (NAFLD) were related to POP exposure in mother bears. There are multiple pathways of adipose-liver crosstalk that contribute to the development and progression of liver disease including the secretion of inflammatory cytokines by adipocytes and the delivery of fatty acids to the liver instead of adipocytes (Parker *et al.*, 2018). POPs are also sequestered in the liver (Gebbink *et al.*, 2008), due to its high lipid content . In humans, serum levels of certain POPs have been associated with NAFLD related conditions such as Type 2 diabetes and obesity (Kuo *et al.*, 2013; D. H. Lee *et al.*, 2014; Taylor *et al.*, 2013). In rats, a single high oral dose of PCBs and OCPs increased the deposition of triglycerides in the liver dependent on the diet (Kohli *et al.*, 1979; Narayan *et al.*, 1990). NAFDL may, however, not be of high concern to polar

bears, because they are known to develop hyperlipidemia while remaining metabolically healthy (Kaduce *et al.*, 1981).

Unique pathways associated with POP exposure observed only in cubs

Concerning non-identical pathways between mothers and cubs, cancer related pathways were particularly associated with the positively correlated genes due to POPs in cubs. A limited number of studies have reported gallbladder carcinoma, primary liver cell carcinoma and hepatoma in adult polar bears, however, similar observations in cubs have not yet been documented (Fortin *et al.*, 2014; Hellmann *et al.*, 1991; R. E. Miller *et al.*, 1985). Since cubs are small and have little fat reserves for POPs sequestration (Blix *et al.*, 1979), POPs are likely more bioavailable and exert toxic effects in cubs than adults.

Several genes that correlated positively with ΣPOP in pups were implicated in the gonadotropin-releasing hormone (GnRH) cascade. GnRH, released by the hypothalamus, stimulates the anterior pituitary gonadotropes to secrete, follicle stimulating hormone (FSH and luteinizing hormone (LH) and thus is the main regulator of male and female reproductive function (Glanowska *et al.*, 2014). In adipose tissue, gonadotropins have been shown to regulate lipid metabolism (McMurray *et al.*, 2005).

Long-lasting consequences due to early-life exposure to POPs in cubs

Exposure to POPs during critical developmental stages has been associated with chronic adult-onset diseases in rodent and human studies (Colborn *et al.*, 1993; Huisman *et al.*, 1995; Lessard *et al.*, 2019; Ulbrich *et al.*, 2004). Evidence supporting the association between early-developmental exposure to POPs and metabolic disorders has been documented thoroughly (Heindel *et al.*, 2017; Mendez *et al.*, 2011; Tang-Peronard *et al.*, 2014; Tang-Peronard *et al.*, 2015; Valvi *et al.*, 2014; C. Yang *et al.*, 2018). While documenting such effects in wild polar bear cubs in a well-designed study is extremely challenging, it is reasonable to anticipate that changes in the insulin signaling pathway, regulation of lipolysis in adipocytes, fatty acid metabolism, fatty acid degradation and oxidative phosphorylation associated with prenatal/early-life ΣPOP exposure increases the risk of developing metabolic dysfunction or disorders later on in life.

In conclusion, there is growing evidence that POPs impair energy metabolism in free-ranging adult polar bears (Routti *et al.*, 2019; Routti *et al.*, 2016; Tartu, Lille-Langoy*, et al.*, 2017). This is the first study to assess the impact of pollutants on polar bear cubs using a genome-wide approach thereby emphasizing physiological differences in response between adults and young. The present study supports our hypothesis that exposure to POPs differentially alters genome-wide gene transcription in adipose tissue from mother polar bears and their cubs. POP exposure was particularly correlated with genes involved in a broad range of metabolic pathways in the adipose tissue of both mothers and cubs. This is a unique demonstration of the sensitivity of the developing adipose tissue transcriptome in polar bear cubs. Future research is needed to better understand the mechanisms and consequences of early-life POP exposure on polar bears throughout their life course, including sex-specific responses.

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4.8 Author contributions

Authors contributed to initial conception or design (HR, JLB); final design (PMH, HR, JLB, MD); data acquisition, analysis, or interpretation (JA, HR, PMH, CJB, AB, DG, MD), drafting the manuscript (PMH); and critically revising the manuscript (PMH, HR, JLB, CJB, AB, JA, MD, DG, AD). All authors gave final approval and agreed to be accountable for all aspects of work in ensuring that questions relating the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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4.10 Figures

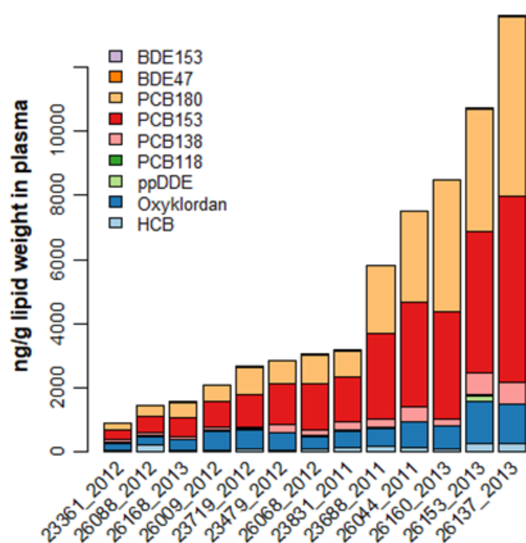

Figure 4.10.1. POP concentrations in adult female bears. Adipose tissue biopsies were collected from adult female polar bears (n=13) and her cub pairs (♂-♂n=5 | ♂-♀n=4 | ♀-♀n=4) in Svalbard, Norway, spring 2011-2013. POP concentrations were measured in plasma and ranged between 897 to 13,620 ng/g wet weight. Total RNA was extracted from all biopsies and subjected to next-generation sequencing to assess RNA presence and quantity.

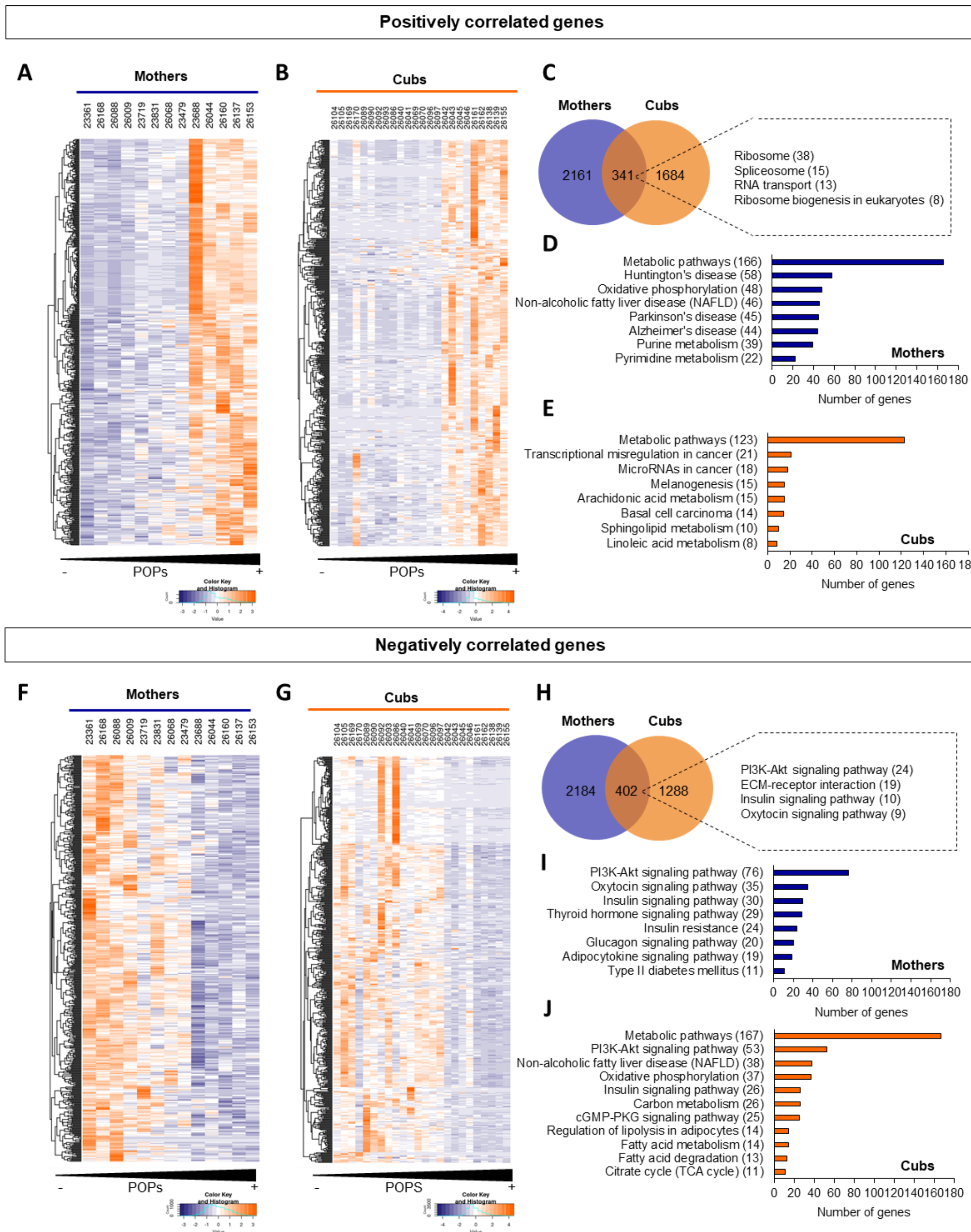

Figure 4.10.2. Adipose tissue gene expression in both adult mother and cub bears is correlated in a dose dependent manner with environmental POPs exposure as revealed by RNAseq analyses. Heatmap comparisons of positive gene correlations (rho ≥ 0.5) and increasing POPs exposure in **(A)** mothers and **(B)** cubs. **(C)** Venn diagram comparing the number and overlap of positively correlated genes (rho ≥ 0.5) in mothers (blue) and cubs (orange). Between mothers and cubs, 341 genes implicated in multiple fundamental molecular GO and KEGG enriched pathways ($p < 0.05$) including, ribosome, spliceosome, RNA transport and ribosome

biogenesis in eukaryotes were shared, number of associated genes in parentheses. Gene-ontology and pathway analyses showing top significant ($p < 0.05$) GOs and KEGG enriched pathways targeted by positively correlated genes in **(D)** mothers and **(E)** cubs, the number of genes associated to each pathway is displayed in parentheses. Heatmap comparisons of negative gene correlations (rho $\leq$ -0.5) and increasing POPs exposure in **(F)** mothers and **(G)** cubs. **(H)** Venn diagram comparing the number and overlap of negatively correlated genes (rho $\geq$ 0.5) in mothers (blue) and cubs (orange). The 402 shared genes between mothers and cubs were implicated in PI3K-Akt signaling, ECM-receptor interaction, insulin and oxytocin signaling GO and KEGG enriched pathways ($p < 0.05$). Gene-ontology and pathway analyses showing top significant ($p < 0.05$) GOs and KEGG enriched pathways targeted by negatively correlated genes in **(I)** mothers and **(J)** cubs. Multiple distinct metabolic pathways were identified by negatively correlated genes in both mothers and cubs, yet they are different from those identified for positively correlated genes.

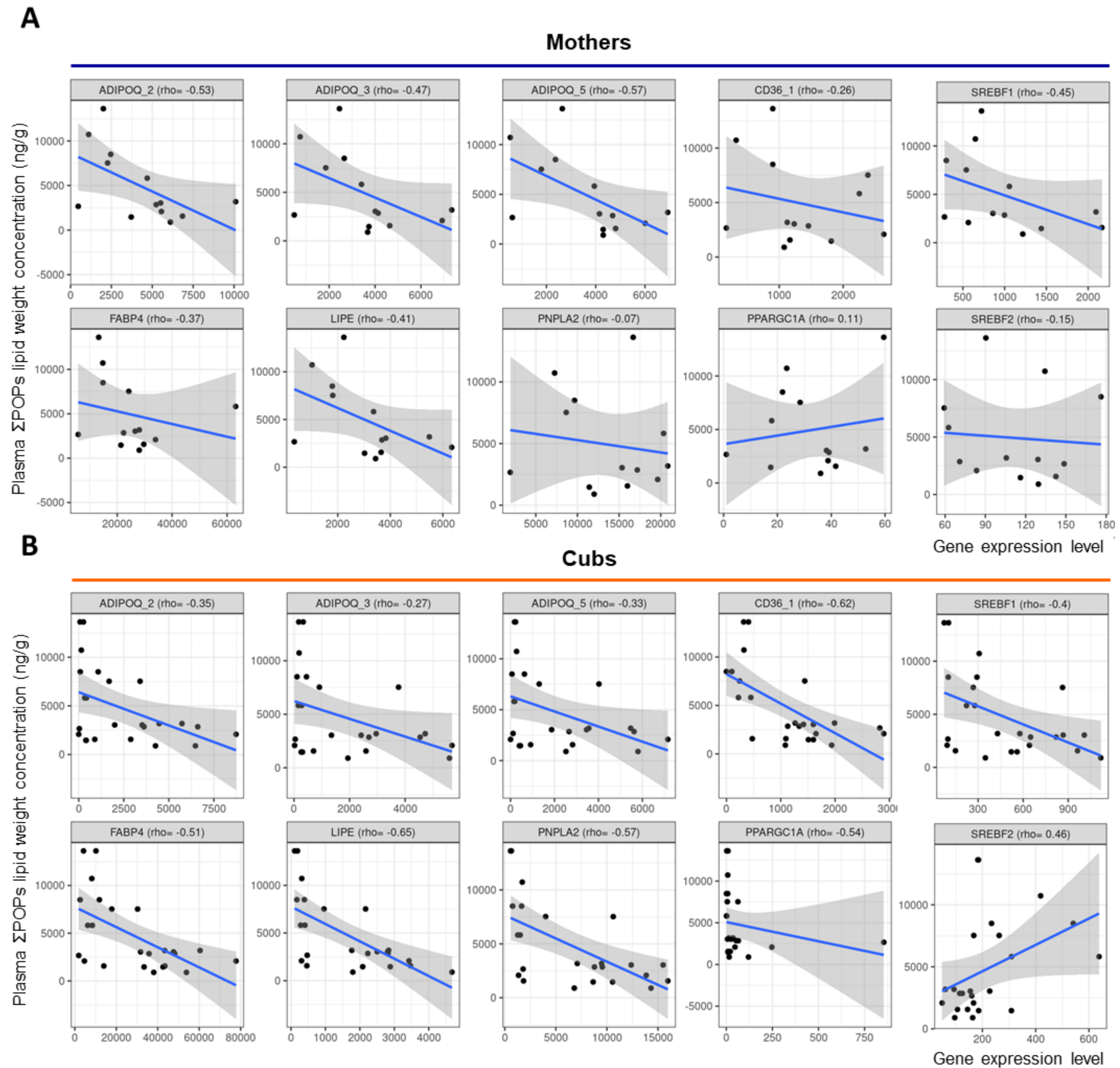

Figure 4.10.3. Correlations between environmental POPs exposure and several key regulators of energy metabolism in (A) mothers and (B) cubs. Grey area represents the 95% confidence intervals of the model.

4.11 Supplementary data

POP analyses

Samples of three adult bears were analysed in 2011 and the remaining 10 adults were analysed in 2014 as described previously (Lippold *et al.*, 2019; Tartu, Bourgeon*, et al.*, 2017) based on methods by Polder et al. (Polder *et al.*, 2014; Polder *et al.*, 2010). Briefly, approximately 2 g plasma was spiked with internal standards (CB-29, -112 and -207, BDE-77, -119 and -181) and added cyclohexane – acetone (3:2), NaCl and water. Lipids were extracted using an ultrasonic homogenizer followed by centrifugation and separation. The lipid content of the samples was determined gravimetrically. Lipids were removed with concentrated H_2SO_4 and the extracts were concentrated. PBDEs (both batches) and PCBs and OCPs in 2011 were analyzed by high resolution gas chromatography (HRGC) Agilent 6890 Series with Agilent 7683 Series autosampler connected to a quadrupole MS detector (Agilent 5973 Series). In 2014, PCBs and OCPs were analyzed using HRGC Agilent 6890 Series with Agilent 7683 Series autosampler coupled to two ^{63}Ni micro (μ) electron capture detectors (Agilent 6890).

The environmental toxicology laboratory at the Norwegian University of Life Science (NMBU, Oslo, Norway) is accredited for testing the analyzed chemicals in biological material according to the requirements of the NS-EN ISO/IEC 17025 (TEST 137). To ensure quality control, three blank samples, one blind, two to three spiked samples and in-house reference material of seal blubber were run for each series of 15 samples. In addition, analytical quality was successfully assured by routinely analyzing certified reference materials (CRM) including CRM 350 (mackerel oil), CRM 2525 (fish muscle), CRM 349 (cod liver oil) and CRM 1946 (Lake Superior fish tissue). Limit of detection defined as three times noise level was 0.14 ng/g wet weight was oxychlordane for the 2014 batch and below 0.07 ng/g wet weight for the remaining analyses. Recovery rate of POPs in spiked reference material varied between 82-118%.

Table S4.11.1. Target and reference genes with accompanying primer sequences. Thermo cycling was performed with preincubation at 95oC for 10 min followed by 45 cycles of amplification including 10 sec denaturation at 95oC, 10 sec annealing with primer specific temperature, and 20 sec elongation at 72oC. Primers have been previously tested and described by Tartu, Lille-Langoy, et al. (2017).

Gene (Name/symbol)	Primer sequences (5' → 3'')	Tan (°C)
Lipase (*LIPE*)	Fwd: GTGAAGGACAGGACAGTGAGG Rev: CTCTTGAGGTAGGGCTCATG	57
Adiponectin (*ADIPOQ*)	Fwd: GATGAGAGTCCTGGGTATAG Rev: CAAAGGGACTCAGAGGTGAAG	60
Sterol regulatory element binding transcription factor 1 (*SREBP1*)	Fwd: CACACCCAGGTCCAGAATG Rev: GAGGTCACTGCGGTTGTT	60
Actin, beta (*ACTB*) * Endogenous control	Fwd: ACCCAGATCATGTTTGAGACC Rev: TGATGTCACGCACGATTTCC	57

Table S4.11.2. Adipokines IL-6, LEP and ADIPOQ were negatively correlated to ΣPOP exposure in mother bears.

Gene	Rho-value	*P-value*
IL-6	-0.539	0.057472354
LEP_1	-0.681	0.012929313
ADIPOQ_5	-0.566	0.047339595

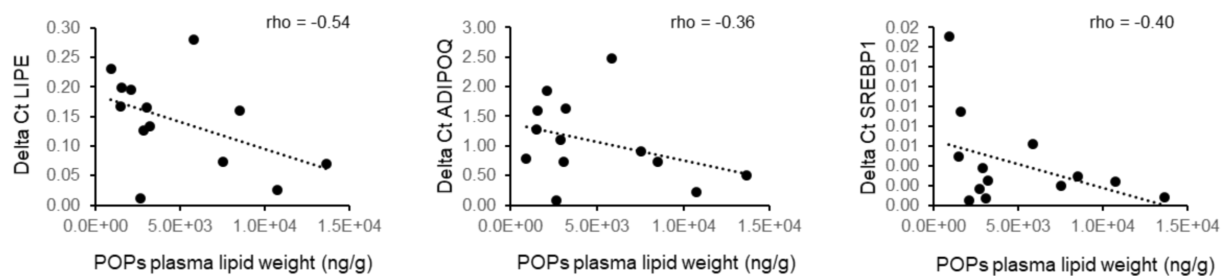

Figure S4.11.1. RT-qPCR validation of gene expression patterns from the RNA sequencing analysis. Total RNA was extracted from adipose tissue of mother bears (n=13) and subjected to RT-qPCR. Spearman's correlation coefficient calculations were performed to obtain rho values. Similarly, to RNA sequencing results, LIPE, ADIPOQ and SREBP1 transcript levels are negatively correlated due to ΣPOP exposure. ACTB was used as an endogenous control.

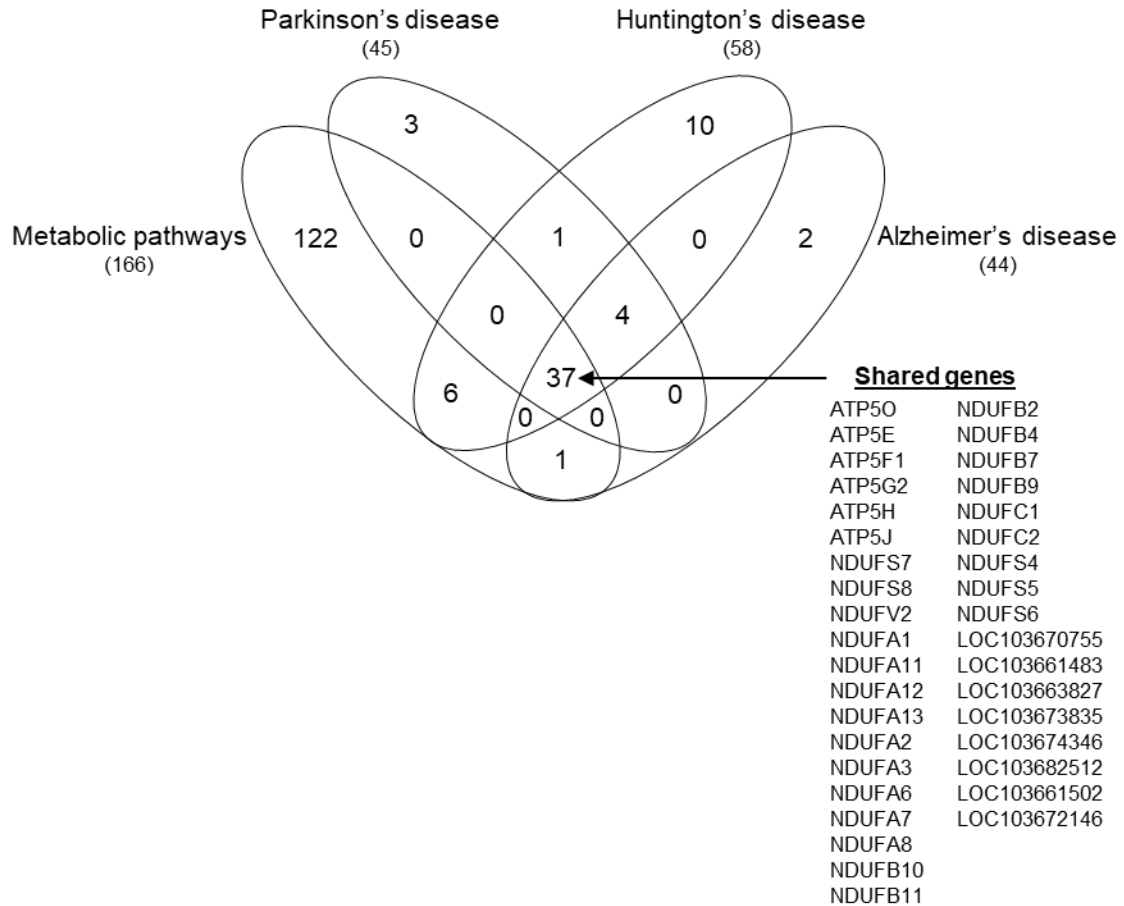

Figure S4.11.2. Venn diagram depicting the number of POPs targeted genes common between metabolic and neurodegenerative disease pathways. Thirty-seven genes, implicated in respiratory oxidation and ATP synthesis, are shared between Metabolic pathways, Parkinson's disease, Huntington's disease and Alzheimer's disease GO terms.

Chapter 5

General discussion and conclusion

For the longest time, research has focussed on the mother's health and environment (incl. contaminant exposure), around the time of pregnancy, in relation with the health of her children (Sharp *et al.*, 2018). Leading organizations of fertility professionals and obstetricians have acknowledged the risk of environmental contaminants (BPA, phthalates and pesticides) on reproductive health and integrated environmental health awareness and assessment as part of preconception and prenatal care (Bellingham *et al.*, 2013; No, 2013). Investigating pregnant women's attitudes and behaviors about environmental contaminants, E. S. Barrett *et al.* (2014) showed that the majority of women are aware of the risks of environmental contaminants and try to avoid and/or reduce their exposures. Little is known, however, about the father's contribution to pregnancy outcomes. Why? After all, during fertilization half of the maternal genome merges with half of the paternal genome.

Male germ cell development including the transformation of PGCs, diploid spermatogonia into haploid sperm requires stage- and testis-specific gene expression, mitotic and meiotic divisions, chromatin remodeling, and epigenetic reprogramming (Carrell, 2012). Due to the epigenome's plasticity, epigenetic changes occurring throughout male germ cell development portray windows of susceptibility towards environmental exposures, potentially causing long-lasting effects and affecting the offspring health.

Recent evidence has raised awareness about the influence of the father's environment, such as diet, psychological stress and environmental contaminants, on the offspring's phenotype (J. M. Braun *et al.*, 2017). Studies showed that the sperm epigenome plays a pivotal role in the transmission of such phenotypes over sometimes multiple generations (Q. Chen *et al.*, 2015; Lambrot *et al.*, 2013; Skinner *et al.*, 2009; Stuppia *et al.*, 2015). Studies on the impact of environmentally relevant pollutant mixtures on different epigenetic mechanisms across multiple generations including a nutritional intervention are still scarce, and often only focus on a single pollutant (Dolinoy *et al.*, 2007; Skinner *et al.*, 2018).

The present thesis highlights the vulnerability of the father's sperm epigenome and demonstrates that *in utero* exposure to an environmentally relevant POP mixture affects three main epigenetic mechanisms that control chromatin structure to serve sperm-specific gene expression including sperm-born miRNAs, DNA methylation and histone activation mark H3K4me3 across multiple direct and indirect exposed generations. Furthermore, the addition of maternal FA supplementation, as a potential nutritional intervention during the same exposure window, partly mitigates / counteracts the POP induced effects in the sperm epigenome. This thesis also shows the influence of the POP affected sperm epigenome on the gene expression of the two-cell embryo across generations. As such, the results presented take steps towards elucidating the dynamics of paternal inter and transgenerational epigenetic inheritance in response to environmental pollution.

Aside, a complementary study shows the impact of Arctic POP exposure on the adipose tissue gene expression of Norwegian mother polar bears and cubs, thereby emphasizing the physiological differences in response between adults and young.

5.1 Different epigenetic components of sperm are affected by POP exposure

5.1.1 Sperm-borne miRNAs

Sperm are packed with many types of small non-coding RNAs (e.g. miRNAs, siRNAs, piRNAs and tRNAs) that have the potential to influence embryonic development (Liu *et al.*, 2012; M. Wang *et al.*, 2017; Yuan *et al.*, 2016). Sperm miRNAs are known for their roles in spermatogenesis, sperm maturation and male fertility (Gunes *et al.*, 2013; Hayashi *et al.*, 2008; Miska *et al.*, 2016). Furthermore, increasing evidence supports that the father's sperm miRNA content responds to paternal environmental stressors (de Castro Barbosa *et al.*, 2016; Fullston *et al.*, 2016; Rodgers *et al.*, 2013; Schuster *et al.*, 2016). It was only until recently, however, that sperm miRNAs were demonstrated to be involved in transmitting paternally acquired phenotypes (Grandjean *et al.*, 2015; Rodgers *et al.*, 2015).

In chapter one, we provide evidence that sperm miRNA profiles are altered across multiple generations due to *in utero* POP exposure, though, the severity of perturbation decreased after the F2 generation. Furthermore, FA supplementation partly counteracted the POP induced effects. POP affected miRNAs were associated with genes involved in multiple

developmental pathways (brain, heart, kidney, embryonic, tissue morphogenesis, reproductive system) particularly in F2. Three miRNAs were consistently significantly altered across F1 and F2 but not beyond, due to POPs, whereas POPs+FA altered but one common miRNA.

These results expand the current knowledge concerning sperm-borne miRNAs and their role in multigenerational epigenetic inheritance (de Castro Barbosa *et al.*, 2016; Fullston *et al.*, 2013; Grandjean *et al.*, 2015; Rodgers *et al.*, 2015), however, many questions remain the be answered. For instance, how long do sperm-borne miRNAs remain active once delivered to the oocyte (Ostermeier *et al.*, 2004)? How can such a small quantity of sperm-borne miRNAs, compared to those already existing in the oocyte, make such a significant difference in early development, and subsequent adult cellular function? What factors determine which miRNAs are retained in sperm during spermatogenesis (Krawetz *et al.*, 2011; D. Miller *et al.*, 2006)? What about other contents of the sperm-borne sncRNAs, such as piRNAs and tRNAs? Sequencing analysis of total sperm ncRNA content in reaction to *in utero* POP exposure could clarify some of these gaps in the literature (Q. Chen *et al.*, 2015; Gapp *et al.*, 2014; Grandjean *et al.*, 2015). Furthermore, the sperm-borne miRNA content is known to change along the length of the epididymis. It was previously shown that cells lining the epididymis release exosomes called epididymosomes, which contain mostly miRNAs and sRNAs, that can spontaneously fuse with mature sperm thereby altering their sncRNA profile (Reilly *et al.*, 2016; U. Sharma *et al.*, 2018). Therefore, it would pertinent to investigate the response of epididymal cells towards POP exposure, and monitor subsequent changes in their molecular cargo which potentially influence the caudal sperm miRNA profile.

5.1.2 POPs and the sperm DNA methylome

A proper balance in DNA methylation is essential for genetic stability and individual health. Global DNA hypo and hypermethylation have been associated with genomic instability, structural abnormalities and developmental defects (Reichetzeder *et al.*, 2016; A. S. Wilson *et al.*, 2007). Previously, transgenerational changes in sperm DNA methylation have been reported in mice and humans due to nutritional effects (Burdge *et al.*, 2011; Painter *et al.*, 2008), and fish due to mercury exposure (Carvan *et al.*, 2017). Transgenerational changes in sperm DNA methylation have also been observed after developmental exposure to PCB,

DDT and dioxin (Gillette *et al.*, 2018; Manikkam *et al.*, 2012; Skinner *et al.*, 2018; Skinner, Manikkam, *et al.*, 2013).

In chapter two, we showed that *in utero* POP exposure affects sperm DNA methylation over multiple (un)exposed generations. POPs, FA and POPs+FA particularly targeted DMSs in distal intergenic and open sea regions. A treatment dilution effect was observed until F3 but not F4 due to all treatments. Compared to POPs and FA, POPs+FA mostly hypo-methylated DMSs in F1-F4. We only observed a partially protective effect of FA against POP-induced DNA methylation alterations across generations. Using permutation analysis, we detected multiple conserved differentially methylated sites (DMSs) due to FA and POPs+FA (Belleau *et al.*, 2018). *In utero* POP exposure induced a transgenerational effect, though no significantly transgenerationally conserved DMSs were detected between F1 and F4.

POP exposure could alter DNA methylation through potentially affecting DNMT activity, which is suggested to be under hormonal control (Garcia-Carpizo *et al.*, 2011), and/or altering SAM availability. *In utero* and lactational exposure to high dose PCBs (100x human level) and methylmercury chloride significantly reduced DNMT-1, and -3b mRNA expression in female rat offspring hepatic tissue (Chu *et al.*, 2008; Desaulniers *et al.*, 2009). PCB exposure and a mixture of PCB, methylmercury chloride, organochloride pesticides significantly reduced universal methyl donor SAM expression in female offspring livers (Desaulniers *et al.*, 2009). Alternatively, some POPs can bind to steroid receptors and affect histone enzymes, e.g. demethylase Jarid1b, and subsequently histone marks (Casati *et al.*, 2015). As a result, these altered histone modifications could indirectly alter DNA methylation.

Next, a probable explanation for the transgenerational preserved DMSs observed in only ancestrally FA and POPs+FA exposed sperm, is the critical role FA plays in one carbon metabolism which is essential during spermatogenesis and early embryonic development (Crider *et al.*, 2012). Previous animal models have shown that folate deficiency or low folate intake result in detrimental effects on male reproduction and the sperm epigenome (D. Chan

et al., 2010; Lambrot *et al.*, 2013). High-dose FA supplementation is associated to negative effects on male reproductive health as well (Aarabi *et al.*, 2015; Ly *et al.*, 2017).

These results were produced using RRBS which, in contrast to WGBS, a historically considered “gold standard” for DNA methylation analysis, focusses solely on CpG-enriched fragments through the digestion of DNA using a methylation insensitive MspI that recognizes CCGG sites covering approximately 1.6 million CpGs (Harris *et al.*, 2010; Meissner *et al.*, 2005). RRBS is accompanied by two weaknesses as it may enrich sequences with high CpG content like CpG islands and detect methylation at non-CpG dinucleotides. Yet, RRBS does interrogate repetitive sequences. Like RRBS, WGBS (MethylC-seq) is based on sequencing bisulfite treated DNA, though, without the use of a restriction enzyme to perform the initial DNA digestion. Although, WGBS provides a higher CpG coverage of 95% compared to RRBS with 12% genome-wide, it remains much more expensive (Harris *et al.*, 2010; Lister *et al.*, 2009; Lister *et al.*, 2008). Other available techniques include methylated DNA immunoprecipitation sequencing (MeDIP) and methylated DNA binding domain sequencing (MBD-seq) which, however, do not have a similar level of resolution compared to the techniques described above. An important limiting factor of the present thesis, that favored the choice of RRBS, is the amount of sperm required for multiple analyses per epigenetic component including the validation and accompanying optimization experiments. RRBS can be successfully performed using a DNA volume as low as 30-50 ng, whereas WGBS, MeDIP and MBD-Seq require 5µg, 300ng - 5µg and 3µg respectively (Bock *et al.*, 2010; Harris *et al.*, 2010; Yong *et al.*, 2016). Therefore, from a practical perspective, RRBS may be the most suitable to primarily identify novel DNA methylation sites that are affected by POP treatment and escape genome-wide epigenetic reprogramming across generations. Furthermore, RRBS was already successfully established in our collaborator’s laboratories. Alternatively, MethylC-Capture sequencing could be used to also evaluate DNA methylation changes in dynamic sperm CpGs (i.e. methylation 20-80%) in the future (Chan *et al.,* 2019).

5.1.3 Sperm histone activation mark H3K4me3

Sperm carry multiple histone modifications (Hammoud *et al.*, 2011; Siklenka *et al.*, 2015). We used ChIP-seq to enrich DNA sequences from chromatin fragments using an antibody derived against the specific histone modification of interest, i.e. H3K4me3. In Chapter two,

we demonstrate that H3K4me3 is affected by *in utero* POPs, FA and POPs+FA exposure across multiple generations as shown by preliminary unsupervised principal component analyses (PCA). Although these results are preliminary and further optimization and subsequent analyses are still ongoing, the PCA plots show certain trends between treatment versus CTRL replicates. Multiple normalization techniques have been applied to the data set, yet a consistent variation between replicates was observed especially when checking significantly differentially expressed H3K4me3 regions on data tracks by Integrative Genomics Viewer. As such, future work will apply a pairwise comparison between samples and extract the top 5% of regions associated to the main source of variability i.e. dietary / pollutant variability. Perhaps variation between treatment replicates is a result of an outbred rat model. Previous research observed similar variations in DNA and histone methylation of ancestrally exposed F3 sperm collected from multiple outbred Sprague-Dawley rats (Skinner *et al.*, 2018).

Due to the low abundance of nucleosomes in sperm, the resolution and efficiency of the immunoprecipitation (IP) was improved by implementing an enzymatic approach, particularly micrococcal nuclease (MNase), to prepare and digest the native sperm chromatin (Hisano *et al.*, 2013). For MNase to access the DNA, however, sperm was pre-treated with a reducing agent, in our case DTT, to loosen up the tightly packed chromatin. As such, MNase was able to digests the exposed linker DNA while the nucleosomal DNA wrapped around the histone octamer remained protected, resulting in a thorough chromatin fragmentation and the retention of only mono-nucleosomal DNA (~150 bp). It has been previously suggested, using somatic cell models in *Drosophila* and yeast, that MNase has a potential sequence bias as it digests A/T-rich nucleosomes faster compared to G/C-rich nucleosomes (Chereji *et al.*, 2016; Chereji *et al.*, 2017; Dingwall *et al.*, 1981). This proposed bias, however, can be tuned by reducing the incubation temperature (Chereji *et al.*, 2016).

To avoid reproducibility issues, we selected the H3K4me3 antibody based on results published by R. N. Shah *et al.* (2018) who evaluated the specificity of 52 commercial “ChIP grade” H3K4me antibodies in human female K562 cell lines using histone peptide microarrays and internally calibrated ChIP (IceChIP) (R. N. Shah *et al.*, 2018). All ChIP

experiments were carried out using H3K4me3 antibodies with identical catalogue and lot numbers. Batch effects were anticipated though could not be completely avoided due to the large number of samples to be analyzed. Samples could be divided into as low as three batches, each batch containing a pool of each treatment and generation.

5.1.4 Other histone marks

Besides investigating activation mark H3K4me3, future studies could focus on repressive marks such as H3K27me3 using ChIP-seq analyses in the same sperm samples. Multiple studies have shown that H3K27me3 is associated with genes involved in development and metabolism (Brykczynska *et al.*, 2010; Hammoud *et al.*, 2009; Zheng *et al.*, 2016). Integrating H3K4me3 and H3K27me3 and ChIP-seq results will clarify which regions of the epigenome escape epigenetic reprogramming and are potentially transmitted to subsequent generations.

5.2 Integration of multiple epigenetic mechanisms and missing links

The analysis of DNA methylation, histone mark H3K4me3 and miRNA profiles in purified sperm from individuals exposed to four treatments, including CTRL, POPs, FA and POPs+FA across F1-F4 within the same model provides a comprehensive insight in the epigenetic alterations associated with environmentally induced transgenerational germline epimutations. A comparison of F1, F2, F3 and F4 generations allowed us to assess the differences between direct and indirect exposure and thereby observe intergenerational (F1-F2) versus transgenerational effects (F3-F4). The F2 phenotype, however, may be the result of the combination of direct and indirect exposure, yet it is not possible to distinguish between both. In addition to the above, we also investigated the potential influence of *in utero* exposed sperm on early embryonic gene expression, i.e. two-cell embryos.

To obtain better understanding of the interaction between different epigenetic mechanisms in response to *in utero* contaminant exposure across generations, it is desirable to perform an integrative omics analysis of the DNA methylation, histone mark and miRNA data sets in combination with two-cell embryo gene expression in association with reproductive phenotypes observed. Multiple data analysis approaches have been already developed to

integrate various omics layers to understand epigenetic mechanisms of complex diseases, including obesity and cancer (Cazaly *et al.*, 2019). Furthermore, Pacheco *et al.* (2011) used integrative analysis of sperm DNA methylation and mRNA expression and identified CpG methylation profiles and mRNA alterations associated with low sperm motility in men (Pacheco *et al.*, 2011).

Contrary to our expectations, we did not observe a consistent diluting treatment effect across all generations. Instead, F3 males showed the poorest pregnancy outcomes, generating F4 two cell embryos with the most significantly differentially expressed genes, and F4 sperm with a 1.8-fold increase in the number of differentially methylated sites (DMSs) due to **all treatments**. We initially hypothesized that this sudden "increase/impact" observed in the F4 was due to an accumulation of epimutations including DNA methylation, histone modifications, and or non-coding RNAs that escaped remodelling during early embryonic development or spermatogenesis. As such, these epimutations would remain uncorrected, resulting in substantial alterations in F4 two-cell embryo gene expression and sperm epigenetic expression. F3 males, however, did not appear to have affected sperm motility parameters. As for the F3 sperm epigenome, neither miRNA profiles nor DNA methylation seemed particularly affected in F3 compared to other generations. Perhaps, histone mark H3K4me3 plays a significant role, which for now, remains to be elucidated due to the preliminary state of the results. The sperm chromatin, however, consists out of countless of different histone marks **which together** orchestrate chromatin structure (Meyer *et al.*, 2017). It would be surprising if one histone mark would justify the transgenerational impact observed. Aside the above, DNA sequence changes cannot be not ruled out (Heard *et al.*, 2014). For instance, epimutations may increase the rate of nearby spontaneous genomic DNA mutations in e.g. epigenetic regulators e.g. lysine (K)-specific demethylase 1A (KMD1A) which can lead to aberrant erasure, establishment, and maintenance of epigenetic marks in the same region (Siklenka *et al.*, 2015; You *et al.*, 2012). Future studies might consider incorporating tests to measure DNA damage in sperm by e.g. Comet assays (Simon *et al.*, 2013).

While this study is ideal for identifying adverse epigenetic outcomes due to *in utero* contaminant exposure in sperm across multiple generations, it does not focus on the mechanism of action. Therefore, it will be of great interest to determine whether and which of these modifications are causally associated with the phenotypes observed. *In vitro* stem cell models are a promising avenue to assess perturbations of the developing sperm and male reproductive organs, as they are able to differentiate into different cell types which could help predict developmental and reproductive toxicity (Chapin *et al.*, 2009). In the present context, stem cells like spermatogonial stem cells would be of high interest. Recent development of CRISPR-Cas9 and other genome editing tools may provide an efficient way to introduce epigenetic alterations, similar to the ones induced by *in utero* POP exposure, without changing the DNA sequence (Liao *et al.*, 2017). Epigenetic alterations may also continue to occur throughout spermatogenesis, as process initiated at the beginning of puberty through increased LH and testosterone levels via the pituitary-gonadal axis (Arzuaga *et al.*, 2019). In the present study design, two rounds of spermatogenesis (2 x ~54 days) after puberty initiation, sperm were collected from male rats, i.e. PND 150 (Sengupta, 2013). Besides collecting caudal sperm, it would be interesting to perform epigenetic analyses *in vivo*, e.g. DNA methylation, on isolated cells of different spermatogenic stages including spermatogonia, spermatocytes, spermatids, and caput epididymal sperm. This would provide insight in the developmental origins of POPs-induced multigenerational sperm epigenetic alterations/epimutations. Perhaps most epigenetic changes develop during spermatocytogenesis and/or spermiogenesis. Exposure to a pharmacological dose of DDT (1% of the lethal oral dose 50%) has been previously shown to affect DNA methylation patterns in particularly prospermatogonial and spermatogonial stage in a rat model (Ben Maamar *et al.*, 2019). Besides a direct impact, it is tempting to speculate that *in utero* POP, FA or POPs+FA exposure also affects the germline indirectly by altering the function of its supporting cells (Xin *et al.*, 2015). Sertoli and Leydig cells are well known to be sensitive to endocrine disrupting chemicals (Campion *et al.*, 2012; Rhouma *et al.*, 2013).

Other questions that require further investigation include, what part of the sperm epigenome is sensitive towards environmental exposure after puberty and what do these epigenetic marks in sperm mean for later life development?

5.3 Two-cell embryo gene expression sensitive to POP exposure

The use of a rat model afforded examination of not only the consequences of *in utero* POP exposure on multiple epigenetic mechanisms involved in inter/trans generational inheritance but also the impact on early embryo gene expression. We predicted that the *in utero* paternal treatment effect would dilute across generations, instead, we observed an opposite effect with an increased of number of significantly differentially expressed genes due each treatment compared to control in F4. What would have caused this sudden increase aside accumulated sperm epimutations? Perhaps, performing a chromatic integrity analyses on the actual sperm could give us insight (Hekmatdoost *et al.*, 2009; Sadeghi *et al.*, 2009), or measurements of oxidative stress in sperm as POPs are known to cause oxidative stress whereas FA has protective properties against free radicals (Agarwal *et al.*, 2018; Joshi *et al.*, 2001; Kumar *et al.*, 2014). Furthermore, it would be beneficial to sequence total RNA two-cell embryo content instead of mRNA only, in order to capture which sperm-specific transcripts are transferred to the oocyte post-fertilization.

5.3.1 Early and late stage two-cell embryos

The two-cell embryo stage was initially selected as paternal epigenetic marks escaping reprogramming would presumably be first observed at this stage. Throughout early embryonic development, two phases of zygotic genome activation (ZGA) take place including minor activation (minor ZGA) before cleavage and major activation (major ZGA) at the two-cell embryo phase (Hamatani *et al.*, 2004). Minor ZGA, which mostly occurs in the male pronucleus, promotes zygotic transcription, yet is weak and initiates synthesis of a small number of polypeptides from zygote-to-early two-cell embryo. Major ZGA, taking place in late two-cell embryos, promotes a more effective form of gene expression reprogramming, along with the generation novel transcripts that were not expressed in oocyte (Hamatani *et al.*, 2004; Zhang *et al.*, 2016). According to the ZGA, the two-cell embryo phase can be divided into early and late stage. Using RNA sequencing, differences in gene expression have been observed for specifically transferase, kinase, mitochondrion, cell death and tRNA metabolic process GO-terms between early and late stage mouse two-cell embryos (Zhang *et al.*, 2016). Other GO-terms including GTPase regulator, calcium ion binding, synapse, cytoskeletal protein binding, neuron differentiation, organelle lumen,

ribonucleoprotein, mitochondrion, cell cycle and RNA processing remained overall similar between both two-cell embryo stages (Zhang *et al.*, 2016). To avoid early and late two-cell embryo bias as much as possible, the timing of all superovulation inductions was kept the same across generations. Furthermore, previous studies identified early and late stage mouse two-cell embryos at respectively 30 and 43 hours after hCG injection (Zhang *et al.*, 2016). As for rats, the formation of two-cell embryos starts at 24 hours after fertilisation and reaches a plateau of two-cell embryo stage from approximately 36 until 48 hours after it transforms into three- and subsequently four-cell embryos (Ansai *et al.*, 1994). The results, shown in the present thesis, are based on rat two-cell embryos consistently collected 43-44 hours after hCG injection and 36 hours after copulation, thus late stage two-cell embryos.

5.3.2 Super-ovulation bias

To retrieve two-cell embryos successfully in synchronization, untreated female rats were induced to super-ovulate once by intraperitoneal injections of PMSG followed by hCG 48 hours later. Previous studies have shown that artificial reproductive technologies including super-ovulation and *in vitro* culture can induce epigenetic changes in the embryo thereby potentially altering gene expression (Fauque *et al.*, 2007; Liang *et al.*, 2013; Tang *et al.*, 2019). Alterations in H4K12ac and H3K9ac in the inner cell mass and trophectoderm as well as DNA methylation changes of LINE-1 retrotransposon elements have been observed due to super-ovulation in mouse blastocysts (Bakhtari *et al.*, 2014; Liang *et al.*, 2013). Repeated super-ovulation has been shown to alter histone modifications particularly H3K9me2 and H3K27me3 in four-cell embryos and blastocysts. In addition, increased mRNA levels of histone deacetylases (*Hdac1* and *Hdac2*) and methyltransferase (*G9a*); and decreased expression of histone demethylase-coding genes (*Kdm6a* and *Kdm6b*) were observed as well (Tang *et al.*, 2019). Based on these studies, it is possible that our super-ovulation treatment affected the embryo epigenome and perhaps gene expression. Despite super-ovulation, however, we still observed significantly differently expressed genes due to *in utero* POP, FA and POP+FA exposure compared to control across F2-F4 generations. To avoid the impact of super-ovulation itself, it would be favorable to generate oocytes and subsequent two-cell or four-cell embryos without super-ovulation, though, this would make the experiment rather complex especially concerning the timing of 48 individuals and resulting batch effects. In addition, the number of generated two-cell embryos might be lower.

5.3.3 Sex-specific two cell embryo transcriptomic analysis

Future studies could investigate sex-specific differences by sexing the two-cell embryos and separate the female from the male embryos followed by transcriptomic analyses (Gardner *et al.*, 2010). POPs exposure has perhaps a different impact on the gene expression dependent on the sex. Furthermore, instead of pooling multiple embryos from different individuals, it would be advantageous to sequence single two-cell embryos to maintain the individual variations.

5.4 Folic acid used as an epigenetic diet against environmental pollutants

Various studies indicate that bioactive compounds play critical roles in epigenetic regulation including DNA methylation, histone modification, and miRNA regulation. Some bioactive compounds, e.g. methyl donors, may counteract or prevent the pollutant induced epigenetic damage. The time of intervention, however, is crucial for the efficacy of epigenetic perturbation to counter abnormal epigenetic development (Tammen *et al.*, 2013). For instance, maternal supplementation of methyl donors was previously shown to reverse DNA hypomethylation induced by early developmental BPA exposure (Dolinoy *et al.*, 2007). Multiple bioactive compounds such as vitamin C, D, choline and certain polyphenols have been previously shown to affect the epigenome (S. Li *et al.*, 2019). Omega 3, an abundant compound in POP contaminated traditional foods that Northern Canadian Indigenous populations consume, has been linked to differential DNA methylation as well (Aslibekyan *et al.*, 2014; Hussey *et al.*, 2017). Using Omega 3 as a nutritional intervention, however, would lead to a confounding conclusion, especially since POPs and Omega 3 are derived from similar nutritional sources. In the present thesis we incorporated methyl donor FA as a nutritional intervention to potentially mitigate/counteract the detrimental epigenetic effects of POP exposure. FA was favoured as its supplementation has already been fortified in many grain products across Canada, it is recommended to pregnant women, it is critical during embryonic development including the two-cell embryo stage and it plays an essential role in DNA methylation and synthesis which is of specific interest as the sperm epigenome was investigated. The two different FA doses, of 2 mg/kg diet (1X) and 6 mg/kg diet (3X), were selected to simulate environmental conditions, i.e. basal FA intake after the introduction of mandatory FA fortification and/plus a daily FA vitamin supplement respectively (Crider *et*

al., 2012). FA supplementation was added directly to the animal feed thereby mimicking the variability in food intake of the general population.

5.4.1 Adverse effects due to FA supplementation

The present thesis shows that FA supplementation partly mitigated / counteracted the POP induced effects in the sperm epigenome. FA supplementation by itself, however, induced various unexpected outcomes observed in not only the sperm epigenome but also subsequent phenotypes. FA supplementation let to multiple significantly altered sperm characteristics as well as an increased number of F3 postimplantation losses (Chapter 3). Female fetuses (GD 19.5) showed an increased number of external and visceral malformations due to FA supplementation across F1-F4 (data prepared for publication). These include multiple congenital malformations such as hydrocephalus and exencephaly, which exceeded the expected incidence of fetal malformations for the Sprague-Dawley strain (Bentz *et al.*, 2015; Mulla *et al.*, 2012). Although the precise underlying mechanisms remain unknown, a large number of differentially methylated sites and significantly differentially expressed genes in respectively sperm and two-cell embryos were involved in various neurodevelopmental gene-ontology pathways due to only FA supplementation across four generations. According previous studies, alterations in the brain morphology following FA supplementation in rats are not uncommon. Moderate FA supplementation (8 mg/kg diet) has been shown to compromise cerebellar morphology in growing outbred Sprague Dawley rats (40% of tested rats showed cerebellar alterations) (Partearroyo *et al.*, 2016).

One could consider masked vitamin B12 deficiency by FA supplementation as an underlying cause. Vitamin B12 deficiency significantly affects functions of blood, nervous system and inflammatory response. Demands of vitamin B12 are particular high in nervous tissue (Pavlov *et al.*, 2019). In sperm, a deficiency has been significantly lower sperm count and increase abnormal sperm characteristics in a rat model (Watanabe *et al.*, 2003). The FA diets used in the present thesis, however, contain the recommended dietary concentrations of vitamin B12 for rats i.e. 25 µg/kg diet (Reeves *et al.*, 1993). Furthermore, the FA experimental diets have been successfully used in previous studies (B.G. Swayne *et al.*, 2012). A vitamin B12 deficiency seems therefore unlikely.

5.5 POP exposure and polar bear adult/cub adipose tissue gene expression

Being on top of the Arctic food chain, apex predators like polar bears display some of the highest POP concentrations among Arctic wildlife (Letcher *et al.*, 2010). Although global POP concentrations have declined over the past decade, newer trends show that concentrations of certain compounds have actually increased in polar bears (Dietz, Desforges, Gustavson*, et al.*, 2018; Lippold *et al.*, 2019). Compared to their mothers, polar bear cubs are exposed to even higher POP levels during lactation (Bernhoft *et al.*, 1997; Bytingsvik, Lie*, et al.*, 2012). Over 90% of the POPs body burden is stored in white adipose tissue, a dynamic metabolic and endocrine organ (Gebbink *et al.*, 2008). The present thesis emphasizes the physiological differences between young and adult polar bear adipose tissue gene expression in response to environmental POP exposure. Besides, this is a unique demonstration of the sensitivity of the developing adipose tissue transcriptome in polar bear cubs. Various metabolic pathways were associated with increasing POP concentrations in cubs and mothers. It is tempting to speculate that early-life POP exposure potentially increases the risk of developing metabolic dysfunction disorders later in life.

5.5.1 Sex-specific responses towards POPs in polar cub pairs

Further research is required to understand the underlying molecular mechanisms of early-life POP exposure in polar bear developing adipose tissue. In addition, focus should be laid on sex-specific responses, especially since the biological attributes of males and females affect not only molecular and cellular responses towards POPs but also health and disease (Rich-Edwards *et al.*, 2018). Initially, we anticipated to investigate sex differences between male and female adipose tissue transcriptomes, as the samples received included biopsies from three male-female cub pairs. Upon sample preparation for RNA extraction, however, one male biopsy of a male-female cub pair consisted mainly out of skin and blood, as such no adipose tissue could be retrieved. Therefore, only two complete male-female cub pair samples besides the other samples were submitted for RNA sequencing. Both male-female cub pairs descended from mothers with very different plasma lipid weight concentrations of POPs i.e. 1564.43 versus 8498.98 ng/g lipid weight (~5.4 times difference). For now, no definite conclusions can be drawn from these male-female cub pairs concerning sex-differences, mainly due to the small sample size.

5.5.2 Global warming and the Arctic

Increasing global surface temperatures potentially enhance POPs' long-range transport towards remote areas, including the Arctic, by influencing the volatilisation behaviour from primary and secondary sources including their partitioning between soil, sediment, water and atmosphere (Teran *et al.*, 2012). Habitat loss, due to decreasing sea-ice extent, affects seasonal cycles for energy metabolism (e.g. fasting) by increasing the polar bears' intake or catabolism of adipose tissue resulting in elevated circulating concentrations of bioavailable pollutants in the blood, particularly males (Sonne *et al.*, 2017). Indeed, recent observations shows increases of certain pollutant concentrations in polar bears (Dietz, Desforges, Gustavson*, et al.*, 2018; Lippold *et al.*, 2019). Such high exposure poses an increased risk to neonatal cubs during sensitive developmental periods (Sonne *et al.*, 2017).

Future studies should include larger polar bear cohorts, including adult males and females, to confirm a significant correlation between POPs exposure and adipose tissue gene expression. In contrast to females, male bears do not have physiological mechanisms to eliminate POPs, therefore they potentially carry some of the highest POPs concentrations in their adipose tissue (Sonne *et al.*, 2017).

5.6 Science communication

It is essential to communicate the relevance of our scientific ideas and discoveries towards the general and medical non-scientist public, and to make data understandable and accessible. Besides, publishing in open access journals and presenting data on (inter) national conferences, we should incorporate novel communication approaches including, social media, websites, blogs or video channels showcasing data in layman's language, film and illustrations. 24 Hours of science is a great initiative to inform the public of what we do and to spread awareness about sperm's sensitivity towards environmental pollution, particularly POP exposure. We only participated once, ideally this should be done on a yearly basis. Science communication may inform decision making, including political and ethical thinking. Perhaps most importantly, regular science communication counters misinformation and misconceptions which often clutter public debate e.g. the news.

Conclusion

In conclusion, the present thesis confirms that *in utero* paternal POPs exposure perturbs the sperm epigenome and affects embryonic development of subsequent generations in an inter- (F1, F2), sometimes, transgenerational (F3, F4) manner via the paternal germline. An early life nutritional intervention of FA, however, partly counteracts the POP's induced sperm epimutations and associated multigenerational phenotypes. Particularly, FA supplementation by itself, led to some debatable results that require further investigation to get a better understanding of the underlying mechanisms and molecular interactions. The results, of this thesis, emphasize the impact of a father's environment on the health and development of his children, great grandchildren and beyond. Lastly, environmental POPs exposure poses an ongoing threat to the Arctic, particularly the ones on top of the food chain like Indigenous people and polar bears. Here, we demonstrate an association between increasing POPs exposure and the adipose tissue gene expression in mother polar bears and her cubs.

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