Identification of Responsible Replacement Plasticizers for Di(2-ethylhexyl) phthalate for Polyvinyl Chloride Based Applications

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Abstract

Phthalates are chemicals that are added during the manufacturing of polyvinyl chloride based plastics to improve its physical characteristics. They are high production volume chemicals that have raised concerns due to their endocrine disrupting properties and deleterious effects on testicular function. This thesis aims to identify functional replacements for phthalates that have a small environmental footprint, are functionally equivalent to di(2-ethylhexyl) phthalate (DEHP), and are better alternatives for the environment and human health. Several alternatives have been proposed, but have yet to be proven better than the chemicals they are meant to replace. Herein, we assess the safety of the commercial plasticizers DEHP and 1,2-Cyclohexane dicarboxylic acid diisononyl ester (DINCH), and new non-commercial plasticizers based on dibenzoate, succinate, fumarate, and maleate functional groups.

Our first aim, following a strategy proposed in *Toxicity Testing in the 21st Century: A Vision and Strategy*, consisted of a high-throughput cell viability assay to screen over 20 chemicals for toxicity in three immortalized Sertoli cell lines. The Sertoli cell was selected for its role in mediating phthalate toxicity, and coordination of early testicular differentiation and development. Based on results from the cell viability screen, mono-(2-ethylhexyl) phthalate (MEHP; the main bioactive metabolite of DEHP), DINCH, dioctyl maleate (DOM) (as a positive control), and two potential alternative plasticizers (1,4 butanediol dibenzoate (BDB) and dioctyl succinate (DOS)) were selected for a toxicogenomic analysis in the TM4 immortalized Sertoli cell line. While treatment with DEHP, DINCH, and DOM caused changes in gene expression, no significant changes were observed in the BDB or DOS treatment groups.

Using the rodent model, a 28-day acute toxicity study with BDB and DOS was designed with common OECD recommended endpoints to screen for systemic toxicity. There were no effects on organ histology and weight, serum analytes, and hematology following treatment. As gestational and neonatal exposure is a time of increased susceptibility to phthalate exposure, this acute exposure study was followed by a gestational-lactational exposure study including DEHP, DINCH, BDB, and DOS. Treatment with DEHP affected markers of endocrine disruption (male anogenital index and female vaginal opening), caused abnormal testicular histology (multinucleated gonocytes and testicular hemorrhage), and caused previously undescribed effects on dam heart weight. Treatment with DINCH was associated with an increase in hemorrhagic

testes. Interestingly, treatment with BDB and DOS did not have any deleterious effects on animal health.

This thesis is the first to present alternatives for DEHP that are not only safe, but also functionally equivalent for use in the manufacturing of PVC plastics.

Résumé

Les phtalates sont des produits chimiques produits en grande quantité. Ils sont ajoutés lors de la fabrication des plastiques à base de polychlorure de vinyle (PVC) pour améliorer leurs propriétés. Depuis plusieurs décennies, la communauté scientifique s'inquiète de leur capacité à perturber le système endocrinien et causer des effets nocifs sur la fonction testiculaire. Cette thèse vise à identifier des substituts pour les phtalates : des molécules ayant une faible empreinte écologique, fonctionnellement équivalents au di-2-éthylhexyle (DEHP) et n'ayant pas d'effets négatifs sur l'environnement et la santé. Pour ce faire, nous avons évalué les plastifiants commerciaux DEHP et 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH), ainsi que des familles de nouveaux plastifiants à base de dibenzoate, succinate, fumarate, et maléate.

Pour notre premier objectif, conformément à la stratégie proposée dans *Toxicity Testing in the 21st Century: A Vision and a Strategy*, nous avons utilisé un test de viabilité cellulaire pour évaluer la toxicité de plus de 20 molécules candidates dans des cellules de Sertoli immortalisées. Ces dernières jouent un rôle clé dans la différentiation et le développement testiculaires, et constituent une cible potentielle du DEHP. A la suite de ces études, le MEHP (métabolite bioactif du DEHP), DINCH, dioctyl maleate (DOM) (comme contrôle positif) et deux alternatives potentielles (1,4 butanediol dibenzoate (BDB) et dioctyl succinate (DOS)) ont été sélectionnés pour une étude toxicogénomique dans des cellules TM4. Nous avons alors pu constater que si l'exposition au DEHP, DINCH et DOM produisait des changements dans l'expression des gènes, le BDB et le DOS présentaient des profils d'expression comparables au contrôle.

Nous avons ensuite effectué une étude de toxicité systémique du BDB et du DOS de 28 jours chez le rat avec des critères suggères par le OCDE. Nous n'avons observé aucun effet de ces deux composés sur l'histologie et le poids des organes, ainsi que sur les analytes sériques ou sanguins. Vu que la période de gestation et de lactation sont des périodes ou le fétus et le nouveau-né sont plus susceptible aux effets des phtalates, cette étude a été suivi d'une étude gestationnelle et lactationnelle visant à comparer les effets du DEHP, DINCH, BDB et DOS. L'exposition au DEHP a engendré des phénotypes classiquement décrits dans les phénomènes de perturbation endocrinienne, tels qu'une diminution de l'index anogenital et une histologie testiculaire anormale (gonocytes multi-nucléés et hémorragie testiculaire) chez les mâles, ou une

ouverture vaginale précoce chez la femelle. Chez les mères, le DEHP a produit des effets précédemment non décrits sur le poids du cœur. De façon intéressante, nous avons observé une augmentation de la fréquence des testicules hémorragiques après exposition au DINCH. Le traitement par BDB et DOS, en revanche, n'a eu aucun effet sur la santé des rats.

Cette thèse est la première à présenter des remplacements pour le DEHP qui soient fonctionnellement équivalents à ce dernier pour la fabrication de plastiques en PVC et n'ayant pas d'effets sur la santé.

Acknowledgements

I would like to start by thanking my supervisor Dr. Bernard Robaire for taking a risk many years ago by giving a young undergrad with little research experience a chance to pursue graduate studies. You have been a great mentor and have challenged my way of thinking. Through our interactions, I have learnt a lot about myself, and I am a better person for it. Along with Dr. Barbara Hales, you have both created a great environment to learn and develop as a researcher, and as a person.

I would also like to thank my family for their encouragement and support over the years. None of this would have been possible without them, and I dedicate all my future successes to them.

To my advisory committee (Martine Culty, Cindy Goodyer, and Jason Tanny), thank you for helping shape my project and making sure I stayed on track. To Claudia Lalancette and Trang Luu for teaching me various methods and protocols I needed to complete my research.

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Finally, but certainly not least, to all the wonderful people I have met on this adventure called graduate school. I can't possibly name everyone, but a few people have made a huge impact on my life, and I am proud to call them friends: Stephen you are my life-long partner in crime; Shar for taking me under your wing and showing naïve Tom the ways of the world; Rory and Jace for the stories we've shared and the adventures we've had; David for teaching me everything I will ever need to know about a well-tailored suit and for 3AM oven-roasted ribs; Ryan for making sure I stayed on track with writing, listening to my rants, and for being someone I could turn to at any time of day for help; Sophie-Anne for being the first friendly face I met in graduate school; Anne Marie, Serena, and France for accepting me for the weird person I am; Heather for the daily memes; and Océane for translating my abstract and being an overall great person to work.

Format of the Thesis

This thesis is a manuscript-based thesis, conforming to section I.C. of the "Guidelines for Thesis Preparation" of the Faculty of Graduate Studies and Research of McGill University. The manuscripts are presented in the order in which they were published or submitted for publication. We retain the right to include these manuscripts in this thesis according to the copyright agreements of the respective publishers (PLoS One, Toxicological Sciences, Nature Scientific Reports) provided that this thesis is not published commercially or used for commercial purposes.

Chapter 1 starts with a brief background on male reproductive physiology, abnormalities of male reproductive function, and the influence of the environmental factors in mediating these effects. It then focuses on a class of chemicals (phthalates) that have been described to affect male reproductive function. In the last section, we explore the regulations relating to new chemicals and the new frameworks used to screen and identify deleterious chemicals. Chapter 2 is a published manuscript that uses an *in vitro* and toxicogenomic approach to identify safe chemicals. Chapter 3 is an *in vivo* acute toxicity study. Data from this study were published as part of a conceptual paper. Chapter 4 is a gestational-lactational exposure study that has been published. Chapter 5 is a general discussion of the thesis. References for the introduction and discussion are found at the end of the thesis. Chapters 2-4 have their own reference sections. Connecting text has been included for cohesion.

Contribution of the Authors

Chapter 2: Toxicogenomic Screening of Replacements for di(2-ethylhexyl) phthalate (DEHP) Using the Immortalized TM4 Sertoli Cell Line

(Published: PLoS One; October 7th 2015)

The doctoral candidate did all the experimental manipulations, data analysis, and wrote the original draft of the manuscript. Thomas Nardelli, Hanno Erythropel, and Bernard Robaire all reviewed the final manuscript. The chemicals were synthesized by Hanno Erythropel. Bernard Robaire conceived and supervised the experiments and is the principle investigator of the study.

Chapter 3: Assessment of the Safety of Two Replacement Plasticizers for di(2-ethylhexyl) phthalate (DEHP) Using a Repeated Dose 28 Day Acute Toxicity Study in Adult Male Rats.

(Some aspects of this chapter are featured in a conceptual manuscript published in Toxicol Sci. August 2nd 2017).

The doctoral candidate designed the experiment, did animal manipulations (gavage, necropsy, sample preparation), analyzed the data, and wrote the original draft of the manuscript. Bernard Robaire conceived, supervised, and was the principal investigator of this study.

This study was also made possible with the technical help of Claudia Lalancette, Sheila Ernest, Trang Luu, and Océane Albert who assisted in rodent necropsies. The Histology Core at the Goodman Cancer Research Centre prepared histological sections that were analyzed and interpreted by Marilène Paquet. Barbara Hales provided guidance in the experimental design.

Chapter 4: In Utero and Lactational Exposure Study in Rats to Identify Replacements for Di(2-ethylhexyl) Phthalate

(Published: Nature Scientific Reports; June 20th 2017)

The doctoral candidate was involved in the design and conception of experiments, did the animal manipulations, analyzed data, maintained a data repository, and prepared the original draft of the manuscript. Océane Albert was involved in the design and conception of experiments, did the animal manipulations, analyzed data, and prepared the original draft of the manuscript. Claudia Lalancette was involved in the design and conception of experiments and did animal

manipulations. Martine Culty designed the ex vivo testicular culture experiments. Barbara Hales contributed towards the design and conception of the experiments. Bernard Robaire designed, conceived and supervised the experiments, and is the principle investigator of these studies. All authors contributed towards the final manuscript.

This study was also made possible with the technical help of Annie Boisvert (ex vivo culture), Sheila Ernest and Élise Kolasa (animal necropsies), and Qudsia Saadat (gonocytes counting in PND3 testes sections).

In all cases, the doctoral candidate has obtained permission from all co-authors to include these manuscripts in this thesis.

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List of Abbreviations

17β-HSD	17β-Hydroxysteroid dehydrogenase
AGD	Anogenital distance
AGI	Anogenital index
AMH	Anti-mullerian hormone
AR	Androgen receptor
BBP	Benzyl butyl phthalate
BDB	1,4 Butanediol dibenzoate
cAMP	Cyclic adenosine monophosphate
CSL	Cranial suspensory ligament
DBP	Dibutyl phthalate
DEHP	Di(2-ethylhexyl) phthalate
Dhh	Desert hedgehog
DHT	Dihydrotestosterone
DINCH	1,2-Cyclohexane dicarboxylic acid diisononyl ester
DOM	Dioctyl maleate
DOS	Dioctyl succinate
FasL	Fas ligand
Fgf9	Fibroblast growth factor 9
FSH	Follicle-stimulating hormone
GCNA1	Germ cell nuclear antigen 1
GD	Gestational day
GDNF	Glial cell-derived neurotrophic factor
GnRH	Gonadotropin-releasing hormone
IL1	Interleukin-1
INSL3	Insulin-like 3
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
MEHP	Mono-(2-ethylhexyl) phthalate
OECD	Organisation for Economic Co-operation and Development
Pdg2	Prostaglandin D2
PGC	Primordial Germ Cell
PKA	Protein kinase A
PLA2	Phospholipases A2
PND	Postnatal day
PP	Peroxisome proliferator
PPAR	Peroxisome proliferator-activated receptors
PPRE	PPAR response elements
PTCH1	Protein patched homolog 1
PVC	Polyvinyl chloride
REACH	Registration, Evaluation, Authorisation, and Restriction of Chemicals
RXR	Retinoid X receptor
SF1	Splicing factor 1
SOX9	SRY-Box 9
SRY	Sex-determining region Y
StAR	Steroidogenic acute regulatory protein
sTNF-α	Soluble tumour necrosis factor α
TNF	Tumor necrosis factor

Chapter 1: Introduction

1. Testicular Function and Morphology

The male reproductive system is comprised of several glands and organs that are crucial for sexual development and reproductive function (**Figure 1**). The testes are of particular importance as they produce male gametes (spermatozoa) and steroid hormones. The testes are a paired organ suspended in the scrotum by the spermatic cord, but the cremaster muscle and tunica dartos will determine their position by raising or lowering the testes to regulate their temperature for proper spermatogenesis to occur[1]. The testes are surrounded by the tunica albuginea, a fibrous sheath of connective tissue, and the visceral and parietal layers of the tunica vaginalis[1] **(Figure 2).**

In humans, the tunica albuginea forms invaginations called septae, which divide the testis into lobules containing the seminiferous tubules **(Figure 3)**. The seminiferous tubules are long convoluted tubules that are surrounded by myoid cells. These tubules contain the Sertoli and male germ cells. The interstitial space between the tubules contains immune and Leydig cells. The tubules are separated from the interstitial space by the basal lamina[1]. While the organization of rodent testes differs slightly, the cellular components and their relative organization are identical. These components are described in the following subsections.

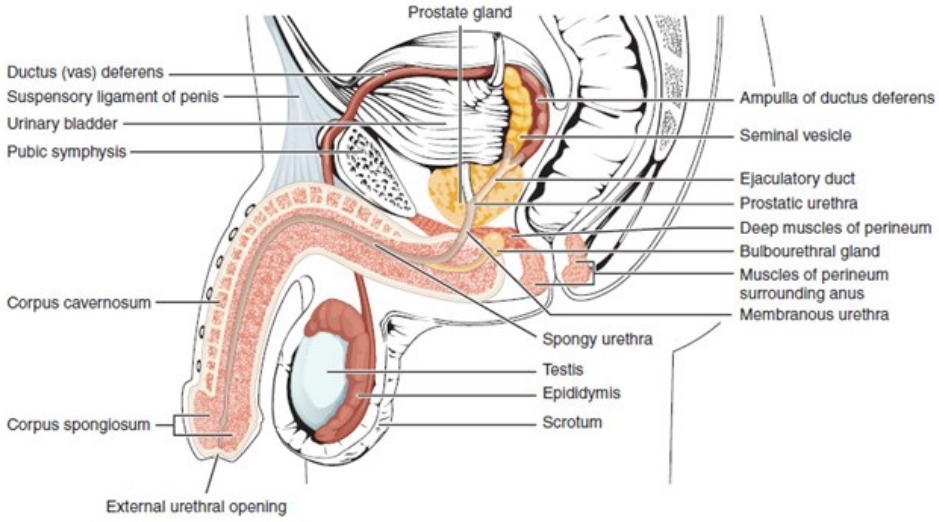

Figure 1: The Male Reproductive System. *The male reproductive system comprises multiple glands and organs that are necessary for normal reproductive function. The testes produce spermatozoa that transit through the efferent ducts and are stored in the epididymis. It is here that spermatozoa gain motility and become capable of fertilization. Upon ejaculation, the sperm travel through the ductus deferens and are mixed with secretions from the seminal vesicles, prostate, and bulbourethral gland before being ejaculated. From: OpenStax, Anatomy & Physiology. Licensed under Creative Commons Attribution License 4.0.*

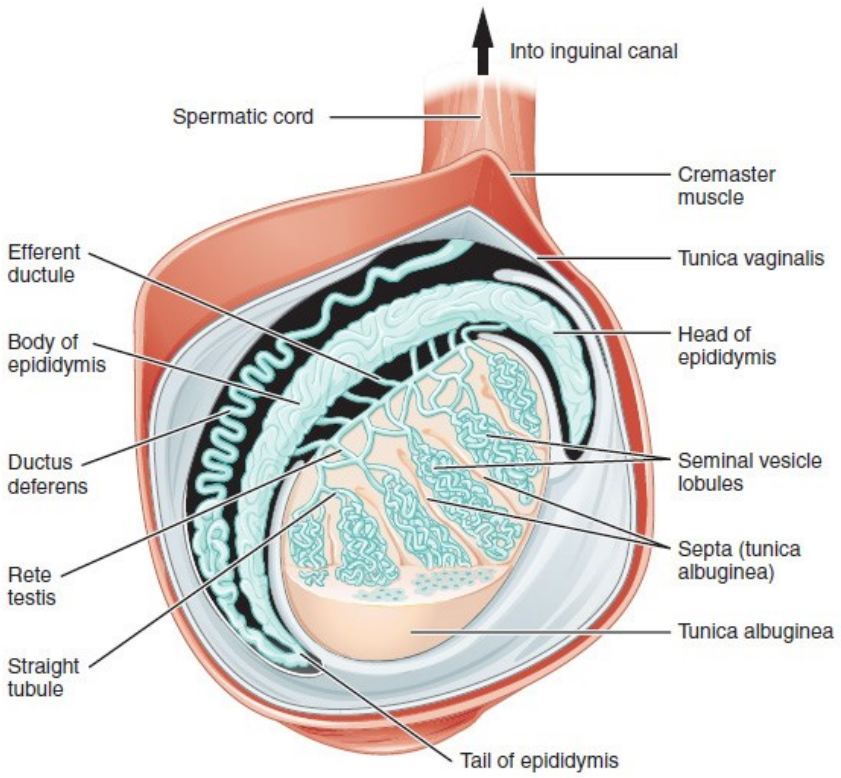

Figure 2: Anatomy of the Human Testis. *The seminiferous tubules are long convoluted tubules found within the testes. These tubules anastomose in the rete testes and connect to the epididymis where sperm are stored. From: OpenStax, Anatomy & Physiology. Licensed under Creative Commons Attribution License 4.0.*

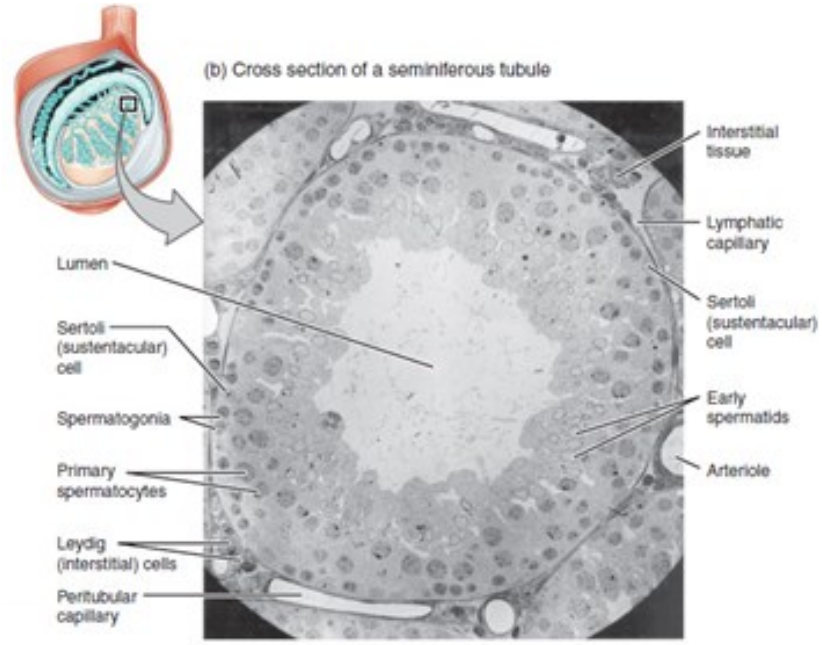

Figure 3: Organization of the Seminiferous Tubules. *The epithelium of the seminiferous tubules contains Sertoli cells and the developing germ cells. The Sertoli cell supports the developing germ cells and secretes fluid that collects within the lumen of the tubules. Outside of these tubules are myoid, immune, and Leydig cells along with blood and lymphatic vessels. From: OpenStax, Anatomy & Physiology. Licensed under Creative Commons Attribution License 4.0.*

1.1. Leydig Cells

In rodents, there are two functionally and morphologically distinct populations of Leydig cells[2]. Fetal Leydig cells differentiate from mesenchymal fibroblasts shortly after Sertoli cell differentiation at around gestational day (GD) 12.5 in mice[3]. These cells form random clusters within the interstitial space and are surrounded by a basement membrane formed by spindle-shaped fibroblasts. By GD16, these cells have some steroidogenic capacity and begin to produce androstenedione. There cells also resemble steroid producing cells with extensive smooth endoplasmic reticulum, tubulovesicular mitochondria, and contain small lipid droplets in their cytoplasm. Unlike adult Leydig cells, fetal Leydig cells do not express 17β-HSD, therefore they rely on Sertoli cells to convert androstenedione to testosterone[4].

While fetal Leydig cells have an important role in masculinization of the early embryo, adult Leydig cells replace fetal Leydig cells as the main steroidogenic cell type of the testes. Adult Leydig cells are different in terms of their ultrastructure, capacity for androgen synthesis, and mechanisms of regulation[2]. They can first be observed in rat testes at PND10-13[5], but are only fully mature after PND56. Unlike fetal Leydig cells, they depend on gonadotropin stimulation for normal differentiation and proliferation[6]. While the majority of fetal Leydig cells atrophy in the adult testes, some persist for unknown reasons[7]. Similar to fetal Leydig cells, the main role of the adult Leydig cell is to produce steroid hormones (testosterone) to support spermatogenesis and androgen dependent tissues throughout the body (reviewed in [8]).

1.2. Myoid Cells

Myoid cells surround the seminiferous tubules and provide structural support, generate peristaltic contractions of the seminiferous tubules, and secrete important paracrine signalling factors[9]. Myoid cells are thought to have an important role in the colonization and maintenance of spermatogonial stem cells within the stem cell niche by secreting GDNF upon activation of the androgen receptor[10]; as animals with cell-specific knockout of the androgen receptor experience a progressive loss of spermatogonia[11]. They also secrete a number of extracellular matrix components and growth factors that can alter Sertoli and Leydig cell function[12,13], which in turn also contributes to the spermatogonial stem cell niche.

1.3. Immunogenic Cells of the Testes

The immune cells of the testes provide protection from infections, establish immune tolerance from self-antigens generated by haploid germ cells, and interact with other testicular cell types. Regulatory T-cells are important for the establishment of tolerance to male gametes[14]. Spermatocytes and spermatids are immunogenic, therefore without the physical barriers provided by Sertoli cells, and active immunomodulatory processes mediated by regulatory T-cells and Sertoli cells, the host would mount an inflammatory autoimmune response against these cells resulting in infertility[15].

Immune cells also influence the steroidogenic function of Leydig cells. Testicular macrophages are found in close proximity to Leydig cells and form physical contacts with them[16]. In non-inflammatory conditions, the absence of testicular macrophages impairs the development and function of Leydig cells. In pro-inflammatory conditions, testicular macrophages secrete cytokines including IL-1 and TNF that act as transcriptional repressors of steroidogenic enzymes. They also generate reactive oxygen species such as hydrogen peroxide that inhibit StAR protein expression. These secretory products created by testicular macrophages in inflammatory conditions ultimately impairs Leydig cell testosterone production (reviewed in [17]).

1.4. Sertoli Cells

The Sertoli cells are essential for testicular differentiation during development (reviewed in [18] and discussed further in Section 2), and spermatogenesis following puberty (reviewed in [19]). They are the predominant cell type within the seminiferous tubules until the first wave of spermatogenesis when germ cells outnumber them. In humans, Sertoli cells undergo rapid mitotic divisions during the fetal/neonatal period and again during the peripubertal window[20] that will ultimately determine final Sertoli cell number and spermatogenic output[21]. This rapid expansion is under the control of many factors including FSH[22] and thyroid hormone[23] which stimulate cellular proliferation or regulate the window of proliferative capacity respectively.

Around the time of puberty, Sertoli cells undergo a functional maturation process whereby marked changes in their gene expression and functional roles take place[24]. They stop proliferating and begin to form tight junctions between adjacent cells. This forms an immune

privileged site for the developing meiotic germ cells[25]. The Sertoli cells also express immunomodulatory factors to further protect the developing germ cells[15].

The dynamic nature of the tight junctional complexes is essential for germ cell migration into the immune-privileged site and requires androgen signalling[26]. As spermatogonia differentiate towards spermatocytes with each spermatogenic cycle, there is complete dissolution and reformation of these tight junctions around the syncytium of germ cells[25]. While it is not understood how androgens regulate tight junctional remodelling, Sertoli cell androgen receptor knockout animals have a modest decrease in the gene expression of many tight junctional complex members[26].

The tight-junctions formed by Sertoli cells limit the diffusion of substances into the adluminal space of the seminiferous tubules[27]. For this reason, the germ cells are dependent on Sertoli cells for nutrients, regulatory factors, and to transduce signals that are required for their development[19]. Sertoli cells also provide structural support to germ cells by forming junctions that facilitate their movement towards the lumen of the seminiferous tubules, and controls their release during spermiation[19]. For these reasons, the Sertoli cell has been dubbed the “nurse” cells of the testes **(Figure 4).**

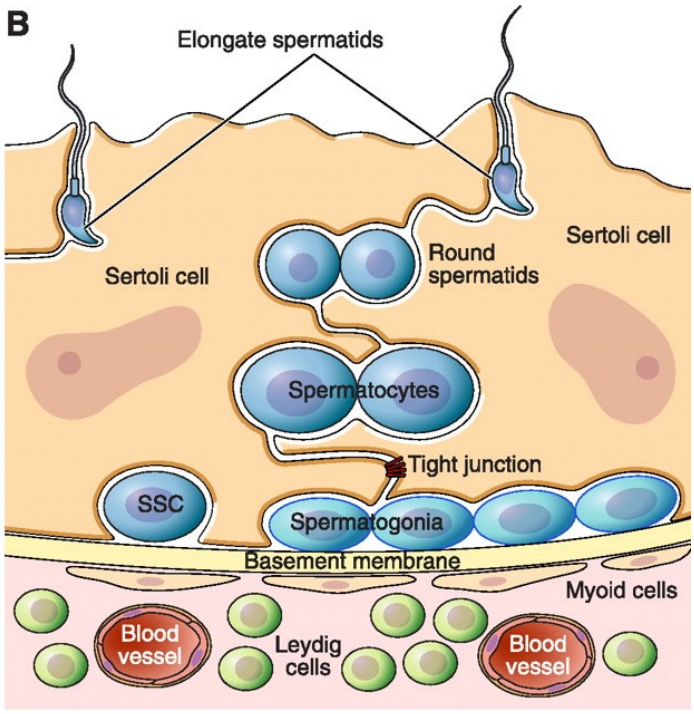

Figure 4: Sertoli cells as "nurse" cells. *The schematic shows a portion of a seminiferous tubule surrounded by the basement membrane. Spermatogonial stem cells and spermatogonia are found along the basement membrane of the seminiferous tubules in close contact with Sertoli cells. Once spermatogonia begin the process of spermatocytogenesis, leptotene spermatocytes start expressing immunogenic factors. The Sertoli cell "protects" these cells by expressing immunoregulatory factors and limiting access to these cells by forming tight junctions. The dynamic cytoskeleton of the Sertoli cells is constantly remodelled to accommodate new spermatocytes during the process of spermatogenesis. From* [28].

1.5. Spermatogenic Cells

1.5.1. Gonocytes and Spermatogonial Stem Cells

During fetal development, primordial germ cells (PGC's) migrate to the gonadal ridge[29] where they undergo several rounds of proliferation and differentiation. Once they colonize the developing fetal testes, PGC's stop expressing alkaline phosphatase[30], gain expression of GCNA1[31], and are found in the central portion of the newly formed testicular cords. It is at this time that PGC's are referred to as gonocytes[32]. Gonocytes (or prespermatogonia) are a transient population of cells that will migrate to the basement membrane of the seminiferous tubules and will contribute towards either the life-long population of spermatogonial stem cells, or differentiate directly into spermatogonia that will support the first wave of spermatogenesis[33].

In mice, spermatogonial stem cells make up only 0.02-0.03% of all testicular cells, and can only be identified by functional transplantation assays[34,35]. Despite their small number, their ability to self-renew and amplify through multiple rounds of mitotic proliferation ensures a constant production of gametes for the lifetime of an individual. Not much is known about the mechanisms regulating the lineage commitment of spermatogonial stem cells, but interactions with other germ cells, somatic cells, and growth factors within the stem cell niche are likely to have an important role[28,36].

1.5.2. Spermatogenesis

In the rodent, A_{single} spermatogonial stem cells eventually proceed through the spermatogenic lineage by producing A_{paired} and $A_{aligned}$ spermatogonia **(Figure 5)**. A_{paired} spermatogonia are connected by cytoplasmic bridges due to incomplete cytokinesis. While still considered "undifferentiated", these cells undergo successive rounds of mitosis with incomplete cytokinesis, and are known as transit amplifying progenitors that will give rise to $A_{aligned}$ spermatogonia. $A_{aligned}$ can have as many as 16 cells connected by cytoplasmic bridges[1]. Under the influence of retinoic acid, these cells will enter the pool of differentiated spermatogonia (A1 in rodents)[37].

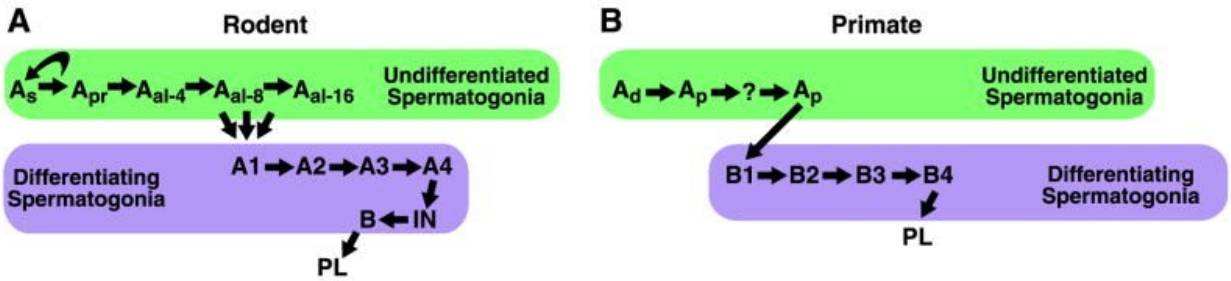

Figure 5: Lineage Commitment in Undifferentiated Spermatogonial Stem Cells. *An inidentified population of spermatogonial stem cells progresses towards differentiated spermatogonia. In rodents, these cells remain connected by cytoplastic bridges and can appear as 2,4,8, or 16 cells are are named* A_{paired}, $A_{aligned-4}$, $A_{aligned-8}$, *or* $A_{aligned-16}$ *respectively. These cells eventually become differentiating spermatogonia and undergo a fixed number of mitotic divisions. While the cell appearance of undifferentiated spermatogonia in humans is slightly different, the concept remains the same. From* [28].

As A1 spermatogonia progress to B spermatogonia, they undergo a fixed number of mitotic divisions before entering meiosis and initiate the process of spermacytogenesis. Meiosis generates haploid male gametes known as spermatids. Spermatids must undergo a final maturation process known as spermiogenesis before they are released from the seminiferous tubules as spermatozoa[1].

1.5.3. Spermiogenesis

Spermiogenesis is a differentiation process where a spermatid gains the capacity to become a functional spermatozoon. During this process, a spermatid elongates and sheds the majority of its cytoplasm as a residual body, develops specialized components such as a flagellum and acrosome, and undergoes a reorganization and compaction of nuclear chromatin whereby most histones are replaced by transition proteins and subsequently protamine.

1.5.4. The Hormonal Control of Spermatogenesis

The function of Leydig and Sertoli cells, are controlled by several endocrine feedback mechanisms that are regulated at the level of the hypothalamus, anterior pituitary, and testes (***Figure* 6**). Neurons in the hypothalamus are master regulators of the axis as they integrate signals that dictate the synthesis of gonadotropin-releasing hormone (GnRH) and its pulsatile

release. These neurons release GnRH in the portal circulation of the pituitary where GnRH will signal to gonadotropes in the anterior pituitary to produce and secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These two glycoproteins travel through the systemic circulation to the testes where they will act on Sertoli or Leydig cells respectively[38].

In response to FSH, immature Sertoli cells in the neonatal testes proliferate to establish the resident Sertoli cell population[22]. In adult animals, Sertoli cells are the only cells that express the FSH receptor in the testes, and its expression varies with the stage of the seminiferous tubule[39]. While the necessity of FSH in spermatogenesis is debated, it controls the expression of genes in Sertoli cells that have supporting roles in spermatogenesis (reviewed in [40]), and is required for quantitatively normal spermatogenesis to occur in humans and non-human primates[41]. The Sertoli cells also produce activin and inhibin, which signal back to the anterior pituitary in a positive or negative feedback manner respectively, to regulate FSH expression[42].

Testosterone is produced from Leydig cells in response to LH stimulation[43]. While testosterone is important for spermatogenesis, germ cells do not express the androgen receptor, and therefore rely on Sertoli, Leydig, and peritubular myoid cells to mediate the actions of testosterone[40,44]. Intra-testicular testosterone concentration is much higher than serum levels[45]. Despite lower levels in serum, testosterone and dihydrotestosterone have important roles outside the testes in the development of secondary male sex characteristics, and for its anabolic effects on skeletal muscle[46] and bone[47]. Serum testosterone also acts at the level of the anterior pituitary and hypothalamus to downregulate the axis thereby keeping serum testosterone within a normal physiological range[48].

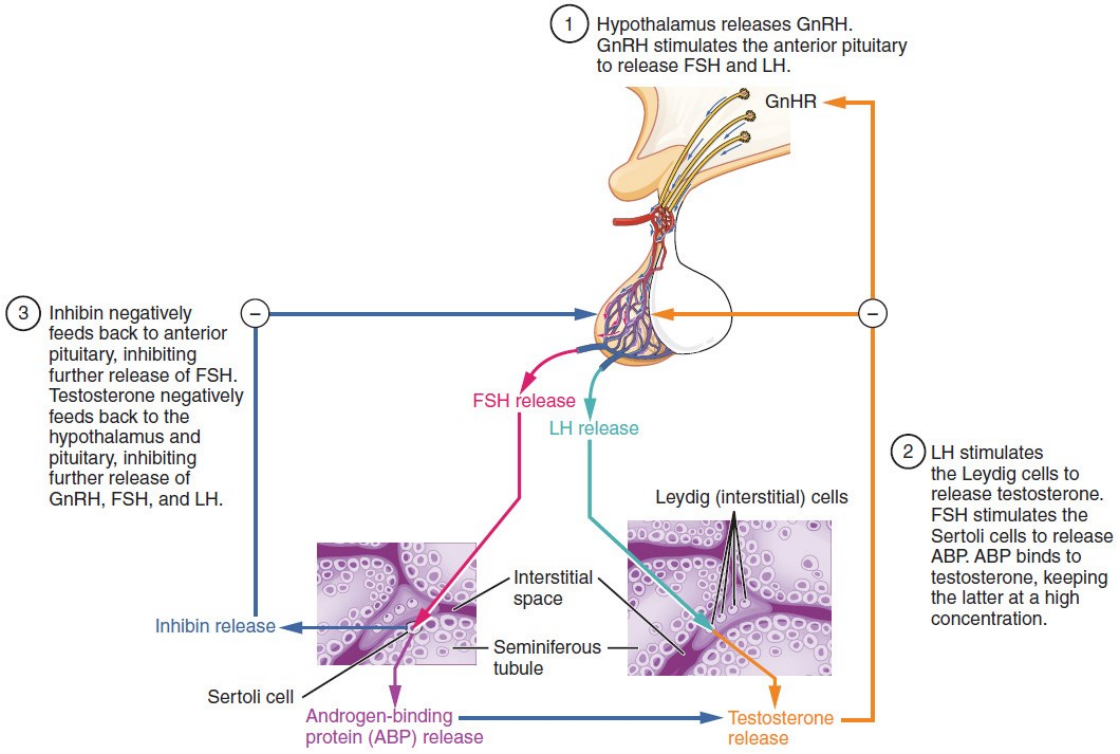

Figure 6: Endocrine Control of Spermatogenesis. *The hypothalamic-pituitary-gonadal axis is under tight control of several signalling factors that ensure the normal function of the axis. Hypothalamic neurons integrate environmental and physiological cues and release GnRH in response. GnRH acts on gonadotropes in the anterior pituitary to produce FSH and LH. These gonadotropins have important roles in Sertoli and Leydig cell function. From:*[38]

1.5.4.1. The Importance of Androgens in Germ Cell Development

Animal studies with androgen receptor knock-out in Sertoli cells report the presence of a compromised blood-testis barrier[49], failure of spermiogenesis[50], and the retention and phagocytosis of germ cells by Sertoli cells[51]. Functional assays suggest androgen receptor (AR) expression by germ cells is not required[52], but rather the Sertoli cell is responsible for mediating androgen signalling for normal spermatogenesis. This dependence on androgens appears to be stage specific as expression of the AR is cyclical and highly expressed during stages VI-VII[53] of spermatogenesis, which corresponds to when perturbations in androgen signalling have the greatest effect on reproductive function.

2. Establishment of the Gonads and Sexual Differentiation

Sexual determination and differentiation are important aspects of fetal development that require a series of tightly regulated and coordinated events[54]. In humans, genetic sex is determined by the paternal contribution of either an X or Y chromosome to the zygote. Without the expression of male-determining factors from the short arm of the Y chromosome in eutherian mammals, the developing fetus will activate a gene transcription program that will lead to female sexual characteristics[55]. SRY, a member of the SOX family of DNA-binding proteins, is transiently expressed from pre-Sertoli cells of the bipotential gonad during fetal development, and is both necessary and sufficient for male differentiation[56]. SRY complexes with SF1 to form a transcriptional activation complex that promotes the expression of SOX9. SOX9 promotes Sertoli cell differentiation, and in addition to activating downstream transcripts including *Pdg*2[57] and *Fgf9*[58], exerts a positive feedback effect on SOX9 expression; thereby ensuring its expression throughout the entire testis[59].

The Sertoli cells also initiate a differentiation cascade for other testicular cell types. Factors downstream of SRY expression are important for the migration of precursor endothelial and myoepithelial cells into the testes from neighboring mesonephric cells[60]. Failure of these cells to migrate to the gonad can result in disorganized seminiferous tubule structure and infertility. Disorganization of the seminiferous tubules also disrupts the spermatogonial stem cell niche. At this stage of development, the Sertoli cells form contacts with germ cells and rapidly metabolize retinoic acid[61,62]. This arrests the differentiation of the germ cell lineage in a pre-

meiotic state, thereby promoting its survival and mitotic capacity for the amplification of germ cell numbers at the onset of spermatogenesis. Sertoli cells also influence fetal Leydig cell differentiation[63,64] and impaired signalling between these cells can result in decreased androgen synthesis and external feminization of a male fetus[65]. Dhh is expressed by Sertoli cells in the early gonad, and its receptor, PTCH1, is expressed in the interstitial space[64]. Dhh knockout in mice leads to a feminized phenotype, restricted spermatogenesis, loss of adult Leydig cells, and impaired formation of the basal lamina of the seminiferous tubules in adult animals[63]. Impairment of fetal Leydig cell development also affects Sertoli cells due to extensive developmental cross-talk between these two cell types within the testes[7,66].

Both the Sertoli and Leydig cells are important in the development of secondary reproductive organs. Sertoli cells express AMH to promote the regression of the Müllerian duct; a structure that forms the fallopian tubes, uterus, cervix, and superior portion of the vagina in female animals. Fetal Leydig cells produce androgen precursors that masculinize the fetus and promote the development of external genitalia, epididymides, and seminal vesicles in male animals.

3. Perturbations of Male Reproductive Function

Both environmental factors and genetic information will determine the phenotypic outcome of the fetus. Understanding the role of environment is particularly important when it negatively influences fetal development. The intrauterine environment established by the mother will determine fetal development and pregnancy outcome[67]. This has been recognized by the medical profession for years, and is the basis for antenatal guidelines for healthy pregnancies[68].

Failure of the male developmental program can result from genetic, environmental, or a combination of both factors[69]. While most mothers will try to create an optimal environment for their child *in utero*, some factors resulting in congenital abnormalities are unknown or unavoidable due to ubiquitous environmental contamination. The following sections describe reproductive abnormalities in males that may result from gestational exposure to environmental factors.

3.1. Cryptorchidism

Cryptorchidism is the failure of the testes to descend to a scrotal position[70]. It is one of the most common genital disorders identified at birth in male patients. The proper positioning of the testes in the scrotum keeps the testes 2-3°C cooler than core body temperature. This cooler environment is essential for spermatogenesis to occur[71]. Two ligaments direct the movement of the testes from the abdominal cavity to the scrotum. The cranial suspensory ligament (CSL) inserts at the apical aspect of the testes and the dorsal abdominal wall while the gubernaculum inserts at the basal aspect of the testes and the inguinal canal. Signalling molecules from Leydig and Sertoli cells and the genitofemoral nerve will ultimately determine the success of testicular migration to the scrotum.

This process of testicular migration is divided into two hormonally distinct steps[72]. The first relies mainly on the secretion of INSL3 from fetal Leydig cells[73]. INSL3 encourages mitotic activity and deposition of hyaluronic acid at the caudal end of the gubernaculum. This process, termed gubernacular swelling, works to secure the fetal testes in proximity of the internal inguinal ring[74]. The swelling process is potentiated by AMH and androgens produced from Sertoli and Leydig cells respectively. Androgens also cause regression of the CSL, which favours the movement of the testes in the direction of the gubernacular ligament.

At around 25 weeks of gestation in humans, the inguinoscrotal stage of testicular migration begins[75]. While contributions from multiple sources assist in testicular descent, this process is not well understood. Animal and human studies have shown androgens are important for this phase of testicular migration. Pregnant dams administered flutamide[76], an androgen receptor antagonist, and infants with androgen insensitivity syndrome[77] both have testes that fail to descent beyond the inguinal region. Androgen stimulation of the inguinoscrotal fat pad is thought to have an important role in the masculinization of the genitofemoral nerve[78]. This causes the genitofemoral nerve to release calcitonin gene related peptide from sensory neurons which is thought to act as a chemotactic regulator of gubernacular migration towards the testes[75].

Cryptorchidism must be treated by orchidoplexy because if left untreated it can result in infertility, testicular torsion, and testicular malignancy[70]. Despite surgery, these individuals still have decreased fertility and increased risk of testicular cancer in adulthood[74].

3.2. Hypospadias

Hypospadias is a defect in the formation of the penis that results in the misplacement of the urethral meatus along the ventral side of the penis, scrotum, or perineum. The occurrence of hypospadias remains unexplained in most cases, but defects in androgen production or signalling can explain a subset of cases[79,80]. Environmental influences have also been proposed to be an important contributior[80].

3.3. Testicular Cancer

Testicular cancer is the most common form of cancer in young men aged 15-34[81,82]. While the exact etiology of testicular cancer is unknown, in addition to hereditary factors, environmental factors that lead to abnormal testicular development and gonocyte division are thought to have a role in the development of testicular cancer **(**Error! Reference source not found.**)**[83–85].

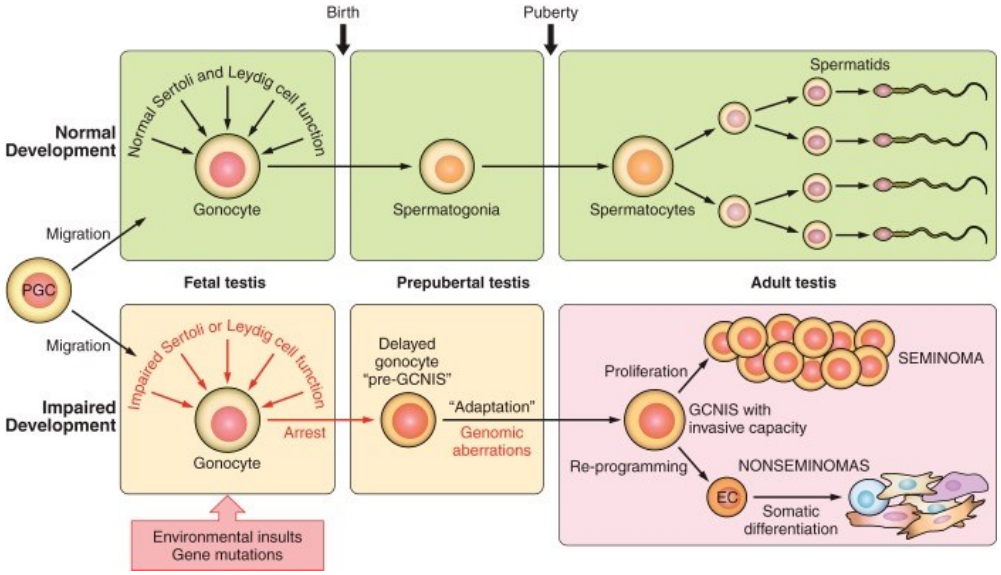

Figure 7: Proposed Hypothesis of the Origins of Testicular Cancer. *During development, gonocytes receive signals from Sertoli and Leydig cells that guide their differentiation towards spermatogonia. Abnormal signalling may cause abnormal differentiation of gonocytes leading to a maintenance of pluripotent gene expression and testicular cancer. Image from* [86].

Of all testicular cancers, those of germ cell origin account for 95% of all cases[87]. The role of atypical germs cells in the development of testicular cancer has been described from testicular

biopsies of patients who subsequently developed testicular cancer[88]. Ultrastructural and immunohistochemical investigations further support the idea that undifferentiated gonocytes may contribute the development of testicular cancer as these cells maintain characteristic features of pluripotent stem cells which are also observed in cases of carcinoma in situ[89].

Despite the relatively early onset of testicular cancer, the prognosis and survival of these patients is generally high[90]. Unfortunately, patients afflicted with testicular cancer are affected either prior to, or at peak reproductive age. Many concerns have been raised about the effects of fertility and sperm quality following chemotherapy and radiotherapy[87,91]. Infertility is a known side-effect of chemotherapy, therefore fertility preservation is important for individuals who plan to have a family[91].

3.4. Infertility

Infertility is defined as not being able to conceive a child after one year of trying without the use of contraceptives[92]. Approximately 8% of men of reproductive age seek medical attention for infertility in the United States[93] and the value is similar in Canada[94]. Male infertility is complex as there are many contributing factors and idiopathic cases. Some known factors include varicocele, cryptorchidism, gonadotoxin exposure, genetic conditions, infections, hormonal dysfunction, immunological conditions, sexual dysfunction, cancer, and systemic diseases[95].

3.5. Anogenital Distance: A Marker of Perturbed Androgen Action

Anogenital distance (AGD) and anogenital index (AGI) are sexually dimorphic measures of genital development. During male fetal development, the perineum elongates in response to local dihydrotestosterone signalling (DHT)[96]. Experimental manipulations with anti-androgenic chemicals can decrease anogenital distance/index in male rats, and is correlated with hypospadias, cryptorchidism, and decreased penile length[97].

A short anogenital distance in males is not deleterious, but rather is a marker of impaired fetal androgen action that is independent of postnatal androgen exposure[98]. This marker is particularly valuable for retrospective studies when attempting to determine fetal androgen

exposure in adults. In humans, reduced AGI is positively associated with incidences of hypospadia[99,100], cryptorchidism[100,101], and infertility[102,103].

4. Endocrine Disrupting Chemicals

Endocrine disrupting chemicals are substances that can interfere with endocrine signalling pathways that can lead to physiological effects[104]. For the purpose of this thesis, endocrine disruptors are chemicals that can mimic, block, or alter normal cellular signalling leading to unintentional or undesirable effects.

Endocrine disruptor targets include, but are not limited to, steroid (estrogen, progesterone, and androgen) and thyroid hormone signalling pathways. Some systems are more vulnerable than others due to low physiological ligand concentrations (typically in the nano- to picomolar range) and efficient amplification of receptor signal transduction in normal physiological conditions[105]. Perturbed feedback caused by inappropriate activation, inhibition, or temporal changes in signalling can have deleterious effects on human health. As described previously, establishment of phenotypic sex is a complex process involving the fidelity of spatial and temporal regulation of a variety of signalling molecules. Foreign chemicals (xenobiotics) can function as endocrine disruptors by activating, repressing, or altering the response of a receptor to its endogenous ligand leading to physiological abnormalities.

4.1. Testicular Dysgenesis Syndrome

The term "testicular dysgenesis syndrome" coined by Skakkebaek refers to a common but unknown developmental etiology that is at least partially encompasses hypospadias, cryptorchidism, testicular cancer, and male infertility. This hypothesis originated from the clinical observation that patients often have concomitant reproductive abnormalities[106,107]. For instance, individuals with testicular cancer often have a low sperm count[108], or have sperm with impaired sperm motility or morphology resulting in infertility[109,110]. Other examples include newborns with cryptorchidism who reportedly have a higher risk of infertility[111] and testicular cancer; even in the unaffected testis[112].

These abnormalities may be due to a combination of genetic susceptibility factors and environmental influences on the development of the fetal testes[69]. While the exact etiology of

testicular dysgenesis syndrome is unknown, the ubiquitous presence of environmental contaminants that affect the male differentiation program may have a role in this syndrome[69] **(Figure 8)**. The proper functioning of fetal Sertoli and Leydig cells appears to be a critical component as testicular biopsies from patients with testicular abnormalities have focal regions of disorganized seminiferous tubules, immature Sertoli cells[113], and impaired Leydig cell function[108]. Failure of these cells to differentiate into mature cell types is consistent with the development of reproductive abnormalities of the male reproductive system.

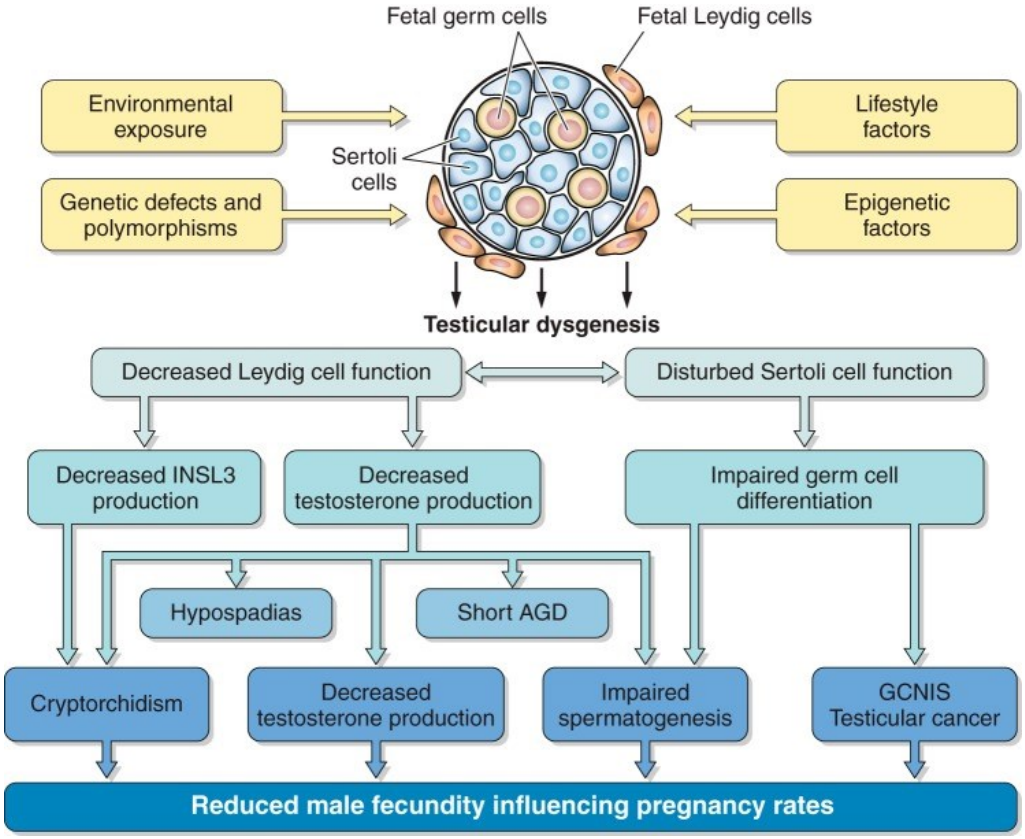

Figure 8: The Testicular Dysgenesis Syndrome Hypothesis. *According to this hypothesis, several genetic and lifestyle factors contribute to abnormal fetal differentiation of Leydig and Sertoli cells. This in turn, contributes to abnormalities of the male reproductive system observed at birth and in adulthood. Image from* [86].

One family of chemicals used in the manufacturing of polyvinyl chloride has been associated with testicular abnormalities in animal models. The following sections will discuss the

uses of polyvinyl chloride, the chemicals used in its manufacturing, human exposure to these chemicals, and the evidence for its deleterious effects.

5. Polyvinyl Chloride and Plasticizers

Polyvinyl chloride (PVC) is the third most widely produced plastic polymer due to its inexpensive cost and virtually unlimited versatility[114]. PVC polymers of high molecular weight are used for rigid plastics in pipes, conduits, and window profiles. Midrange polymers are used for flexible sheeting, flooring, wallpapers, cable coverings, hoses, tubing, and medical products. The lowest weight polymers are used for electrical plugs and items made by blow molding[115].

PVC is mixed with heat stabilizers, lubricants, plasticizers, fillers, and other additives to facilitate its processing and alter its physical and mechanical properties. Plasticizers are used to lower the glass-transition temperature of PVC; thereby disrupting the intermolecular forces between adjacent polymers allowing them to move freely[116]. They are usually a major component of the final product, as large quantities are needed to bypass the effects of anti-plasticization observed when using lower quantities of plasticizer[116]. There are many considerations to account for when selecting a plasticizer. From a manufacturing perspective, plasticizers must be compatible with a polymer, cost-effective, and depending on the end-use have good dielectric properties, and ultraviolet or thermal stability. Socio-political factors expect plasticizers to be sourced from renewable resources, biodegradable, and safe[117]. The following section, will discuss the extensive use of phthalates as plasticizers for PVC.

6. Phthalates

Waldo Semon discovered the commercial application of phthalates for PVC in the late 1920's. Since then, manufacturers have found additional uses for phthalates as solvents, emulsifiers, adhesives, and lubricants[118]. Phthalates are a diverse family of chemicals due to the many side-chain permutations that are orthogonally conjugated to phthalic anhydride. Low molecular weight alcohols are conjugated to produce solvents and emulsifiers, while high molecular weight alcohols are used to synthesize plasticizers for PVC. Because of their diverse applications, phthalates are use in the production of films, sealants, adhesives, bottle cap gaskets, upholstery, cosmetics, food packaging, inks, coated fabrics, and foam[119].

DEHP is the most commonly synthesized plasticizer, and is an industry benchmark for plasticizer characteristics. Consumption estimates suggest DEHP alone accounts for 37% of the entire plasticizer market, which in 2014 was 8 million metric tons[120]. DEHP is found in wires and cables, building and construction materials, medical devices, automotive parts, flooring, and until recently children's toys[121].

6.1. Environmental Footprint of Phthalate Plasticizers

There are several caveats associated with the use of phthalates in consumer products. One of the major problems with phthalate use is their ability to leach out of plastics over time. Phthalates are not covalently bound to PVC, which means they can contaminate the environment as a plastic degrades. PVC based products can contain 15-60% phthalates by weight, which represents a significant environmental concern in a product life-cycle[122]. Although phthalates are not environmentally persistent, and can biodegrade with the assistance of soil microorganisms and fungi in aerobic conditions in a matter of days[123], their extensive use has made them ubiquitous environmental contaminants for several decades to come[124]. Environmental surveys have detected phthalate contamination in soils[125] and rivers[126,127]. Traces of DEHP can also be found in products meant for human consumption and medical purposes[123]. While attempts to covalently bond phthalates to PVC to reduce leaching have been attempted, the end-product generally lacks flexibility and is more expensive; therefore this is not considered an effective strategy[122].

6.2. Phthalate Exposure and Assessment

Different strategies have been used to estimate human exposure to phthalates with varying degrees of success[128]. Exposure assessments can estimate phthalate exposure from surveys about lifestyle, product use, and food consumption. These studies have identified oral exposure as the most common route of DEHP exposure, representing greater than 90% of exposure in children, teenagers, and adults[118,129]. Exposure to phthalates by dermal, inhalation, and parenteral exposures is also possible. While this method highlights important routes of exposure, it is not meant to quantitatively determine body burden[130]. For this reason, human biomonitoring is commonly used. This method ignores the route of exposure, but quantitatively

determines exposure to phthalates from metabolites found in biological samples such as urine[131–133], serum[134], breast milk[134], amniotic fluid[131], and saliva[134].

One of the main limitations of biomonitoring studies is prior knowledge of the metabolite of interest. Phthalate metabolism occurs in two stages and is dependent on the size of the parent compound. The first step is the conversion of the diester into a bioactive monoester by lipases and esterases in the intestine[135]. Following this step, low molecular weight phthalates are excreted in urine primarily as a monoester. High molecular phthalates are more lipophilic and require additional hydroxylation, oxidation, and glucuronidation before they are excreted **(Figure 9)**. In either case, phthalates do not bioaccumulate, and are generally eliminated from the body within a few days[136].

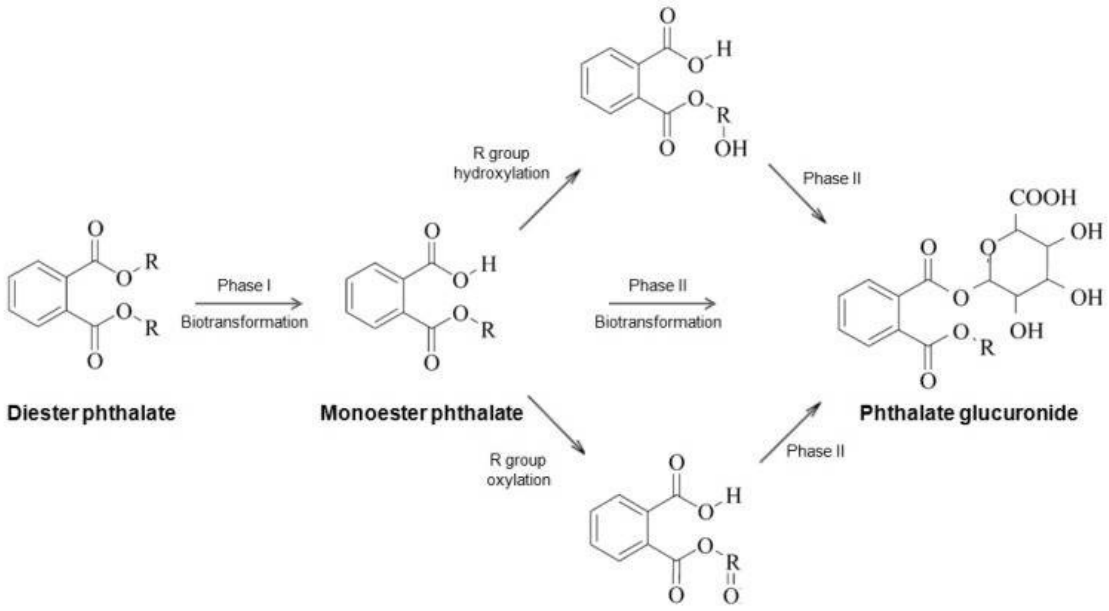

Figure 9: Metabolism of Orthogonally Conjugated Phthalates. *Phthalates are rapidly metabolized to monoesters by esterases within the gastrointestinal tract. In some cases, more hydrophobic metabolites are further metabolized by Phase II enzymes in order to facilitate their excretion. Image from [137], used under the terms of the Creative Commons Attribution Non-Commercial License.*

While phthalate metabolism is qualitatively similar among species[138], predicting the appropriate metabolite for human biomonitoring studies is not always obvious. Monoester

measurements have been routinely used for low molecular weight phthalates, but quantification of oxidized metabolites in urine is more representative of DEHP and high molecular weight phthalate exposure[128,139]. The most appropriate metabolite can also differ by biological matrix. In pregnant rats administered DEHP by oral gavage, MEHP was primarily unconjugated in amniotic fluid, but conjugated to glucuronide in maternal urine[140]. In humans, selection of the appropriate metabolite is further complicated by inter-individual differences in metabolism that vary by age, sex, race, and other demographic factors[138]. Neonates exposed to DEHP as a result of medical intervention have different phthalate urinary profiles compared to the general population[135]. This may be due to immature expression of several hepatic enzymes[141] resulting in the excretion of different metabolites at slower rates than the general population.

Humans are exposed to multiple phthalates simultaneously by direct or indirect exposure [118], but exposure is highly variable and depends on gender[142,143], age[143], and occupation[144]. Women have higher phthalate body burdens than men due to extensive use of personal care and cosmetic products containing phthalates[145]. Individuals undergoing extensive medical interventions also have higher exposures to DEHP by leaching from blood bags and medical tubing[146,147]. Of all demographics, neonates undergoing extensive hospitalization have the highest phthalate body burden[146,148] due to their small size, impaired metabolic pathways, and extensive contact with DEHP containing products[129]. Neonatal exposure to phthalates also occurs from feeding. Phthalates have been detected in breastmilk[149], infant formulae, and baby food[150]. Phthalate body burden generally decreases with age as adolescents and adults generally have lower concentrations of urinary phthalate metabolites than children below 11 years of age [151].

Since phthalate exposure is incredibly variable and in some cases higher than the reference dose of 44μg/kg/day set by Health Canada[152], identifying individuals with high exposure to this class of chemicals is critical for prophylactic intervention and changes in habits or lifestyle.

7. Evidence of Phthalate Toxicity

Xenobiotic exposure can have undesirable effects on human health, even many years following initial exposure. There are few epidemiological studies on the effects of phthalates, and those that are available have conflicting results or are limited in their conclusions. These

limitations are partially due to retrospective exposure assessment, small sample size, window of exposure, and the potential for combinatorial effects of other chemical exposures[130]. Basic, clinical, and epidemiological evidence suggests many human disorders resulting from phthalate exposure have antenatal origins, and suggest gestational and neonatal exposures are periods of increased vulnerability to these effects[69,153].

7.1. Epidemiological Studies

Recent findings suggest that phthalate exposure during gestation in humans has been correlated with impaired pulmonary function[154], immune hypersensitivity[155], asthma[156], and metabolic[157] and cognitive disorders[158–160] in children. Classically, DEHP and other phthalates have been extensively studied for their anti-androgenic properties. There is limited evidence of the effects of phthalates in humans. One study by Swan et al. measured urinary phthalate metabolites during pregnancy to calculate a "phthalate score" which was predictive of a decrease anogenital distance in boys suggesting impaired fetal androgen signalling during the male programming developmental window[161,162].

Some of the most convincing evidence for phthalate toxicity is from rodent models that replicate many features of testicular dysgenesis syndrome following exposure to DEHP and other phthalates. The following section will highlight the findings of "phthalate syndrome" in rodents following exposure to DEHP.

7.2. Animal Studies

Animal studies allow for a better mechanistic understanding of phthalate toxicity than epidemiological studies as they can control for chemical exposure, use a genetically homogeneous population, and allow for the investigation of endpoints that are not feasible in humans. While there are many potential animal models, the laboratory rat is a standard in the field of toxicology due to its small size, prolific breeding, and short life-cycle[163]. It is often used by the pharmaceutical industry for drug screening[164], and has been routinely used to study the toxicity of phthalates. Most importantly, the rat is sensitive to the endocrine disrupting effects of phthalates[165], making it an appropriate model to study the effects phthalates observed in humans.

Few studies have assessed the effects of DEHP in female rodent models, but there is evidence for estradiol suppression and anovulation at very high doses[166] that is most likely due to inhibition of aromatase expression[167]. Most studies have focused on endocrine disruption in male rats and the reproductive abnormalities that result. The following sections will discuss these findings and the similarities to testicular dysgenesis syndrome in humans.

7.2.1. Effects on Leydig Cells

While the exact mechanism of phthalate toxicity remains unknown, fetal rat models have shown that mixtures of phthalates have dose-additive, inhibitory effects on androgen activity[168]. This effect is due to a decrease in fetal testicular steroid hormone production[168,169], as complementary studies report a downregulation of steroidogenic enzyme gene expression[170]. DEHP and its metabolites do not act directly on the androgen receptor as receptor competition assays suggest they have very low affinity for the androgen receptor[171].

Testosterone is a steroid hormone produced from Leydig cells with important roles in virilisation of the fetus[172,173] and maintenance of spermatogenesis[174]. Gray et al. reported effects on newborn pups that are characteristic of androgen insufficiency following DEHP treatment. These pups had shorter anogenital distances, reduced testis weights, maintained female-like areolas, and had reproductive organ malformations[175].

Other studies have supported the Leydig cell as a target of phthalate toxicity. MEHP can inhibit LH stimulated testosterone production in Leydig cells[176] and multifocal areas of Leydig cell hyperplasia can be observed in rats[171]; the latter has been associated with testicular germ cell tumours[177].

7.2.2. Effects on Sertoli Cells

Rodent models have shown that phthalates can also disrupt Sertoli cell function, but many of these studies are limited to higher exposures. An *in vitro* co-culture experiment with neonatal Sertoli cells, gonocytes, and 0.01-1.0μM MEHP showed increased gonocyte detachment from Sertoli cells, and decreased the Sertoli cell response to FSH stimulation and proliferative activity. [178]. Another study suggests that MEHP can induce the expression of sTNF-

α from germ cells which initiates a feedforward mechanism through Sertoli cells that upregulates FasL expression, and germ cell apoptosis[179–181].

7.2.3. Effects on Germ Cells

Phthalates also have deleterious effects on gonocytes, but it is unclear whether these effects are direct or mediated by another cell type. Following exposure to DEHP during the gestational period, multinucleated gonocytes can be found in the testes of fetal rats[170]. While the formation of multinucleated gonocytes is poorly understood, a consensus suggests they represent the aberrant differentiation of gonocytes, and are often concomitant with cryptorchidism and testicular cancer[182].

7.2.4. PPAR Agonist Properties

There is a large body of evidence to suggest that phthalates are peroxisome proliferators. In an endogenous setting, peroxisome proliferator-activated receptors (PPARs) bind diverse macromolecules that resemble long-chain fatty acids. These molecules contain a hydrophobic backbone and a hydrophilic acidic group. Upon binding to their ligand, PPAR's form heterodimers with retinoid X receptor (RXR) which recruits them to DNA response elements, resulting in differential gene expression[183]. All three characterized PPAR isoforms are expressed in the testes[184,185].

Phthalate monoesters are peroxisome proliferators, and the extent of their ability to activate PPAR receptors is correlated with the potency of their detrimental effects[186]. To confirm whether phthalates are peroxisome proliferators, luciferase expressed under PPAR response elements (PPRE) was transfected into COS-1 cells. Stimulation with MEHP activated PPRE controlled luciferase expression in cells expressing PPARγ and PPARα receptors[187]. Based on multiple transactivation assays, the aliphatic side-chain of the phthalate monoester metabolite is the determinant factor of peroxisome proliferator activity. Longer aliphatic side-chains were more effective at activating the PPAR signalling response than shorter ones[186]. In order to confirm a direct interaction of MEHP with PPAR, a scintillation proximity assay was used to show MEHP, but not DEHP binds directly to PPARα/γ[188].

Signalling through PPAR receptors and a reduction in testosterone production have been described with other potent PPAR agonists[189–191]. After treatment with phthalates, PPARα knock-out mice do not have reduced testosterone production compared to their wild-type controls[190] further supporting a role of PPARα in Leydig cell steroidogenesis. These findings are contradictory to another study that showed a potent PPAR agonist (Wy-14,643) did not reduce fetal testosterone production in rats while several phthalates did[192]. There still remains much to be understood about the mechanism of phthalate toxicity as PPARα knock-out mice still develop testicular lesions when exposed to phthalates, but these observations are delayed when compared to wild-type animals[193]. Similar studies examining the role of other PPAR isoforms are not available at this time. PPARγ knockout is embryonic lethal, and a testis specific knockout does not exist. Despite these limitations, immunostaining for PPARγ in Leydig cells is reduced following DEHP exposure suggesting it may also have a role in mediating phthalate toxicity[170].

PPAR agonists also perturb Sertoli cell function. Studies using immunolocalization techniques show both PPARα[184,194] and PPARγ[184] are translocated to the nucleus following treatment with PPAR agonists. Because PPAR signalling requires RXR, PPAR activation sequesters RXR to the nucleus. RXR forms heterodimers with many nuclear receptors including RAR before translocating to the nucleus[195]. These studies also show abnormal RARα localization following PP stimulation[194]. RARα and RARγ knockout mice lose cyclical gene expression in Sertoli cells, related to the spermatogenic wave and have testicular degeneration[196]. This suggests that impairment of RAR signalling by PP, including phthalates, could have deleterious effects on the testes. Despite these findings, Sertoli cell RXR knock-out animals did not recapitulate any of the effects of RAR knockouts[196].

8. Regulatory Decisions

8.1. Restrictions on the use of Phthalates

The ubiquitous use of, and unavoidable exposure to phthalates has been a concern to regulatory agencies for many years. The European Union was the first to a implement temporary restriction on the use of phthalates on December 7th, 1999[197]. This decision restricted the use of seven phthalates (di-isononyl phthalate (DINP), di(2-ethylhexyl) phthalate (DEHP), di-n-octyl phthalate (DNOP), di-iso-decyl phthalate (DIDP), butyl benzyl phthalate (BBP), dibutyl

phthalate (DBP)) in soft toys and childcare products made from PVC intended to be placed in the mouth of children less than three years of age.

The European Chemicals Agency considers some phthalates, including DEHP, substances of very high concern, and therefore requires manufactures to obtain authorization to use them in some products[198]. European restrictions on phthalates are now under the jurisdiction of Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH). Under Annex XIV of these regulations, DEHP, DBP, and BBP are subject to authorization for use, but products that are imported containing these phthalates do not require authorization unless they are listed in Annex XVII that sets limits on phthalates in childcare articles. Recently, these restrictions have been expanded to include flooring, coated fabrics and paper, recreational gear and equipment, mattresses, footwear, office supplies and equipment, and other articles moulded or coated with plastic, that could result in exposure from dermal contact or inhalation[199].

Both the United States and Canada have similar policies regarding the use of phthalates in children's toys. Phthalates are part of the Environmental Protection Agency's action plan under the Toxic Substances Control Act and in some cases are banned from children's products under the Consumer Product Safety Improvement Act of 2008[200]. In Canada, some phthalates are listed in the Chemical Management Plan, and this listing has recently been expanded to account for "the potential for cumulative risk from combined exposure to [multiple phthalates]"[201]. Similar to the United States, the Canadian Consumer Product Safety Act bans the use some phthalates in children's toys[202]. A final assessment from Health Canada is scheduled for early 2018[201].

8.2. The Challenges of Implementing the Precautionary Principle

The precautionary principle aims to prevent harm from unknown risks, and is used in regulatory policy when there is concern over the slow pace of research on a topic compared to the potential risks[203]. It can be divided into four components: taking preventive action; shifting the burden of proof to the proponents of an activity; exploring alternatives; and increasing public participation in decision-making[204]. Many groups representing the chemical industry argue regulatory agencies are misguided in their application of the precautionary principle[205,206].

The validity of regulatory policy concerning phthalates is not a point of discussion for the purposes of this thesis, but opponents to this theory and its application raise important concerns that should be addressed. By restricting the use of phthalates, companies may turn to chemicals with unproven safety[207] or may be even more deleterious than those currently in use. Replacements may also be more expensive or potentially less adaptable, therefore would require some type of sacrifice on behalf of a society who is accustomed to a certain standard. Restricting or banning chemicals without suitable alternatives is not always a viable plan and therefore we should have "responsible replacements" available should phthalates be banned from the consumer domain.

8.3. Challenges Facing Regulatory Agencies

Part of the reason for the necessity of the precautionary principle is due to several impediments regulatory agencies face while executing their mandate in a timely manner. Data gaps in the literature are among the largest problems facing regulators; and there is a lack of incentive on the chemical industry to generate these data[208]. Another issue is the incredible size of the chemical universe and the laborious and costly animal testing required for a critical evaluation of a chemical[209]. In light of these challenges, regulatory agencies have had to develop strategies to utilize limited resources in the best possible way to make decisions with the largest socioeconomic impact.

8.3.1. Prioritizing Chemicals

To deal with the many data gaps of the chemical universe, regulatory agencies have used different strategies to prioritize chemicals.

8.3.2. Predictions Based on Structural Similarities

Structural similarities and computational toxicology are useful for predicting toxicity where there are data gaps in the literature, and are the basis of the "read-across" approach. These concepts rely on the idea that chemicals with structural similarities will have similar properties on human health[210]. Functional groups, core molecules, the number of carbons atoms, and incremental carbon-chain length are all important structural features. Structural analysis is

limited in that it requires some form of judgement and is not based on empirical data for a given chemical. Despite these limitations, it still has value in prioritizing chemicals for future studies.

8.3.3. Toxicology in the 21st Century

The National Research Council (NRC) of the US National Academy of Science has proposed a plan that will help regulators be more effective in executing their mandates. In a report titled *Toxicity Testing in the 21st Century: A Vision and a Strategy,* the NRC presents a long-term plan that utilizes advances in high-throughput assays and novel *in vitro* approaches for toxicity characterization and prediction[211]. This plan aims to reduce the number of animals, cost, and time of chemical testing while increasing the mechanistic understanding of chemical toxicity.

The foundation of this concept relies on the idea that the potential for harm can be inferred from how chemicals interact with effector molecules within a cell to trigger a response. By shifting away from whole animal testing, this non-hypothesis based method of screening relies on *a priori* characterization of many cellular processes to make predictions about toxicity based on targeted effector molecules. This concept has been used by the EPA as part of their ToxCast program. ToxCast has data from over 9,000 chemicals that have been screened through 1,000 high-throughput assays[212]. The data generated from this program has been used as a part of the EPA's Endocrine Disruption Screening Program to rank and prioritize chemicals for further study based on biological activity instead of production volume and potential exposure[212].

While data from these high-throughput assays have been useful in guiding policy, they have not replaced standardized animal testing for toxicity screening. Part of the reason is institutional inertia, the complexity of modeling toxicokinetics using an *in vitro* or *in silico* model, the loss of cell-cell interactions *in vitro*, and the uncertainty of data extrapolation from alternative models[213,214].

9. Replacement Plasticizers

While the use of phthalates is slowly being restricted, there has been an extensive amount of work to find replacements for these chemicals. A 2007 market survey identified several alternative plasticizers including diisononyl cyclohexane-1,2-dicarboxylate, di(2-ethylhexyl)

terephthalate, citrates, dibenzoates, and polyadipates used to manufacture children's toys[215]. One bourgeoning field of research is the development of plasticizers derived from renewable sources[216]. Bioplasticizers, like Dow Chemical Ecolibrium™ or Danisco's Grindsted Soft-n-Safe™, are derived from sustainable sources[217,218]. Marketing materials from both companies suggest they are safe and have little to no environmental footprint. While specialty plasticizers are promising replacements for specific applications, they can be subject to proprietary formulations, and premium prices that are not cost competitive with phthalates[219]. The following sections will discuss a popular commercial alternative (DINCH), and other alternatives that were recently developed, are inexpensive, and have potential to replace DEHP in PVC based plastics.

9.1. Di(isononyl)cyclohexane-1,2-dicarboxylate (DINCH)

DINCH is a recently developed plasticizer marketed for sensitive "close-contact" human applications including enteral and hemodialysis tubing, bags, respiratory tubes, catheters, gloves, and breathing masks where DEHP and other phthalates are normally used[220]. The production of this plasticizer has increased from 25,000 tons in 2002 to 100,000 tons in 2007[221].

Despite very promising statements, there is a lack of peer-reviewed studies about the safety of DINCH. Information from internal documents provided by BASF in the SCENIHR report[222] mention DINCH has a slower rate of leaching than DEHP, suggesting it could result in less environmental contamination and inadvertent human exposure. These documents also mention physiological effects including an increase in liver, testicular, and thyroid gland weight after a 90-day exposure study; in some cases at doses as low as 100mg/kg. Effects on liver and thyroid were observed in a two-generation study in both the F0 and F1 generation. While these reports do not mention any reproductive abnormalities, both male and female pups had reduced AGI. Of the peer-reviewed literature available, one study reports effects on adipocyte differentiation[223] and another reports biphasic effects on steroidogenesis in fetal and MA-10 immortalized Leydig cells[224]. Together, these findings suggest DINCH may not be as innocuous as previously thought and emphasizes the need for further investigation.

9.2. Novel Plasticizers

A novel series of plasticizers based on maleic anhydride, which resembles phthalic acid, along with several structural isomers derived from fumaric and succinic anhydride are potential,

non-commercial, alternative plasticizers[225]. Studies suggest two families in particular (succinate and maleate based plasticizers) have excellent plasticizer properties, and that succinates readily biodegrade into metabolites that can be used as a biological source of carbon[225,226]. Furthermore, succinate plasticizers can be sourced from by-products of fermentation, thereby decreasing our dependence on fossil fuels and the associated environmental impact[216]. Dioctyl succinate also has decreased leaching from PVC compared to DEHP[227] which, similar to DINCH, could result in less environmental contamination and inadvertent human exposure.

Another promising family of plasticizers are the dibenzoates, that have been used commercially for over 40 years[228]. Blends of diethylene glycol dibenzoate and dipropylene glycol dibenzoate are good plasticizers for PVC[229], but are resistant to biodegradation as they form persistent, toxic, monobenzoate metabolites due to the presence of a central ether function[230,231]. An alternative dibenzoate plasticizer without an ether function, 1,5 pentanediol dibenzoate, was shown to readily biodegrade and has comparable plasticizing properties to DEHP[232]. Similar biodegradation studies with 1,6 hexanediol dibenzoate resulted in similar findings[230]. Furthermore, 1,6 hexanediol dibenzoate did not leach as readily as DEHP in aqueous environments[227].

10. Regulatory Requirements for New Chemicals

While alternative plasticizers have promising physical characteristics, they are new chemicals and are therefore subject to legislative measures meant to assess the risks associated with their use and the potential for detrimental effects on human health and the environment.

10.1. Introducing New Chemicals to the Canadian Marketplace

Regulatory agencies have an important role in determining chemical policy, as they must make decisions that protect vulnerable segments of the population while not hindering development and innovation. In order to accomplish this task, a risk assessment of relevant data is necessary for the critical evaluation of new chemicals. The Domestic Substances List, maintained by Environment Canada, is the sole basis for determining whether a chemical is new or not in Canada. This list includes approximately 23,000 chemicals manufactured, imported, or used in Canada from January 1st, 1984 to December 31st 1986[233].

In Canada, new chemicals with annual usage of more than 100kg are subject to critical evaluation under the Canadian Environmental Protection Act[234]. Under the New Substances Notification Regulations, manufacturers must fulfill certain requirements for a chemical dossier depending on production volume. At the lowest tier, these requirements can be as limited as chemical name, trade name, CAS registry number, and anticipated uses. The highest tier (greater than 10,000 kg/year) requires a more comprehensive package including detailed physical properties of a chemical, aquatic toxicity, skin sensitization tests, a 28-day acute toxicity test, mutagenicity data, and other data relevant to human health and exposure[234].

Chemicals can also enter the Canadian marketplace if they have been previously approved for use in the United States for five years. The Non Domestic Substances List, also maintained by Environment Canada, includes more than 58,000 entries from the Environmental Protection Agency's Toxic Substances Control Act. These chemicals are subject to less stringent requirements when being introduced to the Canadian marketplace[235].

References for this section are found at the end of the thesis.

Formulation of the Project

Phthalates have been designated as chemicals of high concern by regulatory agencies due to their widespread exposure and potential effects on human health. Despite these concerns, phthalate based chemicals still comprise a large portion of the market as alternatives have been either too expensive or are limited in application. Furthermore, the effects on human health following exposure to alternative plasticizers has not been studied in-depth. Our goal was to screen a series of alternative plasticizers in a high-throughput context to identify lead candidates for more thorough investigation using classical rodent models. This would allow for the identification of "responsible replacements" for DEHP and other phthalates found in products made from PVC.

Within the context of a team grant to assess the potential toxicity of replacement plasticizers on reproductive function, immortalized Leydig, Sertoli, germ, and prostate cell lines were used. As discussed above, the Sertoli cell plays an important role in development by orchestrating signals between the many cell types of the developing testis and in maintaining the germ cell niche. Using a surrogate marker for cell viability, the MTT assay identified chemicals with overt toxicity resulting in cell death. From these data, succinate and dibenzoate plasticizers had the least effect on cell viability. Two candidates dioctyl succinate and 1,4 butanediol dibenzoate were selected for further screening based on a combination of desirable biological and physical properties. Using a system biology approach, changes in gene expression were assessed by microarray to assess the adaptive response, and whether plasticizer exposure targeted pathways that were relevant to the normal physiology of the cell.

The second and third aims of this project involved screening for toxicity using classical rodent models. The first was a sub-acute 28-day toxicity study and the second was a gestational exposure study. A Sprague-Dawley rat model was selected in both cases as this animal model is most prevalent in reproductive toxicology. Doses were selected to be representative of the rodent equivalent of high human exposure that can occur in neonates requiring extensive hospitalization (15-30mg/kg), and that of the lowest dose in the literature previously reported to have observable effects following exposure to DEHP (300mg/kg). In both cases, the health of the animals was

assessed by observational, developmental, and terminal endpoints (with an emphasis on reproductive and endocrine effects).

Conceptual developments in the field of toxicology have changed the framework of safety assessment and chemical screening. In addition to classical rodent models, an *in vitro* and toxicogenomic approach was used to screen for unwanted toxicity. By fulfilling these aims, this thesis aims to identify whether alternatives for phthalate plasticizers are a viable option, or whether they will be another case of "regrettable replacements" with similar deleterious effects.

DEHP and other phthalate plasticizers are slowly being phased out of products because of regulations aimed to protect humans from their potential deleterious effects on the reproductive system. Several candidates have been proposed, but their safety has not been assessed. Within the context of a team grant, several immortalized cell lines representative of the male reproductive system (Leydig cells, spermatogonia, prostate) were screened for toxicity using in vitro models. This first chapter describes the work done in three immortalized Sertoli cell lines.

Chapter 2: Toxicogenomic Screening of Replacements for di(2-ethylhexyl) phthalate (DEHP) Using the Immortalized TM4 Sertoli Cell Line

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The doctoral candidate did all the experimental manipulations, data analysis, and wrote the original draft of the manuscript. Thomas Nardelli, Hanno Erythropel, and Bernard Robaire all reviewed the final manuscript. The chemicals were synthesized by Hanno Erythropel. Bernard Robaire conceived and supervised the experiments and is the principle investigator of the study.

Abstract

Phthalate plasticizers, including di(2-ethylhexyl) phthalate (DEHP), are being phased out of many consumer products because of their endocrine disrupting properties and ubiquitous presence in the environment. The concerns raised by the use of phthalates have prompted industry to find alternatives that are safe, biodegradable, and are functionally equivalent. We examined the toxicogenomic profiles of: mono(2-ethylhexyl) phthalate (MEHP, the bioactive metabolite of DEHP); the commercial plasticizer diisononyl cyclohexane-1,2-dicarboxylate (DINCH); and three recently proposed plasticizers: 1,4-butanediol dibenzoate (BDB), dioctyl succinate (DOS), and dioctyl maleate (DOM) in the immortalized TM4 Sertoli cell line. Results of gene expression studies revealed that DOS and BDB clustered with control samples while MEHP, DINCH and DOM were distributed further away from the control-DOS-BDB cluster, as determined by principle component analysis. While no significant changes in gene expression were found after treatment with BDB or DOS, treatment with MEHP, DINCH, or DOM resulted in many differentially expressed genes. MEHP upregulated genes downstream of PPARs, without modulating the expression of PPAR's themselves. MEHP also upregulated enzymes involved in cholesterol biosynthesis. DOM upregulated genes involved in the glutathione stress response, DNA repair, and cholesterol biosynthesis pathways. Treatment with DINCH resulted in altered expression of a large number of genes involved in major signal transduction pathways including ERK/MAPK and Rho signalling. These data suggest DOS and BDB may be safer alternatives to DEHP/MEHP than DOM or the commercial alternative DINCH.

Introduction

Plasticizers are compounds that are added to brittle polymers, such as polyvinyl chloride (PVC), to increase their flexibility and malleability. It is estimated that in 2006, total plasticizer production totalled 5.8 million metric tons, of which phthalates made up 75% of production[1]. Di-(2-ethylhexyl) phthalate (DEHP) is the most commonly used phthalate for plasticizing PVC. It is able to plasticize PVC because it contains both polar moieties that ensure compatibility with the polymer, and non-polar moieties that are able to disrupt the polar interactions between adjacent PVC polymer chains[2]. During manufacturing, it is common for PVC products to contain up to 40% plasticizer (such as DEHP) by weight, but the final amount depends on the desired physical properties of a plastic[3]. One caveat of using DEHP is that it does not form covalent bonds with PVC; therefore, plasticizers can leach out over time into the environment, ultimately resulting in human exposure[4, 5].

Although human exposure to DEHP is mainly due to leaching from PVC, it and other lower molecular weight phthalates can also be found in cosmetics where they are used as emulsifiers and solvents[6]. As a result of ubiquitous exposure, phthalates and their metabolites are readily found in urine, breast milk, and serum[7]. Recent restrictions on the use of six phthalates in selected products have been implemented to reduce the phthalate burden of neonates[8,9], as children who would chew phthalate containing plastics while teething or those who require extensive perinatal care had a phthalate burden as high as one to two orders of magnitude greater than adults[6,10,11]. Combined with a smaller body mass and impaired metabolic pathways, neonates are one of the most vulnerable demographics to the effects of phthalates[12, 13].

Phthalates have well documented anti-androgenic effects[14], but the mechanism by which they exert these effects is still not fully understood. Pathways involving members of the peroxisome proliferator-activated receptor (PPAR) family have been identified as potential mediators of phthalate toxicity. MEHP can activate several PPAR isoforms[15] and PPAR-alpha null Sv/129 mice have a milder phenotype than wild-type following treatment with DEHP[16]. In addition to direct pertubation of PPAR signalling, PPAR dysregulation can alter other nuclear receptor signalling pathways, such as the retinoic acid and thyroid hormone signalling, by sequestering endogenous heterodimer binding partners or by biasing heterodimer formation[17, 18].

Nuclear receptor signalling via these and other nuclear pathways is important for proper gonadal development and spermatogenesis[19, 20]. It has been proposed that phthalate exposure may in part be responsible for “testicular dysgenesis syndrome"; an umbrella term for clinical presentations of cryptorchidism, hypospadia, testicular cancer, and decreased sperm production that are believed to be caused by a common developmental etiology[21].

Phthalate reproductive toxicity is complex as multiple cell types have been proposed as targets[22]. The Sertoli cell is considered to be a mediator of phthalate toxicity as it has a critical role in gonadal sex-determination, testicular development, and spermatogenesis[23]. Several strains of immortalized Sertoli cells have been derived for *in vitro* use to simplify complex biological systems involving multiple cell types to pinpoint cell specific mediated toxicity. The 15P-1 Sertoli cell line is derived from transgenic adult mice expressing the large T protein of polyoma virus[24]. 15P-1 maintains the expression of Wilms’ tumor and Steel genes and can support meiotic differentiation of germ cells[24]. The MSC-1 cell line is derived from adult mice using small and large T-antigens from the SV40 virus. While it does not express the follicle stimulating hormone receptor, it maintains characteristic expression of transferrin, clusterin, and inhibin βb[25]. The TM4 Sertoli cell line is derived from 11-13 day old mice and resembles immature Sertoli cells. It is well characterized, has not been transformed, is not tumorigenic, and maintains many important aspects of Sertoli cell physiology. It is a particularly good model for endocrine disruption studies as it maintains the ability to respond to FSH stimulation, and expresses both androgen and estrogen receptors[26–28].

High-throughput *in vitro* and *in silico* methods combined with cell culture methods have provided the tools necessary to screen and identify deleterious compounds in an effective and cost-efficient manner[29]. Previous *in vitro* studies have shown MEHP can decrease pyruvate and ATP production while increasing reserves of intracellular lipids in Sertoli cells[23]. Phthalate monoesters can also disrupt Sertoli-germ cell cross-talk and promote germ cell apoptosis via FASL/FAS signalling pathway by increasing MMP2 activity and downstream cleavage of TNFα[30,31]. Furthermore, phthalates can cause precocious release of germ cells into the lumen of the seminiferous tubules by disrupting ectopic specialization formation and other cytoskeletal components of Sertoli cells involved in germ cell transit[32].

Several plasticizers have been proposed as possible replacements for DEHP, but there is a lack of data regarding their safety. **Table 4** provides a list of several alternatives, and **Figure 10** shows the compounds subsequently chosen for this study. Diisononyl cyclohexane-1,2-dicarboxylate (DINCH) is a commercial plasticizer marketed for applications involving close human contact[33]; however, there are very few peer-reviewed studies on its effects in biological systems[34]. Dioctyl succinate (DOS) and dioctyl maleate (DOM) are part of a series of proposed replacement plasticizers that maintain structural elements of phthalate plasticizers[35–37]. Second generation dibenzoate plasticizers, such as 1,4-butanediol dibenzoate (BDB), have also been proposed as alternatives to address concerns raised by commercial diethylene- and dipropylene-glycol dibenzoate plasticizers, as the latter two compounds lead to the formation of persistent toxic metabolites in the presence of common soil microorganisms[38,39].

With the decrease in cost and increase in sensitivity, microarray experiments have become effective for high-throughput xenobiotic screening[40]. By generating a toxicogenomic fingerprint, a novel compound can be compared to a database of known toxicants to predict unwanted toxicity[40, 41]. A similar toxicogenomic strategy using fetal testes from pups who were exposed to a series of phthalates during gestation successfully segregated developmentally toxic phthalates from inert ones based solely on differential gene expression[42]. Furthermore, all phthalates deemed developmentally toxic targeted genes regulating steroidogenesis, lipid and cholesterol homeostasis, and other important developmental pathways[42]. Structure-function analysis and read-across can also help identify families of compounds that share common mechanisms of toxicity[43]. Together, these strategies can be used to predict toxicity, prioritize screening, reduce development costs, and minimize the use of animals in toxicity screening of novel plasticizers before their commercial use[44, 45].

In this study, the toxicity of MEHP, the bioactive metabolite of DEHP, an alternative commercial plasticizer (DINCH), and three novel plasticizers (DOM, BDB and DOS) were assessed using cell viability and toxicogenomic methods in immortalized Sertoli cell lines.

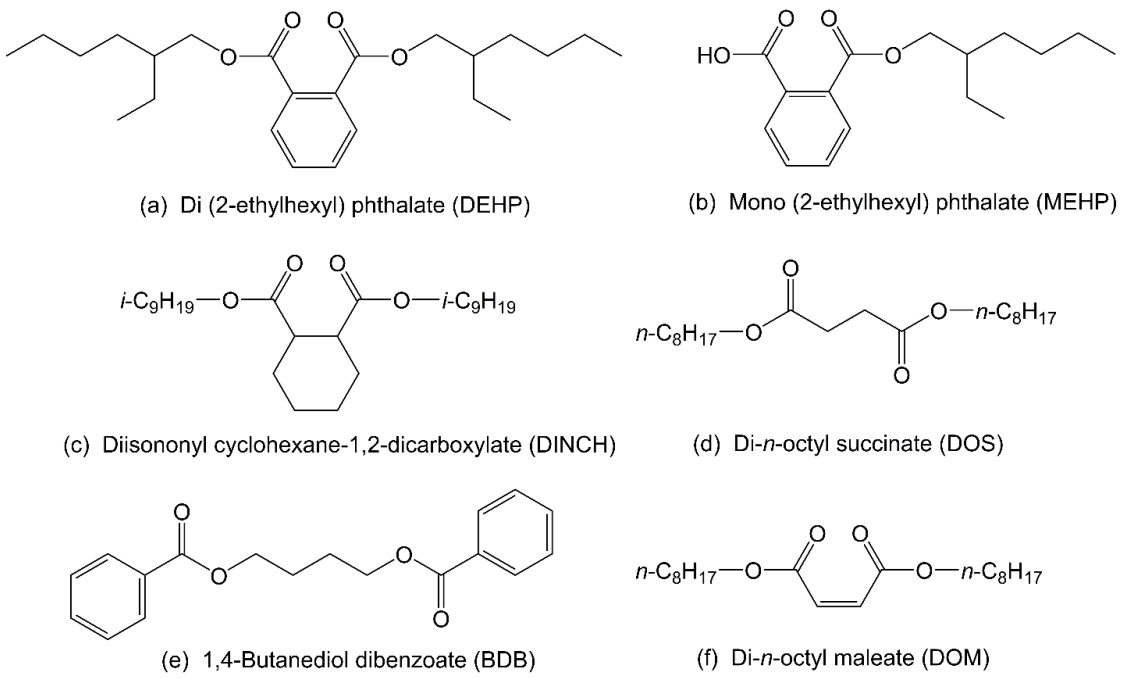


Figure 10: Chemical Structures of Commercial and Non-Commercial Plasticizers *The phthalate plasticizer DEHP (a) and its main bioactive metabolite MEHP (b), a current commercial replacement DINCH (c), and three alternative plasticizers: DOS (d), BDB (e), and DOM (f).*

Materials & Methods

Source of Chemicals

DEHP was purchased from Sigma-Aldrich Corporation (Cat#80030, St. Louis, MO), MEHP was purchased from Wako Pure Chemical Industries (Cat#323-65643, Osaka, Japan), DINCH was supplied by BASF Canada (Mississauga, ON). DOM, DOS, and BDB were synthesized as previously described[35–37]. In addition to these compounds, structural analogues shown in **Supplemental Table 4** were also tested for their effects on cell viability in three immortalized cell lines. The maleates, succinates, fumarates were chosen due to their structural similarity to DEHP[35–37] while the dibenzoates are structurally similar to diethylene glycol dibenzoate plasticizers[38, 46]. These compounds were either synthesized in-house or purchased as indicated.

Cell Cultures

MSC-1 (donated by Dr Robert Viger, Centre hospitalier universitaire de Québec, Charlesbourg, QC, cell line originally derived and characterized in 25) and TM4 (CRL1715, ATCC, Manassas, VA) cells were cultured at 37°C with 5% CO_2 in either DMEM supplemented with 10% FBS, and 0.5% Penicillin-Streptomycin (P/S) or DMEM:F12 (ATCC) supplemented with 2.5% FBS, 5% Horse Serum, and 0.5% P/S respectively. 15P-1 cells (CRL-2618, ATCC, Manassas, VA) were cultured at 32°C with 5% CO_2 in DMEM supplemented with 5% FBS, 1% sodium pyruvate, and 0.5% P/S. All cell culture reagents were purchased from Wisent (St-Bruno, QC) unless otherwise indicated.

Cell Viability Assay

The MTT assay is an indirect measurement of cell viability that measures the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a purple formazan. This conversion occurs primarily in the mitochondria of living cells. Cells were seeded in Costar 96-well plates (Corning, Tewksbury, MA) and allowed to adhere for 24hrs. The culture media was aspirated and replaced with media containing either vehicle or plasticizer at concentrations ranging from 10^{-8} to 10^{-4}M in 10 fold increments. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, Oakville, ON) was used as vehicle at 0.4% at all concentrations except 10^{-4}M where 1% DMSO was used; all values were compared to their respective vehicle control **(Supplemental Figure 16 &Figure** 17**).** After 44hrs, 50µg of MTT (Millipore, Temecula, CA) was dissolved in 1x phosphate buffered saline (PBS; 1.71M NaCl, 0.03M KCl, 0.06M $Na_2HPO_4 \cdot 2H_2O$, 0.02M KH_2PO_4) and was added to each well for an additional 4hr incubation. Following incubation, the cell culture medium was carefully aspirated and the MTT crystals were dissolved using 100µL DMSO. The optical density was measured using a SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA) spectrophotometer. The absorbance at 620nm was subtracted from 570nm to correct for background.

RNA Extraction, Quantification and Purity

TM4 cells were seeded in six-well plates containing 250,000 cells in each well and allowed to adhere for 24 hours. The culture medium was aspirated and fresh media with 10^{-4}M

treatment (DOS, BDB, MEHP, DOM, and DINCH) or vehicle (1.0% DMSO) were added and allowed to incubate for 48hrs.

Following incubation, the RNeasy Plus Mini Kit (Qiagen, Toronto, ON) was used for RNA extraction. The culture medium was aspirated and 600µL Buffer RLT supplemented with 6µL β-mercaptoethanol (Sigma-Aldrich, Oakville, ON) was added to each well. Samples were pipetted several times to mechanically disrupt the cells and stored at -80°C for future extraction. On the day of extraction, lysates were further homogenized using QIAShredder columns (Qiagen). RNA was extracted from the flow-through as per the manufacturer's instructions.

RNA purity was determined using a NanoDrop 2000 spectrophotometer (ThermoFisher, Waltham, MA) to determine 260/280 and 260/230 ratios in order to ensure samples did not contain DNA, and were free of chemical contaminants used during the extraction process that may affect downstream applications. RNA integrity, quantity, and purity were further analysed using the RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA) as per manufacturer's instructions. All samples had integrity (RIN) values > 9.8 as determined using the 2100 Bioanalyzer (Agilent Technologies).

Gene Expression Microarray

RNA was converted to cRNA and labeled with Cy3 using the Low Input Quick Amp Labeling Kit (Agilent Technologies) following the manufacture's protocol starting with 100ng of RNA. Only samples yielding more than 1.65µg and having a specific activity greater than 9.0 pmol Cy3/µg cRNA were hybridized to microarray chips. This was determined using the NanoDrop 2000c (ThermoFisher). cRNA was hybridized to SurePrint G3 Mouse GE 8x60K Microarrays (Agilent Technologies) for 17 hours at 65°C. Microarray chips were scanned using the SureScan G2600D Microarray Scanner (Agilent Technologies). Probe intensities were converted to numerical values using Feature Extraction ver. 11.5.1.1 (Agilent Technologies) software with protocol GE1_1105_Oct12 and grid 028005_D_F_20110614. Probe values were imported into GeneSpring 12.6.1 GA-PA (Agilent Technologies) and normalized using percentile shift normalization to the 75th percentile with a baseline transformation to the median of all samples. Each treatment was analysed independently of each other using 1% DMSO as a baseline sample. Every treatment had four biological replicates except DOS where one replicate

was excluded due to high background. All microarray data have been uploaded to GEO (GSE66812). PCA analysis on conditions was used to determine similarity between samples using GeneSpring 12.6.1 GA-PA (Agilent) software.

RT-PCR Validation of Microarray Data

The StepOne Plus Real-Time PCR System (Applied Biosystems, Burlington, ON) was used to determine relative quantities of mRNA to validate findings from the microarray experiment. Using the QuantiTect Primer Assay (see **Supplemental Table 5**, Qiagen) and Power SYBR Green RNA-to-CT 1-Step Kit (Applied Biosystems), 20ng of RNA was reverse transcribed with ArrayScript UP Reverse Transcriptase at 48°C for 30 minutes. This step was followed by a 10-minute incubation at 95°C to activate AmpliTaq Gold DNA Polymerase. cDNA was amplified and quantified over 40 cycles that each consisted of a denaturing step at 95°C for 15 seconds, an annealing step at 55°C for 30 seconds, and an elongation step at 72°C for 30 seconds. SYBRGreen fluorescence was quantified during the elongation step. A continuous melt curve from 60°C-95°C with 1% temperature increments was used to detect non-specific amplification to ensure accurate transcript quantification. All samples were run in triplicate with five biological replicates. *Hprt* was validated and used as a housekeeping gene to normalize starting RNA quantities. Relative expression was quantified using the ΔΔCt method. A reference sample was generated from a mixture of RNA from one biological replicate of all experimental conditions. Validated genes were selected based on relevant biological function, pathways, or fold changes found in the microarray datasets.

Pathway Analysis

Probes within the 20-100th percentile after normalization, and registered as detected in at least one of two experimental conditions (control or treatment), were kept for analysis. Significance from this reduced list of entities was determined using a moderated t-test with Benjamini-Hochberg FDR correction and an asymptotic p-value computation. Probes with a fold change greater than 1.5 relative to controls and statistically significant were exported to Ingenuity Pathway Analysis v. 21249400 Sept 2014 (Qiagen, Redwood City, CA) for further analysis.

Statistics

For cell viability assays, families of compounds were studied in independent groups of 5-6 compounds (see **Supplemental Table 4** for grouping). Each group shared a common control of either 0.4% or 1.0% DMSO. For each group two statistical analyses were done. For the highest concentration (10^{-4}M) significance was determined by one-way ANOVA (factor being compound) followed by Dunnett's correction for multiple comparisons. This group usually contained 5-6 family comparisons. For lower concentrations, two-way ANOVA (factors being compound and concentration) followed by Dunnett's multiple comparison test was used to determine significance. In this second case, a family was comprised of 20-24 comparisons. This method of analysis was selected due to the shared control DMSO sample. For qPCR experiments, one-way ANOVA corrected by Dunnett's multiple comparison test was used to determine significance. All statistical analyses were computed using GraphPad Prism 6.05 (GraphPad Software, La Jolla, CA).

Results

Cell Viability

Using the MTT assay, several families of plasticizers were studied for their effects on cell viability **(Figure 16 and Figure 17)**. Plasticizers in the succinate and dibenzoate families generally had the least effect on cell viability compared to control while maleates and fumarates greatly decreased viability in most cases. Based on both desirable plasticizing properties and biological impact on cell viability, BDB and DOS were selected as safe alternative plasticizers while DOM was selected as a positive control, i.e., a plasticizer predicted to be toxic. From the supplemental data, the candidates used for further screening are presented in **(Figure 11)**. DEHP significantly decreased viability in all three cell lines examined with the most prominent effect being a 40% decrease in the 15P-1 cell line ($p \leq 0.05$) at 10^{-4}M. DOM significantly decreased cell viability in both MSC-1 and 15P-1 cell lines at 10^{-4}M. MEHP, DBD, DOS, and DINCH treatments did not change cell viability in any Sertoli cell line tested. In order to understand the adaptive processes that take place in the absence of cell death, the TM4 cell line was selected for microarray studies.

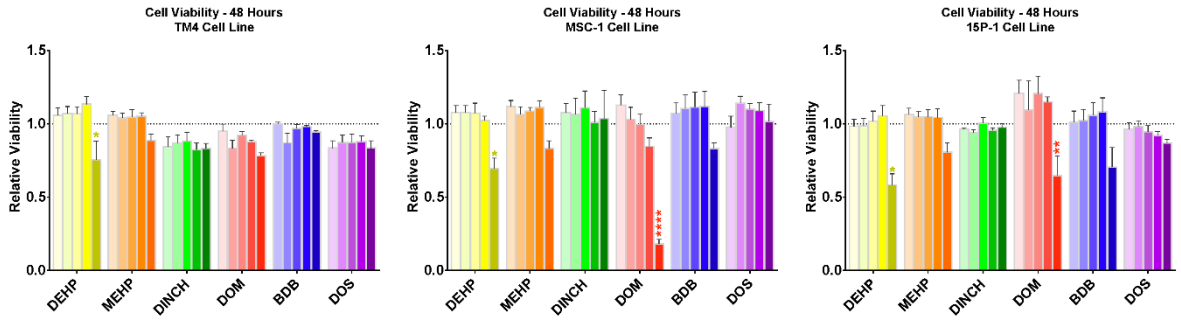


Figure 11: Cell Viability Following Treatment with Plasticizers *Values are expressed as a ratio of either 0.4% or 1.0% DMSO control (see Figures 16 and 17 in Supplemental Data.). Viability was measured by the colorimetric MTT assay using concentrations of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}M (lightest to darkest bars) in (a) TM4, (b) MSC-1, (c) 15P-1 immortalized Sertoli cell lines. * = $p \leq 0.05$; ** = $p \leq 0.01$; **** = $p \leq 0.0001$; n=3-5 plated in triplicate.*

Principle Component Analysis

In order to determine the overall gene transcript relationships in TM4 cells after treatment, a principle component analysis was done. This analysis is a mathematical algorithm that uses variation in datasets to determine principle components. Sample variance is plotted in three-dimensional space with similar treatments in close proximity **(Figure 12)**. PCA analysis determined three major components representing 37.56%, 20.82% and 15.35% of variance between all samples. BDB and DOS clustered closely to the control sample with vector magnitudes of 23,703 and 29,931 units respectively from the 1.0% DMSO sample. In comparison, MEHP (63,217), DINCH (97,601), and DOM (122,994) all clustered further away from control.

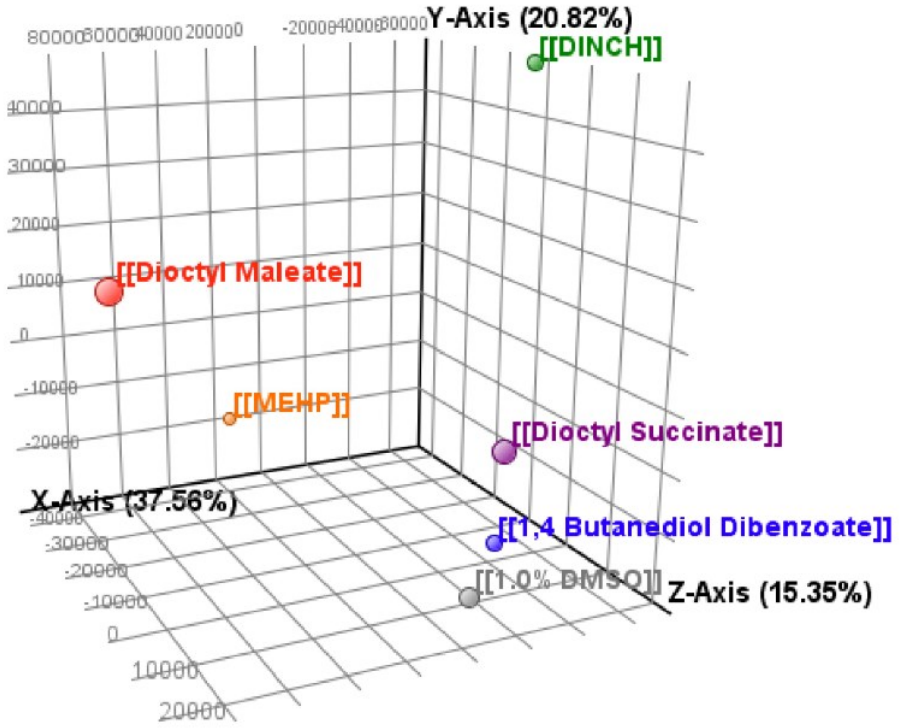


Figure 12: Principle Component Analysis of Microarray Data *TM4 Sertoli cells treated with 10^{-4}M vehicle (DMSO), MEHP, DINCH, DOM, DOS or DBD for 48hr. n=4 in all cases except DOS where n=3.*

Differential Gene Expression

DOS and BDB did not have any differentially expressed genes at 10^{-4}M after a false discovery correction was applied relative to the 1.0% DMSO control **(Figure 13)**. MEHP had relatively few genes changed, while DINCH had 1,261 uniquely mapped genes up-regulated and 753 down-regulated by 1.5 fold or greater. DOM had 2,014 differentially expressed genes with the largest overall magnitude fold-changes compared to MEHP and DINCH. Almost a third (226/648) of the genes significantly changed after DINCH treatment overlapped with those changed after treatment with DOM **(Figure 14)**.

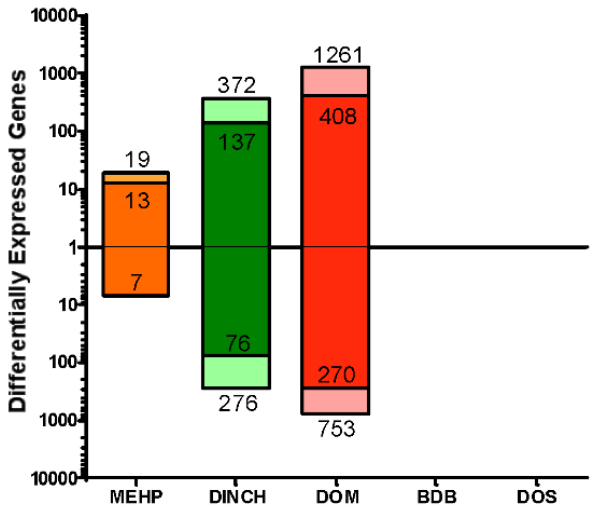
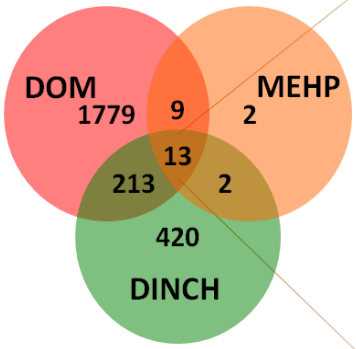


Figure 13: Differential Gene Expression across Treatment Groups: *Saturated color (inner bar) indicates unique mapped genes that were significantly changed by >2.0 fold determined by moderated t-test and Benjamini-Hochberg FDR correction (P>0.05) while lighter bars represent genes that were changed by > 1.5 fold with the same statistical criteria.*



	Fold Change vs. Control		
	MEHP	DOM	DINCH
ALDH1A1	3.6	2.5	2.5
DACH2	-6.1	-6.6	-7.3
DEPTOR	2.1	5.0	1.7
DYRK4	-3.5	-17.8	-2.7
GDAP1L1	-2.1	-2.4	-1.8
GSTO2	1.9	2.0	2.2
MAMDC2	-5.4	-6.1	-3.8
MMAB	1.7	1.8	1.6
MVD	2.4	3.6	2.1
NOS3	2.1	1.6	2.8
PMVK	2.4	2.1	2.1
SAA3	-4.1	-39.1	-2.3
SEMA3B	2.6	2.3	3.1

Figure 14: Commonalities in Gene Expression Across Treatments. *Left panel: Venn diagram of distinct and common genes between DINCH, MEHP and DOM treatment groups (10^{-4}M) with changes in expression greater than 1.5 fold. Right panel: Table showing 13 genes common to the three treatments and the fold changes in response to treatment.*

Thirteen genes were differentially regulated after MEHP, DINCH and DOM treatments. Of these shared genes, *Mvd* and *Pmvk* were validated by qPCR **(Figure 15 d,e)** because of their role in cholesterol biosynthesis. These genes are also downstream targets of SREBF2 [49], which is a transcription factor predicted to be activated by MEHP treatment in our dataset (z-score=2.0; p≤2.48e-6), and has an important role in cholesterol homeostasis. In order to further verify our model, *Pdk4* and *Angptl4* were validated **(Figure 15 a,b)** as both are downstream targets of PPAR, and Pdk4 is known to be upregulated following treatment with MEHP [50]. All four genes were significantly upregulated after treatment with both MEHP and DOM.

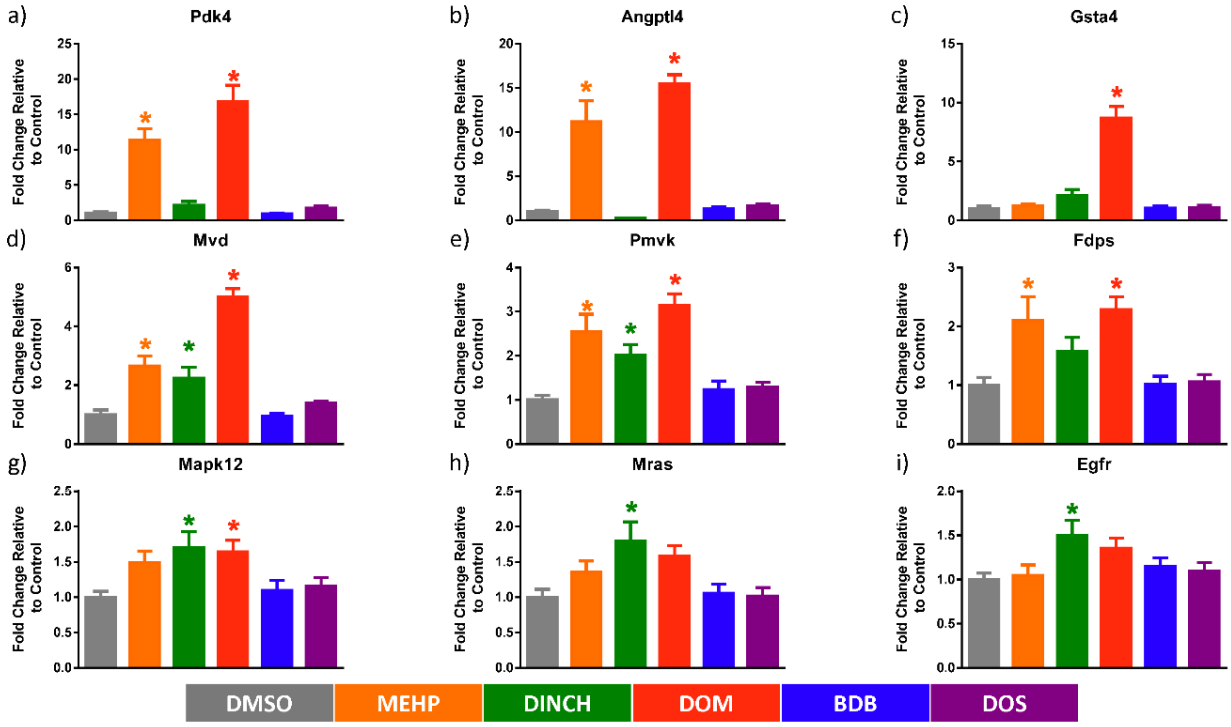


Figure 15: qPCR Validation of Selected Transcripts from Microarray Experiment. *Values are normalized to the DMSO control sample, which was set to one. Graphs have been organized by the magnitude of the scale of the y-axis. Significance was determined by one-way ANOVA corrected by Dunnett's multiple comparison test. n=4-5 biological replicates plated in triplicate. * P<0.05*

Pathway Analysis

To understand the biological relationship of differentially expressed genes, those genes that were significantly changed by 1.5 fold or more were imported into Ingenuity Pathway Analysis. The MEHP dataset was enriched for genes involved in cholesterol biosynthesis and nuclear receptor signalling pathways (**Table 1**). The DOM dataset was downregulated for genes involved in DNA replication, folate metabolism, and cell-cycle progression, and upregulated for genes involved in xenobiotic metabolism, aryl hydrocarbon signalling, glutathione mediated detoxification, oxidative stress responses, and cholesterol biosynthesis (**Table 2**). Findings were confirmed by qPCR by validating *Gsta4* and *Fdps* (**Fig. 15 c,f**) in addition to other targets previously mentioned. In addition to the oxidative stress and cholesterol biosynthesis pathways previously observed, treatment with DINCH generally upregulated genes involved in ERK/MAPK, Ephrin, and Rho signalling pathways (**Table 3**). Furthermore, many of these pathways were assigned z-scores above 2 by the pathway analysis software, suggesting that based on the directionality of our gene expression dataset, these pathways are predicted to be activated. These findings were confirmed by qPCR for *Mapk12*, *Mras*, and *Egfr* (**Fig. 15 g,h,i**). **Tables 1, 2, and 3** show selected pathways that were significantly enriched in our dataset and genes that were differentially regulated by 1.5 fold or greater. More details regarding the proportion of matching entities, the log (p-value), and the directionality of the response for each plasticizer can be found in the appropriate tables.

Table 1: Pathway Analysis of Genes Differentially Expressed by MEHP Treatment[1]

Pathway	-log(p-value)	Ratio	z-score	Upregulated	Down-regulated
PXR/RXR Activation	2.46	2/59 (0.04)	N/A	*Aldh1a1, Cpt1a*	
RAR Activation	2.69	3/172 (0.02)	N/A	*Aldh1a1, Tgfb2*	*Dhrs3*
Aryl Hydrocarbon Receptor Signaling	3.04	3/130 (0.03)	N/A	*Aldh1a1, Tgfb2, Gsto2*	
Superpathway of Cholesterol Biosynthesis	9.43	5/27 (0.19)	N/A	*Mvd, Nsdhl, Pmvk, Acat2, Lss*	

Table 2: Pathway Analysis of Genes Differentially Expressed by DOM Treatment[1]

Pathway	-log(p-value)	Ratio	z-score	Upregulated	Down-regulated
PXR/RXR Activation	2.46	2/59 (0.04)	N/A	*Aldh1a1, Cpt1a*	
Mismatch Repair in Eukaryotes	1.74	5/16 (0.32)	N/A		*Pcna, Rfc4, Msh3, Fen1, Rfc5*
VDR/RXR Activation	1.95	15/78 (0.2)	-1.414	*Foxo1, Runx2, Hoxa10, Ncor1, Sema3b, Cst6, Prkd3, Prkca*	*Bglap, Spp1, Ccnc, Ccl5, Hes1, Klf4, Cxcl10,*
Cyclins and Cell Cycle Regulation	2.5	16/75 (0.22)	-2.53	*Hdac9, Abl1, Ccnb2, Hdac5, Tgfb1, Ppm1l, Tgfb3, Btrc, Gsk3b*	*Pa2g4, Suv39h1, Cdkn2b, Ccnb1, Cdkn2d, Ccne1 ,E2f2*
Xenobiotic Metabolism Signaling	2.96	40/239 (0.17)	N/A	*Aldh4a1, Ftl, Gstm5, Ugt1a6, Gsta5, Arnt, Hmox1, Aldh1a1, Gstm3, Ppm1l, Gstm4, Chst3, Chst11, Aldh3a1, Prkd3, Cited2, Aldh6a1, Gstk1, Camk2b, Prkca, Gstm1, Mgst1, Map3k6, Gstm3,*	*Ndst3 Chst7, Camk2d,Rras2, Aldh1l2, Aldh18a1*

[1] The tables list pathways deemed to be significantly enriched in each dataset according to Ingenuity Pathway Analysis. The first column is the curated name of the pathway. The second column is the –log(p-value) associated with this pathway determined by the software using a Fisher's exact test. The ratio column is the number of entities in a dataset that match the entities in the curated pathway with the value in brackets representing the ratio. The z-score is a value assigned by the software to indicate whether a pathway is predicted to be activated or repressed when sufficient data is available. The last two columns mention genes that are either up- or down-regulated in our dataset that are also members of the curated pathway.

Folate Transformations I	3.02	5/9 (0.56)	N/A	*Nqo1, Hdac5, Esd, Pik3r3, Mgst2, Cat, Aldh3b1, Map3k8, Gsto2, Mgst3, Mthfs*	*Mthfd1l, Shmt1, Mthfd1, Shmt2*
LPS/IL-1 Mediated Inhibition of RXR Function	3.13	35/197 (0.18)	-0.333	*Aldh4a1, Gstm5, Gsta5, Alas1, Abcb9, Aldh1a1, Scarb1, Gstm3, Chst3, Gstm4, Xpo1, Chst11, Cpt1c, Aldh3a1, Hmgcs1, Aldh6a1, Gstk1, Gstm1, Mgst1, Cpt1a, Gstm3, Ly96, Mgst2, Cat, Aldh3b1, Gsto2, Acox3, Mgst3*	*Ppargc1b, Ndst3, Apoe, Chst7, Aldh1l2, Aldh18a1, Acsl1*
Aryl Hydrocarbon Receptor Signaling	4.02	28/130 (0.22)	0.333	*Aldh4a1, Gstm5, Gsta5, Arnt, Ctsd, Aldh1a1, Tgfb1, Gstm3, Gstm4, Aldh3a1, Aldh6a1, Gstk1, Gstm1, Mgst1, Gstm3, Nqo1, Mgst2, Tgfb3, Aldh3b1, Gsto2, Esr1, Mgst3*	*Mcm7, Pola1, Rbl1, Ccne1, Aldh1l2, Aldh18a1*
Cell Cycle: G1/S Checkpoint Regulation	4.07	17/61 (0.28)	0.277	*Hdac9, Smad3, Abl1, Hdac5, Cdkn2d, Foxo1, Tgfb1, Tgfb3, Btrc, Gsk3b*	*Pa2g4, Suv39h1, Rbl1, Cdkn2b, Ccne1, Gnl3, E2f2*
NRF2-mediated Oxidative Stress Response	4.54	35/168 (0.21)	1.5	*Ftl, Gstm5, Gsta5, Dnajb2, Dnaja1, Hmox1, Scarb1, Gstm3, Abcc1, Gstm4, Gclm, Gsk3b, Prkd3, Gstk1, Prkca, Gstm1, Mgst1, Gstm3, Nqo1, Dnajb14, Bach1, Pik3r3, Mgst2, Cat, Dnajc14, Aox1, Gsto2, Ptplad1, Enc1, Mgst3, Ephx1*	*Pmf1, Atf4, Herpud1, Rras2,*
Cell Cycle Control of Chromosomal Replication	4.67	11/26 (0.43)	N/A		*MCM5, MCM3, ORC2, RPA3, MCM2, CDT1, CDC6, ORC6, MCM4, MCM7, RPA2*
tRNA Charging	4.96	14/38 (0.37)	N/A	*Farsa*	*Cars, Cars2, Mars2, Gars, Tars, Farsb, Nars, Lars, Wars, Rars, Aars, Sars, Iars*
Glutathione-mediated Detoxification	6.26	12/23 (0.53)	N/A	*Gstm1, Mgst1, Mgst2, Gstm5, Gstm3, Gstm3, Gsta5, Gstm4, Gsto2, Gsta4, Mgst3, Gstk1*	
Superpathway of Cholesterol Biosynthesis	14.3	20/27 (0.75)	N/A	*Mvd, Sqle, Nsdhl, Pmvk, Acat2, Idi1, Mvk, Hsd17b7, Msmo1, Tm7sf2, Sc5d, Ggps1,*	

				Fdps, Fdft1, Dhcr7, Dhcr24, Lss, Hmgcr, Hmgcs1, Cyp51a1	

Table 3: Pathway Analysis of Genes Differentially Expressed by DINCH Treatment[1]

Pathway	-log(p-value)	Ratio	z-score	Upregulated	Down-regulated
NRF2-mediated Oxidative Stress Response	1.3	10/168 (0.06)	1	*Ftl, Gstm5, Rras, Nqo1, Mras, Dnajb2, Gsto2*	*Abcc4, Dnajc11, Fos*
RhoA Signaling	1.83	9/117 (0.08)	2.333	*Sept8, Ngef, Rhpn2, Ptk2b, Cdc42ep5, Gna13, Sept6, Pkn1*	*Myl4*
VDR/RXR Activation	1.85	7/78 (0.09)	1	*Cxcl10, Serpinb1, Tgfb2, Sema3b, Ccl5, Rxra*	*Ccnc*
ERK/MAPK Signaling	1.87	12/176 (0.07)	2.714	*Ptk2b, , Rras, Hspb2, Mras, Prkar1b, Rps6ka5, Rapgef3, Creb3l4, Rps6ka1*	*Fos, Ywhag, Ppp1r7*
Superpathway of Cholesterol Biosynthesis	1.96	4/27 (0.15)	N/A	*Mvd, Fdps, Dhcr7, Pmvk*	
p38 MAPK Signaling	2.01	9/109 (0.09)	2.646	*Cdc25b, Tifa, Hspb2, Tgfb2, Map4k1, Rps6ka5, Creb3l4, Rps6ka1, Mapk12*	
Signaling by Rho Family GTPases	2.09	15/227 (0.07)	2.496	*Sept8, Arhgef4, Ptk2b, Cdc42ep5, Mapk12, Pkn1, Arfip2, Mras , Gna13, Sept6, Arhgef9, Cdh13*	*Gnaq, Fos, Myl4*
Glutathione-mediated Detoxification	2.21	4/23 (0.18)	N/A	*Gstz1, Gstm5, Gsto2, Gstt1*	
Ephrin Receptor Signaling	2.81	14/171 (0.09)	N/A	*Ngef, Rras, Sh2d3c, Vegfb, Vegfc, Creb3l4, Mras, Figf, Gna13*	*Itsn1, Gnaq, Efnb2, Sdcbp,Efnb3*
ERK5 Signaling	3.75	9/62 (0.15)	1.414	*Rras, Mras, Rps6ka5, Creb3l4, Gna13, Rps6ka1*	*Fos, Ywhag, Gnaq*

Discussion

Despite the large body of evidence indicating that several phthalates such as DEHP can be toxic, regulatory agencies have only recently implemented regulations that limit their use. Our study provides evidence supporting the role of PPAR in mediating phthalate toxicity. While this is the first report of gene expression changes in the TM4 Sertoli cell line following treatment with MEHP, our findings of gene activation downstream of PPAR, lipid metabolism, and nuclear receptor involvement support observations from other cell types and animal models[15,16,42,47]. DEHP decreased cell viability in multiple cell lines as previously reported in MA-10 Leydig cells after 48 hour treatment[48]. While DEHP is not usually considered to be biologically active, Sertoli cells express a hormone sensitive lipase that is capable of metabolizing DEHP into biologically active or toxic metabolites[49, 50]. Previous studies have shown that 2-ethylhexanal, a by-product of DEHP metabolism, can decrease cell viability[48], but we are unable to determine whether DEHP was metabolised in our model and whether DEHP or one of its metabolites other than MEHP was responsible for the observed decrease in viability. Taken together, our results for DEHP and MEHP are consistent with previous findings.

While DOM is a good plasticizer, the accumulation of the toxic metabolite monooctyl maleate in the presence of the common soil bacterium *Rhodococcus rhodocrous* makes DOM a less desirable alternative[38]. In our studies, the maleate family generally decreased cell viability in most cell lines. Diethyl maleate (DEM) is a well-characterized compound used to deplete glutathione and induce cell damage by increasing reactive oxygen species[51]. Few studies have used DOM in a biological context, but DOM and DEM share a common maleate core that conjugates to glutathione in a reaction that is catalyzed by glutathione S-transferases (GST)[52]. Furthermore, it has been reported that inducers of GST gene expression are typically Michael acceptors, which are compounds that contain an unsaturated bond with an electron-withdrawing group[53]. Therefore, it is not surprising that DOM upregulated several GST isoforms and several genes involved in response to reactive oxygen species damage. Taken together, these data suggest DOM or similar maleate based plasticizers with varying side-chain lengths would not be suitable replacements for phthalate plasticizers based on the read-across principle[54].

DINCH production has grown to an annual capacity of 200,000 metric tons in 2013[55]. While DINCH does not leach as much as DEHP from PVC, by-products of DINCH metabolism

previously undetectable in human urine samples collected between 2000 and 2001, can be detected in samples collected from 2007-2012 in increasing quantities, indicating increasing human exposure[56]. Despite the marketing of this compound as a safe phthalate replacement, our microarray analysis suggests that DINCH is biologically active. Interestingly, 30% of genes that were differentially regulated by DINCH were also changed by DOM treatment, which had more pronounced effects on cell viability and gene expression. The DINCH gene expression dataset was enriched for genes involved in cellular movement, glutathione mediated detoxification, and important signalling pathways such as RhoA and ERK/MAPK. Many of these pathways were predicted to be activated, and can be correlated back to biological roles in Sertoli cell proliferation, differentiation, cytoskeleton, and junctional dynamics[57–59]. Whether these changes in gene expression are physiologically relevant or whether compensatory mechanisms can take place to maintain homeostasis at an organismal level remains to be determined.

Two novel plasticizers (BDB, DOS) did not have an effect on cell viability following 48 hour exposure *in vitro*. Furthermore, there was no significant change in gene expression following microarray analysis. While our data suggest that DOS and BDB are potential replacements for phthalate plasticizers, further testing is required to determine whether there is systemic toxicity after chronic or developmental exposure to these compounds.

In our study, we have proactively screened a large list of candidate plasticizers to identify those least likely to have deleterious biological effects. In addition to being functionally equivalent to DEHP, new dibenzoate and succinate based chemicals biodegrade readily[36, 46]. Succinates have the added advantage in that they can be sourced from the by-products of fermentation[36]. Thus, unlike phthalate-based compounds, succinic acid production does not depend on petroleum refinement; thereby minimizing the associated environmental impact and our dependence on petroleum based products[60]. Based on our findings and previously published results on their plasticizing properties, dioctyl succinate and 1,4 butanediol dibenzoate are promising replacements for DEHP and other phthalate based plasticizers.

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Supplemental Figures & Tables

Full List of Plasticizers with CAS Numbers

Full Name	Abb.	CAS RN	Provenance	Analysis Grouping
di(2-ethylhexyl) phthalate	DEHP	117-81-7	Sigma-Aldrich	3
mono(2-ethylhexyl) phthalate	MEHP	4376-20-9	Wako Pure Chemical	3
1,2-cyclohexane dicarboxylic acid diisononyl ester	DINCH	166412-78-8	BASF Canada	1
1,3 propanediol dibenzoate	PrDB	2451-86-7	Synthesized in-house	3
1,4 butanediol dibenzoate	BDB	19224-27-2	Synthesized in-house	3
1,5 pentanediol dibenzoate	PDB	6624-73-3	Synthesized in-house	3
1,6 hexanediol dibenzoate	HDB	22915-73-7	Synthesized in-house	3
diethyl succinate	DES	123-25-1	Sigma-Aldrich	1
dibutyl succinate	DBS	141-03-7	Synthesized in-house	1
dihexyl succinate	DHS	15805-75-1	Synthesized in-house	1
dioctyl succinate	DOS	14491-66-8	Synthesized in-house	1
di(2-ethylhexyl) succinate	DEHS	2915-57-3	Synthesized in-house	1
diethyl fumarate	DEF	623-91-6	Sigma-Aldrich	2
dibutyl fumarate	DBF	105-75-9	Scientific Polymer Products	2
dihexyl fumarate	DHF	19139-31-2	Synthesized in-house	2
dioctyl fumarate	DOF	2997-85-5	Synthesized in-house	2
di(2-ethylhexyl) fumarate	DEHF	141-02-6	Synthesized in-house	2
diethyl maleate	DEM	141-05-9	Sigma-Aldrich	4
dibutyl maleate	DBM	105-76-0	Acros Canada	4
dihexyl maleate	DHM	16064-83-8	Synthesized in-house	4
dioctyl maleate	DOM	2915-53-9	Synthesized in-house	4
di(2-ethylhexyl) maleate	DEHM	142-16-5	Synthesized in-house	4

Table 4: Plasticizers names, Abbreviations, CAS Numbers and Source. *Grouping analysis refers to which compounds were tested together on the same 96-well plate for the MTT assay (and therefore share common control DMSO treated samples).*

List of Qiagen Primers Used for Validation

Product Number	Description	Gene Name	Detected Transcript	Transcript Length
QT00166768	Mm Hprt 1 SG QuantiTect Primer Assay (200)	hypoxanthine guanine phosphoribosyl transferase	NM_013556 (1349 bp)	168 bp
QT00139748	Mm Angptl4 1 SG QuantiTect Primer Assay (200)	angiopoietin-like 4	NM_020581 (1916 bp)	126 bp
QT00119532	Mm Mapk12 1 SG QuantiTect Primer Assay (200)	mitogen-activated protein kinase 12	NM_013871 (1905 bp)	114 bp
QT00127680	Mm Pmvk 1 SG QuantiTect Primer Assay (200)	phosphomevalonate kinase	NM_026784 (1204 bp)	140 bp
QT00115976	Mm Mvd 1 SG QuantiTect Primer Assay (200)	mevalonate (diphospho) decarboxylase	NM_138656 (1761 bp)	103 bp
			NR_028354 (1864 bp)	103 bp
QT02238992	Mm Fdps 2 SG QuantiTect Primer Assay (200)	farnesyl diphosphate synthetase	NM_001253751 (1478 bp)	150 bp
			NM_134469 (1262 bp)	150 bp
QT00106246	Mm Mras 1 SG QuantiTect Primer Assay (200)	muscle and microspikes RAS	NM_008624 (4058 bp)	150 bp
QT00101584	Mm Egfr 1 SG QuantiTect Primer Assay (200)	epidermal growth factor receptor	NM_007912 (2678 bp)	68 bp
			NM_207655 (5983 bp)	68 bp
QT00098987	Mm Gsta4 1 SG QuantiTect Primer Assay (200)	glutathione S-transferase, alpha 4	NM_010357 (970 bp)	77 bp
QT00157248	Mm Pdk4 1 SG QuantiTect Primer Assay (200)	pyruvate dehydrogenase kinase, isoenzyme 4	NM_013743 (3453 bp)	149 bp

Table 5: List of primers used for RT-PCR validation*: Table includes catalogue number, catalogue name, gene name, transcript reference number, and length of the aplified PCR product.*

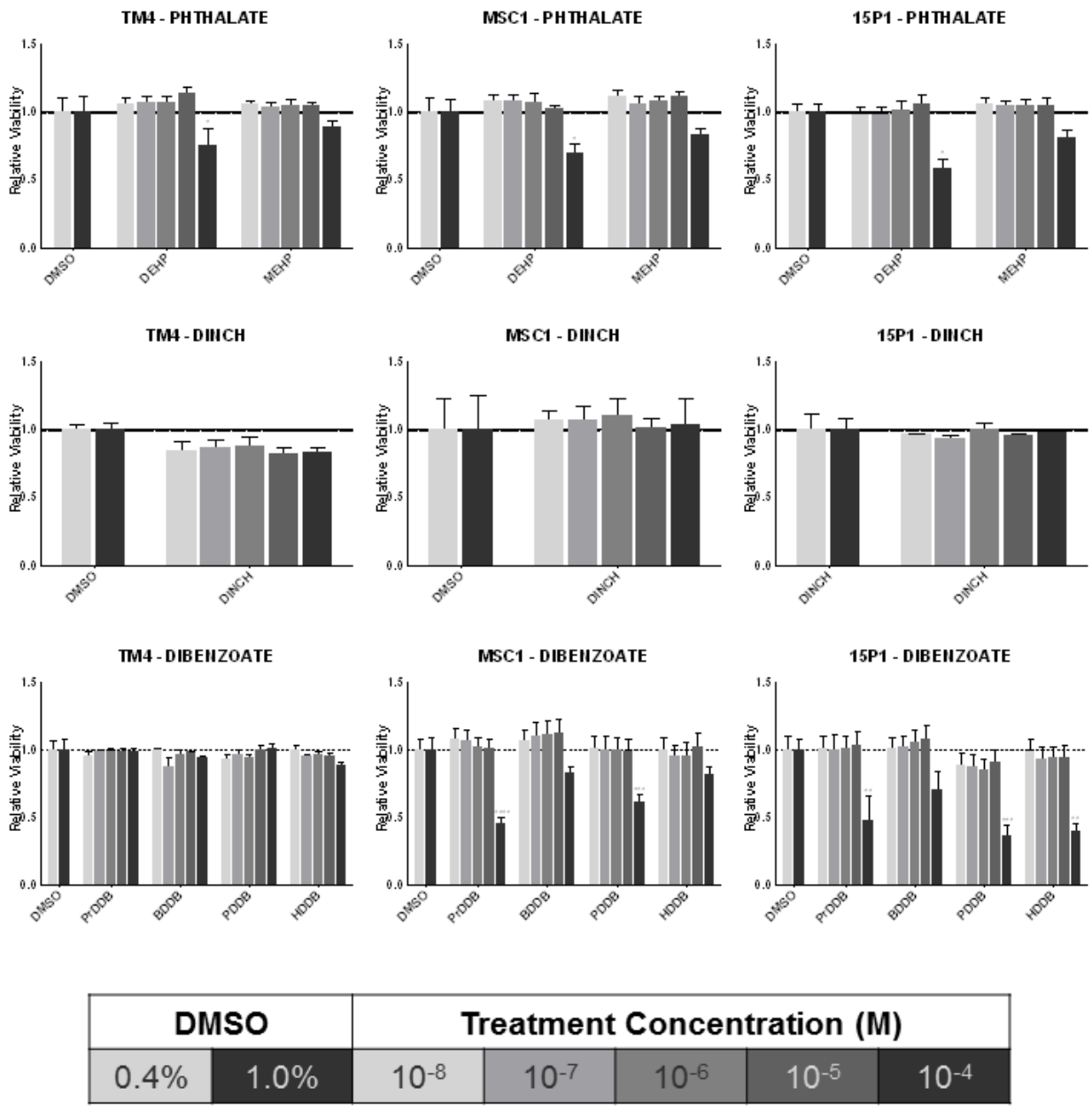

Figure 16: Cell Viability Data for all Chemicals 1/2 Figure shows results for phthalates (DEHP and bioactive metabolite MEHP), a commercial alternative plasticizer (DINCH), and a modified dibenzoate series. Results for other alternative plasticizers can be found in **Figure 11**.

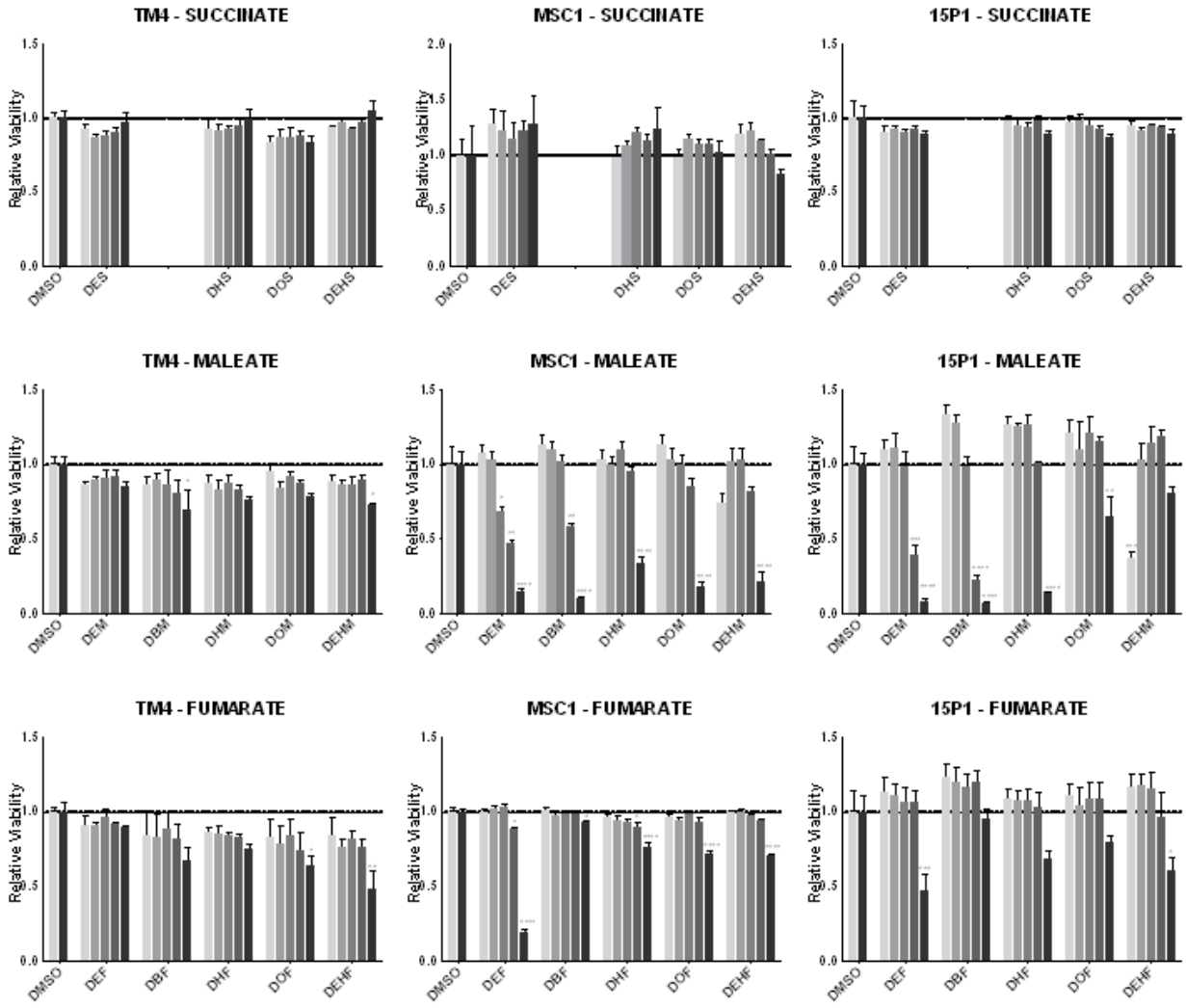

Figure 17: Cell Viability Data for all Chemicals 2/2

Connecting Text

In the previous chapter, utilizing *in vitro* assay systems and a toxicogenomic approach, we screened several candidate plasticizers for their effects on cellular viability and gene expression. Of all the families of chemicals, the dibenzoates and succinates had the best overall characteristics in terms of functionality and safety. From these two families of chemicals 1,4 butanediol dibenzoate (BDB) and dioctyl succinate (DOS) were selected for further studies.

While the field of toxicology is moving towards high-throughput methods to screen for toxicity and prioritize chemicals for investigation, *in vitro* data does not direct regulatory policy. For any new chemical, an acute toxicity study in rodents must be done to assess systemic toxicity and guide regulatory policy. The next chapter describes an acute toxicity study in Sprague-Dawley rats that were treated with 15 and 150mg/kg BDB and DOS by oral gavage. This study was designed based on guidelines in Protocol 407 by the Organisation for Economic Co-operation and Development (OECD), an international conglomerate of regulatory agencies that have agreed upon standardized testing for regulatory purposes.

Chapter 3: Assessment of the Safety of Two Replacement Plasticizers for di(2-ethylhexyl) phthalate (DEHP) Using a Repeated Dose 28 Day Acute Toxicity Study in Adult Male Rats.

Thomas C. Nardelli, Bernard Robaire

The doctoral candidate designed the experiment, did animal manipulations (gavage, necropsy, sample preparation), analyzed the data, and wrote the original draft of the manuscript. Bernard Robaire conceived, supervised, and was the principal investigator of this study.

This study was also made possible with the technical help of Claudia Lalancette, Sheila Ernest, Trang Luu, and Océane Albert who assisted in rodent necropsies. The Histology Core at the Goodman Cancer Research Centre prepared histological sections that were analyzed and interpreted by Marilène Paquet. Barbara Hales provided guidance in the experimental design.

Some data in this chapter are featured as part of the following conceptual paper:

Océane Albert, Thomas C. Nardelli, Barbara F. Hales, Bernard Robaire; Identifying Greener and Safer Plasticizers: a Four-Step Approach, *Toxicological Sciences*, https://doi.org/10.1093/toxsci/kfx156

Abstract

Phthalates are ubiquitous environmental contaminants that are found in consumer products and medical devices made from polyvinyl chloride. In recent years, regulatory agencies worldwide have been moving towards banning these chemicals in consumer products due to their endocrine disrupting properties. Several alternatives that have similar plasticizing properties, but different molecular structures have been proposed as responsible replacements. Two candidates from previous *in vitro* screens emerged as compounds with better biodegradability, plasticizer functionality, and no toxicity in model cell lines. As a first step to determine the relative safety of these chemicals in whole animal models, we assessed the acute toxicity of 1,4 butanediol dibenzoate (BDB) and dioctyl succinate (DOS) in Sprague-Dawley rats at 15 and 150 mg/kg/day for 28 days. There was no significant effect compared to control animals in terms of animal growth or well-being, organ weight and histology, hematology, and serum analytes. This study is the first step in screening for undesirable toxicity *in vivo*, and is meant to set the framework for comprehensive developmental toxicity studies.

Introduction

Plasticizers are additives used to improve flexibility and extend the applications of otherwise brittle plastics such as polyvinyl chloride (PVC). Di(2-ethylhexyl) phthalate (DEHP) is a high production volume phthalate plasticizer used in a variety of consumer products including flooring and wall coverings, food packaging, and medical devices[1]. Retrospective human studies and animal exposure studies have highlighted the deleterious anti-androgenic action of phthalates on human health[2]. Recent literature also suggests the deleterious effects of phthalate exposure can extend beyond the reproductive system[3-6]. These findings have prompted industry to find alternatives that are safe, inexpensive, and amenable to many downstream applications.

There are many factors to consider when developing novel plasticizers. New plasticizers and their metabolites should not leach from plastic, biodegrade easily, and be biologically inert. Furthermore, a plasticizer must be inexpensive, compatible with the plastic resin, and be able to extend the use of a resin for multiple applications[7]. Previous *in vitro* studies propose succinate and dibenzoate plasticizers are potential alternatives for DEHP[8, 9]. Both chemical families are good plasticizers[10, 11], biodegradable[11-14], and have a decreased rate of leaching from PVC in aqueous environments[15]. Plasticizers synthesized from succinic acid have an added advantage in that the raw materials for their synthesis can be sourced from by-products of fermentation[16].

Of the dibenzoate and succinate based plasticizers, 1,4 butanediol dibenzoate (BDB) and dioctyl succinate (DOS) were selected for their desirable physical properties and apparent lack of toxicity using *in vitro* models[8, 9]. While *in vitro* assays can be a powerful method for screening libraries of compounds, the systemic toxicity of these compounds is unknown. An *in vivo*, repeated-dose acute toxicity study in a male rat model was designed following guidelines provided by the Organisation for Economic Cooperation and Development (OECD) in order to identify potential deleterious effects of these chemicals following oral administration of 1,4 butanediol dibenzoate and dioctyl succinate for 28 days.

Materials and Methods

Chemical Synthesis and Purity

1,4 butanediol dibenzoate and dioctyl succinate were synthesized as previously described[10, 11, 14]. All chemicals were synthesized prior to this study and the purity of the lot was determined to be 99% by NMR analysis (unpublished). Chemicals were stored in a vacuum chamber with desiccant at room temperature.

Animal Housing and Administration of Treatment

Twenty male Sprague Dawley rats were ordered from Charles River Laboratories (St-Constant, Quebec) over four days. Male pups were weaned at PND21 at Charles River Laboratories and delivered to the McIntyre Medical Building Animal Facility the next day. Animals were allowed to acclimate for seven days, and were provided with Irradiated Lab Animal Diet (Harlan Laboratories Inc., Madison, WI) and water ad libitum. On PND29, the animals were randomly allocated to one of five treatment groups and gavaged with a maximum of 1ml corn oil containing either 1,4 butanediol bibenzoate or dioctyl succinate. Control animals were administered 1ml of corn oil. Animal weight was measured daily to administer the appropriate dosage. Animals received either no treatment (vehicle), 15mg/kg, or 150mg/kg of BDB or DOS. Gavage was repeated daily between 1:00-2:00pm for 28 days (PND 56). Abnormalities in behaviour were monitored immediately, one hour, and four hours following gavage. Food consumption was monitored weekly until PND 57 at which point food was removed. Animals were fasted for 24 hours prior to necropsy on PND58.

All animals survived to the completion of the study. Over four days, one animal from each group was necropsied on PND 58 between 9:30am-12:00pm. Animals were coded and the order of necropsy was randomized so that the manipulators were blind to treatment. Rats were euthanized by carbon dioxide asphyxiation followed by cardiac puncture. All housing, handling, and terminal procedures were in accordance with guidelines outlined in *A Guide to the Care and Use of Experimental Animals* [17] prepared by the Canadian Council on Animal Care. All studies were described in the animal ethics Protocol #5867 that was approved by the McGill University Animal Research Centre.

Blood Collection and Processing

Approximately 8ml of whole blood obtained by cardiac puncture was collected and divided equally between two BD Vacutainer Blood Collection Tubes (one for whole blood hematology (lavender cap) and one for clinical serum chemistry (red/grey cap)). In either case, tubes were inverted several times to ensure proper mixture of anti-clotting (K_3EDTA) or clotting factors. For clinical chemistry, once a clot had formed (approximately 30 minutes later), tubes were centrifuged for 8 minutes at 1000 x *g* and serum was stored in a new tube for analysis.

Each animal was dissected, and its organs were distributed among four investigators. The first investigator made the initial incision and removed the contents of the abdominal cavity (stomach, small intestine, large intestine, cecum, colon, spleen, and liver. The second investigator simultaneously removed the brain from the cranial cavity of the decapitated animal. The third investigator removed the contents of the thoracic cavity (heart, lungs, thymus). The fourth removed the male reproductive tract (testes, epididymides, prostate, and seminal vesicles). Organs were weighed, trimmed, and immersed in 10% neutral buffered formalin for approximately one week after which tissues were stored in 70% ethanol until further processing.

Tissue Histology

Samples were dehydrated and infiltrated with paraffin using a Tissue-Tek VIP 6 (Sakura Finetek USA, Torrance, CA) with 1 hour steps of 1X 70% ethanol, 1X 80% ethanol, 2X 95% ethanol, 2X 100% ethanol, and 3X xylene at room temperature under pressure and vacuum. These steps were followed by 4X paraffin steps of 1 hour at 60°C with pressure and vacuum. Samples were embedded in paraffin using a Heated Paraffin Embedding Module EG1150H (Leica Microsystems, Concord, ON) and a Cold Plate for Modular Tissue Embedding System EG1150C (Leica Microsystems). From the embedded tissue blocks, 4µm sections were cut using a Fully Automated Rotary Microtome RM2255 (Leica Microsystems). After mounting sections to glass slides, sections were rehydrated and stained using a Multi-Stainer ST5020 (Leica Microsystems) and the ST Infinity H&E Staining system (Leica Microsystems). The rehydration and staining protocol was as follows: 3X Xylene for 2 minutes, 3X ethanol for 1 minute, 1X 80% ethanol for 1 minute, 1X water for 1 minute, 1x ST HemaLast for 30 seconds, 1X ST Haematoxylin for 2.5 minutes, 1X water for 2 minutes, 1X ST Differentiator for 45 seconds, 1X

water rise for 1 minute, 1X ST Bluing Agent for 1 minute, 1X Water for 1 minute, 1X 80% ethanol for 1 minute, 1X ST eosin for 30 seconds, 3X 100% ethanol for 1 minute, and 3X xylene for 1 minute. A coverslip was added to slides using a Fully Automated Glass Coverslipper CV5030 (Leica Microsystems).

Haematology and Clinical Chemistry

Five samples were determined to be slightly haemolysed by visual inspection at the time of analysis, but this was not related to treatment. Whole blood was stained using Camco Quik Stain (Fisher Scientific, Ottawa, Ontario). A complete blood count (erythrocytes, thrombocytes, leukocytes, hemoglobin) with four-part white blood cell (granulocyte, monocyte, lymphocyte, eosinophil) differential analysis was done using the Vet abc Plus+ (scil Animal Care Company, Barrie, ON) using 10µl EDTA treated whole blood.

For serum biochemistry, a Vitros 250 (Ortho-Clinical Diagnostics, Markham Ontario) was used with dry-slide matter to determine measurements of clinical chemistry.

Pathological Evaluation

Slides were coded with letters A-T that corresponded to individual animals. The pathologist was unaware of any treatment until after their final report was produced.

Statistical analysis

Significance was determined by one-way ANOVA followed by Dunnett's post-hoc test. All statistical analyses were computed using GraphPad Prism 6.05 (GraphPad Software, La Jolla, CA).

Results

Effects of Treatment on Animal Behaviour

Animal behaviour was followed up-to four hours after gavage. At no point during the study did animals show signs of convulsions, impaired motility, irregular grooming, vocalizations, hunched back, or respiratory distress.

Effects of Treatment on Animal Well-Being

There was no significant effect on animal weight, or metabolic index (calculated as food consumption divided by change in body weight) in this study **(Figure 18).**

Animal Necropsy

While animals were healthy some abnormalities were observed during necropsy. The sinistral adrenal gland of animal 5 (BDB 15) could not be found by the manipulator and the dextral adrenal was not weighed. Animal 9 (BDB 150) had bilaterally small seminal vesicles and prostate gland but was not excluded from analysis. The sinistral side of Animal 11 (control) was missing an epididymis and had microtestis while the dextral side was normal. Animal 13 (DOS 15) was missing a dextral testis and epididymis. No other abnormalities were observed. Paired organs were averaged prior to normalization to body weight. When a member of a paired organ was missing, it was assumed both members of said pair weighed the same. There was no significant change in any organ weight **(Figure 18)**.

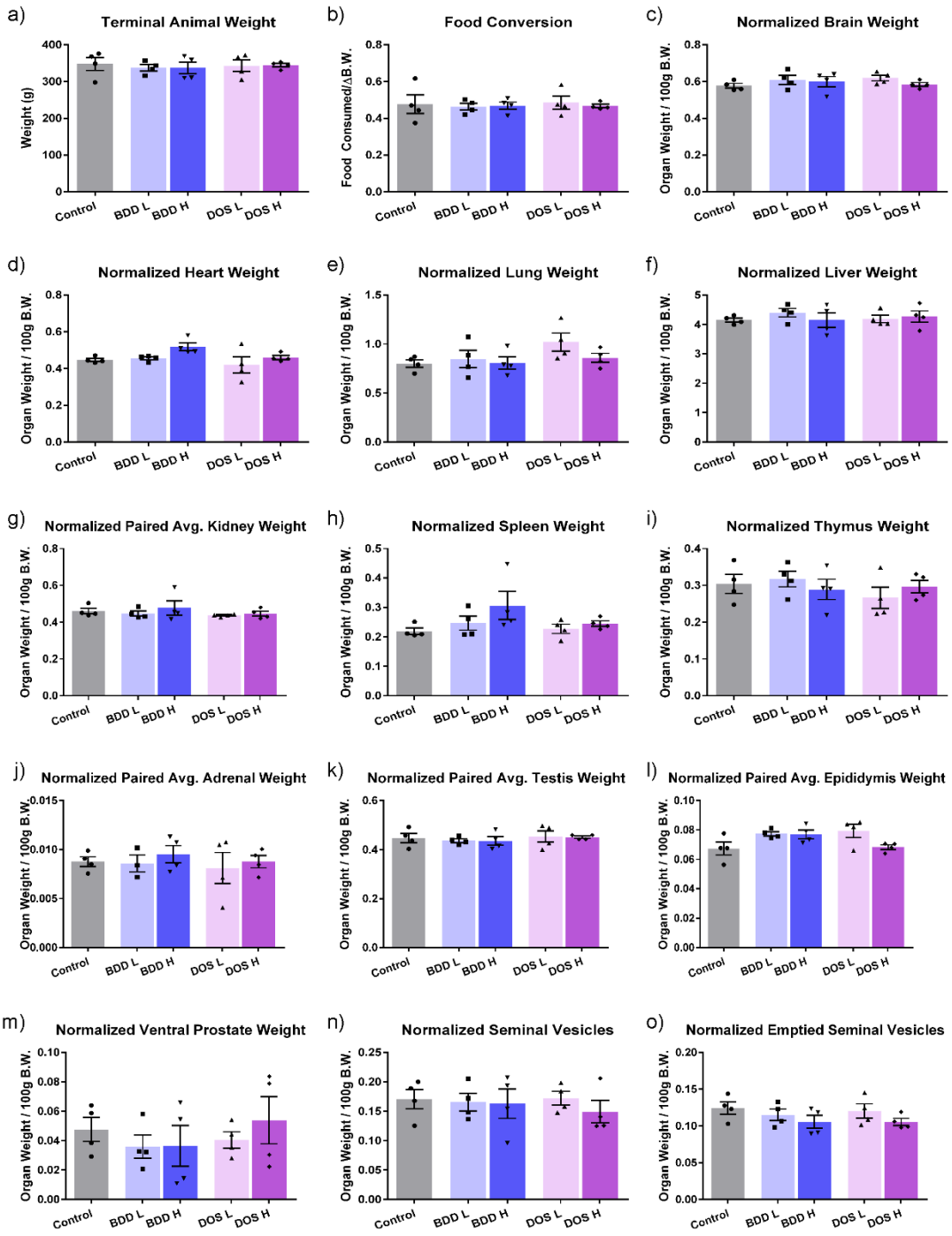

Figure 18: General parameters of animal health and necropsy results: *(a) Weight at time of necropsy. (b) Amount of food consumed normalized to change in body weight. (c-g) Weight of vital organs; (h-j) weight of accessory organs; (k-o) weight of reproductive organs collected. Organ weight was normalized to every 100g of body weight of the animal for non-capsulated*

organs. No endpoints were significantly changed. Bars represent average ± SEM. The points represent individual animals.

Haematological Analysis

Haematological parameters were examined as markers of systemic disease. Primary perturbations of the bone marrow and the resident hematopoietic stem cells are not commonly observed, and most often are secondary to homeostatic mechanisms following exposure to a toxicant[18]. Proper functioning of each component of the haematopoietic system is essential for the survival and well-being of an organism. Briefly, erythrocytes transport oxygen to organs; leukocytes provide a defense from pathogens and toxins; platelets are essential for repairing breaks within the vasculature; and plasma contains nutrients, transport proteins, endocrine signalling molecules, by-products of metabolism, and other components essential for the proper functioning of platelets and white blood cells[18]. Changes in erythrocyte count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular hemoglobin concentration, or the presence of reticulocytes can indicate problems with haematopoietic stem cell proliferation[18]. Changes in leukocyte number, cell-type distribution, and morphology can suggest antigenic stress caused by an infection or toxin[18]. Changes in platelet count may indicate blood loss from internal bleeding or sequestration in the spleen due to an acute stress response. There were no significant effects on any of these parameters in our data **(Figure 19)**.

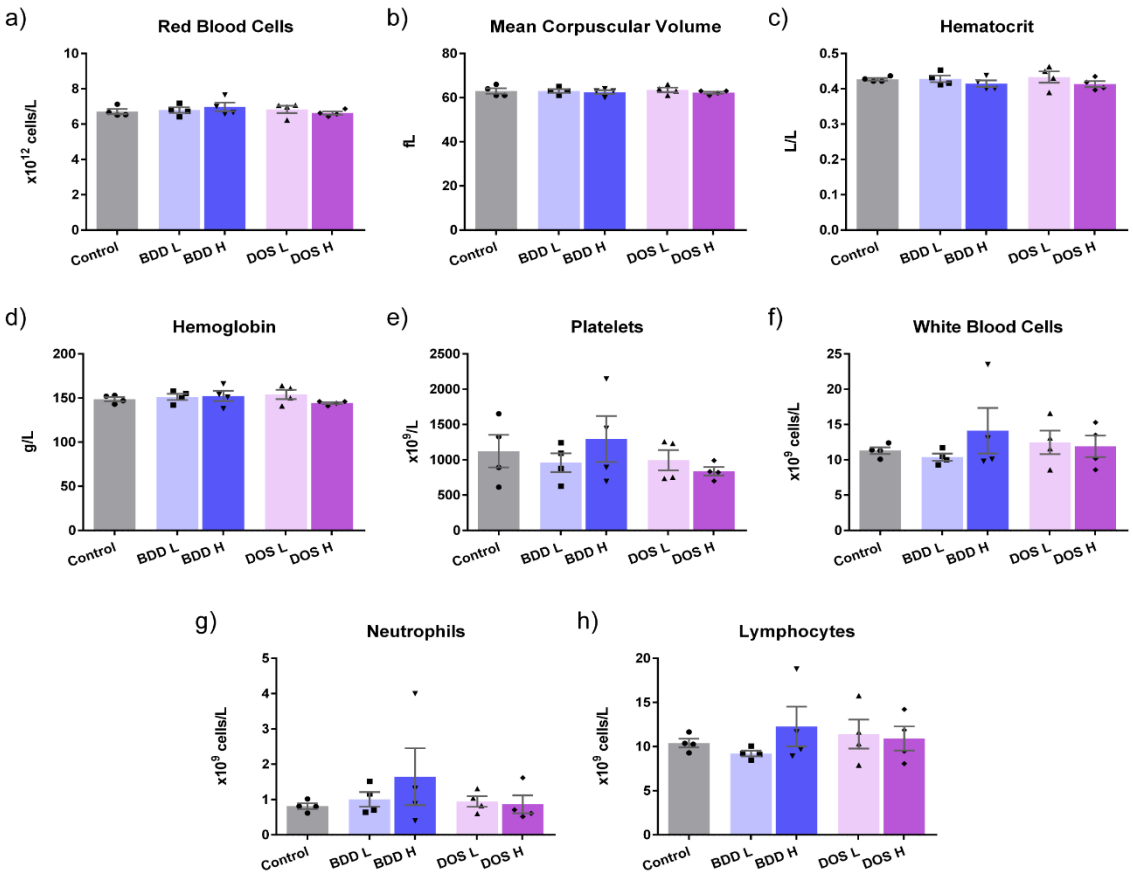

Figure 19: Hematology from blood collected by cardiac puncture. *(a-d) characteristics of erythrocytes, (e) platelets, and (f-h) leukocytes. No significant changes were observed compared to control. Bars represent average ± SEM. The points represent individual animals.*

Clinical Chemistry

Analytes from serum samples were studied as biomarkers of pathogenesis. The selected biomarkers are elevated in disease states and generally follow the progression of their indicator.

Serum albumin and total protein are markers that are used to identify malnutrition and dehydration, but can also indicate hepatic or renal disease[19]. Renal disease is also identified by increases in blood urea nitrogen and creatinine. A change in these analytes would reflect a low glomerular filtration rate as they are by-products of liver and muscle metabolism respectively[19]. Renal failure can lead to hyperphosphatemia and hypocalcaemia. This is caused by impaired phosphate excretion by the renal tubules, and impaired formation of biologically active vitamin D (calcitriol) which is necessary for calcium absorption from dietary sources[19].

Several markers can indicate perturbations in hepatic function. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are primarily used as indicators of hepatic disease[20]. Alkaline Phosphatase (ALP) is present in many tissues with the highest expression in the placenta, small intestine, bone, kidney, and liver. It is commonly used as a marker of bone turnover, but liver ALP can also increase in response to various drugs and liver damage[20].

Metabolic markers can provide insight into the general well-being of an animal. Creatine kinase is involved in energy production and is used as a biomarker for damage to metabolically demanding contractile tissue, such as skeletal muscle or myocardium[20]. Glucose concentration can indicate irregular pancreatic function and stress[19]. Several electrolytes we also measured for their biological importance in various physiological and enzymatic processes.

No significant changes in any of these parameters were found as a consequence of treatment with BDB or DOS **(Figure 20, 21)**.

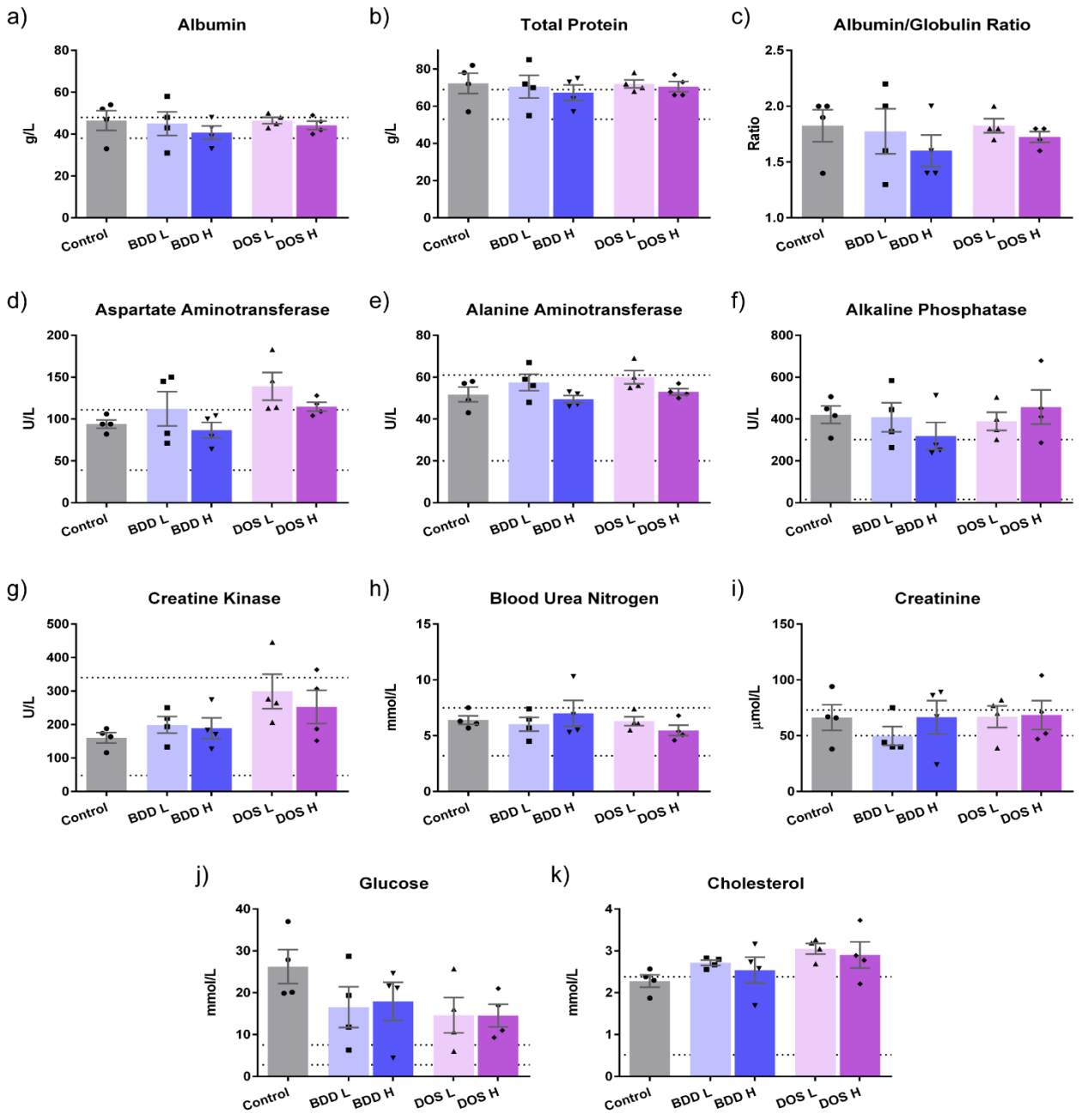

Figure 20: Serum Biomarker Analysis. *Standard clinical biomarkers were monitored to assess the whether treatment impaired liver, kidney, bone, heart, muscle, or basal metabolic function. Dashed lines are clinical reference values. Bars represent average ± SEM. The points represent individual animals.*

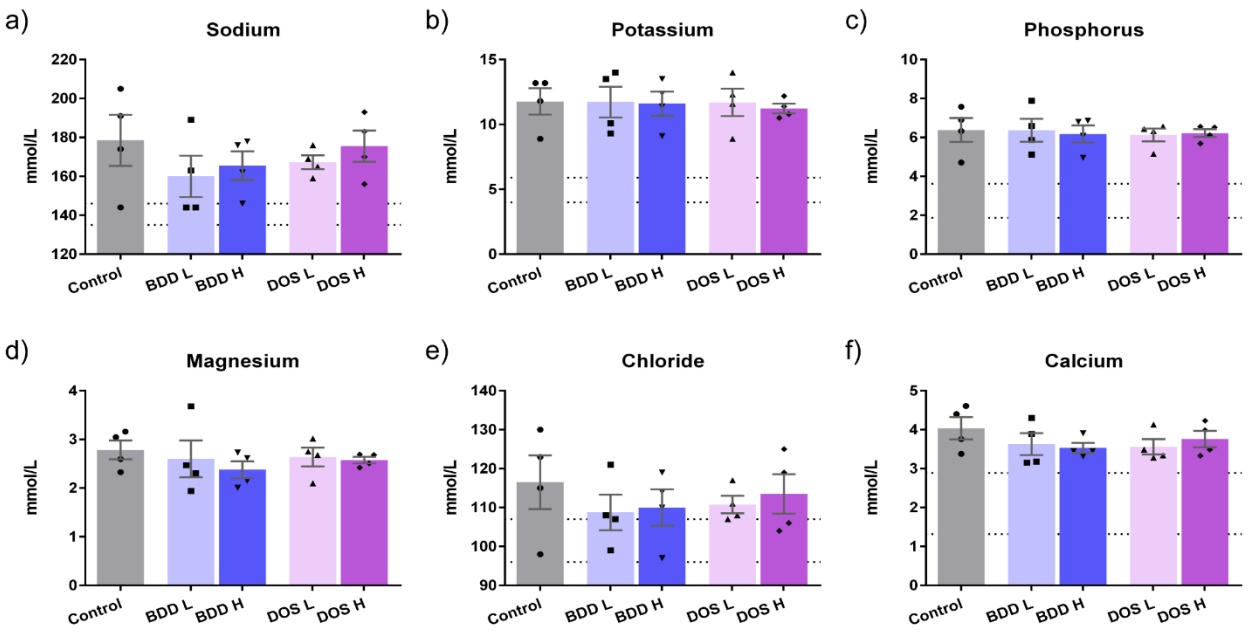

Figure 21: Analysis of Serum Electrolytes. *Serum electrolytes from blood collected by cardiac puncture. Dashed lines are clinical reference values. Bars represent average ± SEM. The points represent individual animals.*

Pathology

While serum biochemistry and haematology can screen for a metabolic disorder, they are rarely diagnostic on their own[21]. Stomach, intestinal segments, kidney, adrenal glands, spleen, liver, lung, heart, and brain were fixed and assessed for clinical histopathology. Few histopathological changes were observed. Several instances of hepatic lymphohistiocytic inflammatory infiltrate in the liver parenchyma and multifocal renal lymphocytic inflammatory infiltrate were observed. Two animals had pyelonephritis (F-BDB 150, P-DOS 15) which was most likely caused by a lower urinary tract infection. Several representative histopathology micrographs are provided in **(Figure 22)**, and a summary of observations and their severity are described in (**Table 6**). These observations have been previously described in control animals[22-24], and were not more frequent in treated animals.

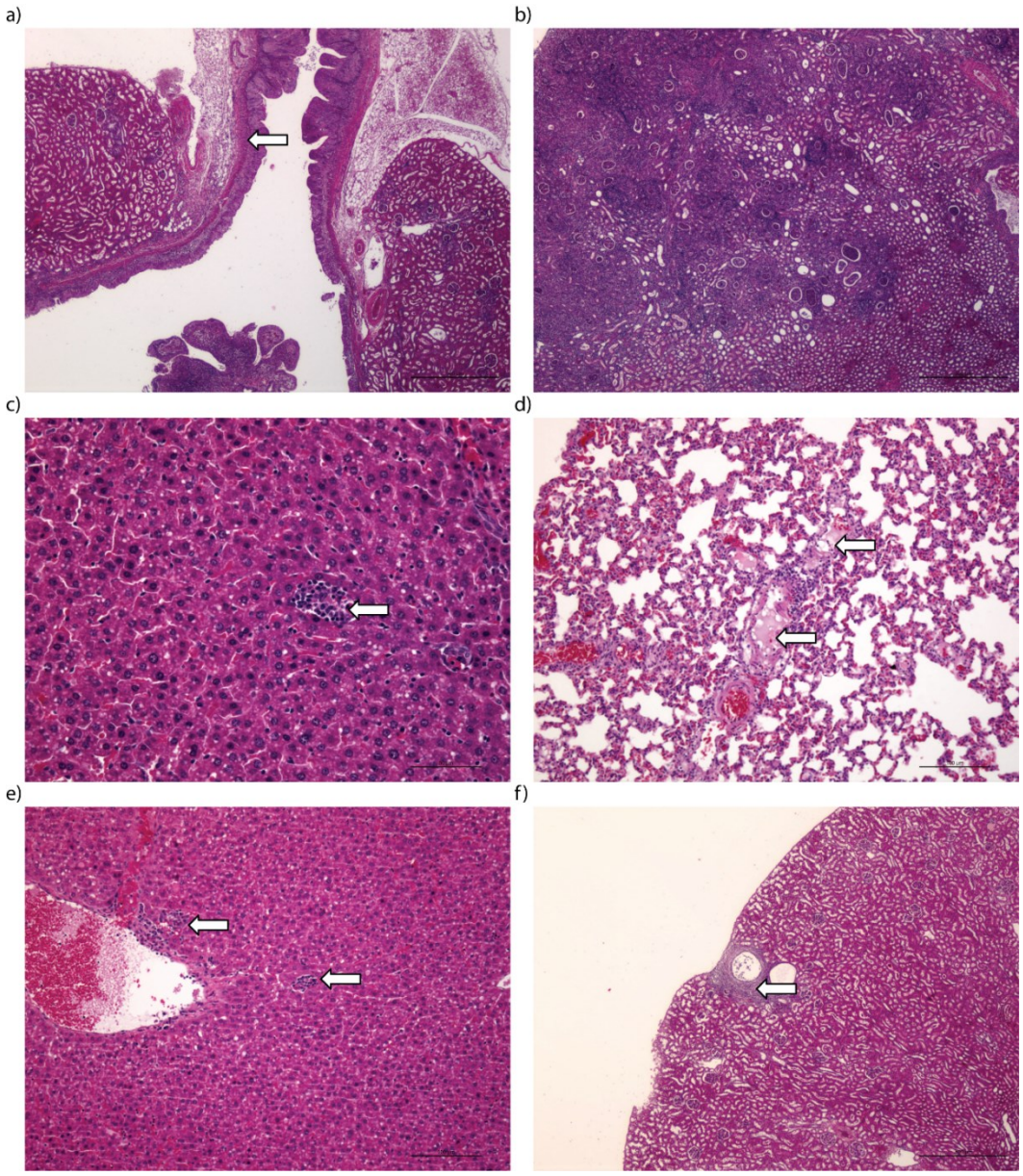

Figure 22: Representative Examples of Lesions Observed. *a) chronic suppurative inflammation of the renal pelvis indicated by arrow (25x magnification, scale bar 500µm, Animal F); b) nephritis, chronic interstitial active inflammation (25x magnification, scale bar 500µm, Animal F); c) focus of hepatic lymphocytic inflammation (200x magnification, scale bar 50µm, Animal F); d) foci of pulmonary perivascular lymphocytic inflammation (100x magnification, scale bar 100µm, Animal F); e) random foci of hepatic lymphocytic inflammation (100x magnification, scale bar 100µm, Animal K); f) renal focus of interstitial lymphocytic inflammation with dilated tubules (25x magnification, scale bar 500µm, Animal K).*

Table 6: Pathological Findings[2]

	CONTROL		BDB (150)			DOS (15)			DOS (150)			
ANIMAL ID	H	L	E	F	S	C	N	P	D	G	K	Q
KIDNEY	A	A	A	A			A	A		A		
INFLAMMATORY INFILTRATE, LYMPHOHISTIOCYTIC, FOCAL, CHRONIC							1					
LYMPHOCYTIC INFLAMMATION, FOCAL, INTERSTITIUM			2									
LYMPHOCYTIC INFLAMMATORY INFILTRATE, INTERSTITIAL, MULTIFOCAL	1	1								1		
PYELONEPHRITIS, LYMPHOHISTIOCYTIC, UNILATERAL CHRONIC								1				
PYELONEPHRITIS, SUPPURATIVE, BILATERAL, CHRONIC				3								
HEART											A	
LYMPHOCYTIC INFLAMMATORY INFILTRATE, MULTIFOCAL											1	
LIVER	A			A	A			A	A		A	A
ARTERITIS PROLIFERATIVE, MULTIFOCAL				2								
INFLAMMATORY INFILTRATE, LYMPHOHISTIOCYTIC, MULTIFOCAL					1			2				1
LYMPHOCYTIC INFLAMMATION, MULTIFOCAL RANDOM				2					2			
LYMPHOCYTIC INFLAMMATORY INFILTRATE MULTIFOCAL RANDOM	1										1	
LUNG				A	A	A						
PERIVASCULAR LYMPHOCYTIC INFILTRATE, MULTIFOCAL				2	2	2						

[2] *Summary of findings reported by pathologist when animals were affected by treatment. A=affected, severity of lesions: (1) modest, rare; (2) mild, infrequent; (3). Each group contained four animals. If not listed, no effects were observed.*

Discussion

There are multiple permutations in a study that can complicate the interpretation of *in vivo* studies[25], but mutually agreed upon guidelines, such as those developed by the OECD, have standardized toxicity screening for regulatory purposes[26]. These universally accepted protocols not only improve the quality of scientific research, but also reduce the need for repeat studies and the use of additional animals[27].

Organ weights, haematological endpoints, and clinical biochemistry are standard endpoints for toxicological risk assessment[28,29]. Liver, kidney, and testis weight generally correlate well with histopathological findings, but some organs require more sensitive examination[28]. Haematology and clinical biochemistry provide a non-terminal method to predict, screen, and track unwanted toxicity that generally correlates well with histopathological findings[30]. There are still some limitations of using solely a clinical biochemistry approach as some biomarkers are expressed from several organs [31], while others are relatively insensitive unless extensive organ damage has already occurred [32]. Histopathology is laborious, but identifying structural abnormalities allows for a better understanding of the pathology for a particular organ[33].

While there were some abnormalities observed in this study, they were not significant, could not be attributed to a specific treatment, and were also observed in control animals. Animals treated with BDB or DOS were healthy, gained weight, and grew at a pace similar to control animals.

Several considerations were made with respect to the animal strain and dose of plasticizer used for this study. The species and strain of the animal will influence the pharmacokinetics of the test compound. The rat has become a standard in toxicology research due to its similarities to human metabolism, small size, and ease of manipulation[34]. The Sprague-Dawley rat model was selected as an outbred model that is commonly used in toxicity testing. Dosages of BDB and DOS were selected based on current human phthalate exposure levels, knowledge of predicted metabolite toxicity (succinic acid, benzoic acid, and corresponding long-chain alcohols), estimates of leaching rates, and pharmacokinetic differences between humans and rodents. Based

on these considerations, the low-dose of 15mg/kg is representative of the maximum likely human exposure after accounting for interspecies differences.

The solution to the phthalate replacement problem is complex. The ubiquitous use of these chemicals makes a ban unfeasible without sacrificing conveniences to which our society has become accustomed. At the same time, replacements may not always be better than the compounds they are meant to replace. Sub-chronic toxicity studies covering a spectrum of endpoints provides a framework to identify safe alternatives, and to design long-term hazard assessment studies which are necessary to fully characterize the toxicity of a chemical[35].

From the data presented in this manuscript, our findings suggest DOS or BDB are safe alternatives to the conventionally used phthalate plasticizers. This manuscript is the first *in vivo* assessment of systemic toxicity of two novel phthalate alternatives in a rodent model, and is a step towards finding responsible replacements for phthalates plasticizers.

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Connecting Text

The previous chapter describes an acute toxicity study designed to assess the safety of 1,4 butanediol dibenzoate and dioctyl succinate. Our results showed that these chemicals have no deleterious effects on animal health.

The next chapter describes a gestational-lactational exposure study where BDB and DOS were compared directly to di(2-ethylhexyl) phthalate (DEHP) and its current commercial replacement DINCH. Gestational and neonatal exposure is a critical time in development when organogenesis is particularly sensitive to xenobiotic perturbations. In the case of DEHP, the most vulnerable time of exposure is during the male programming window in utero. Exposure during this time is believed to lead to several testicular abnormalities that share a common developmental etiology and has been coined “the testicular dysgenesis syndrome”. In the following study, pups were exposed during this sensitive time in development and assessed into young adulthood. Unlike the previous study, in addition to organ weights, a series of developmental and endocrine sensitive biomarkers were used to understand whether exposure to any of these chemicals could affect not only general physiology, but also endocrine function.

Chapter 4: In Utero and Lactational Exposure Study in Rats to Identify Replacements for Di(2-ethylhexyl) Phthalate

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The doctoral candidate was involved in the design and conception of experiments, did the animal manipulations, analyzed data, maintained a data repository, and prepared the original draft of the manuscript. Océane Albert was involved in the design and conception of experiments, did the animal manipulations, analyzed data, and prepared the original draft of the manuscript. Claudia Lalancette was involved in the design and conception of experiments and did the animal manipulations. Martine Culty designed the designed ex vivo testicular culture experiments. Barbara Hales contributed towards the design and conception of the experiments. Bernard Robaire designed, conceived, and supervised the experiments, and is the principle investigator of these studies. All authors contributed towards the final manuscript.

This study was also made possible with the technical help of Annie Boisvert (ex vivo culture). Sheila Ernest and Élise Kolasa provided technical assistance with animal necropsies and Qudsia Saadat counted gonocytes in PND3 testes sections.

Abstract

Di(2-ethylhexyl) phthalate (DEHP) and other phthalates are ubiquitous environmental contaminants with endocrine disrupting properties. Two novel plasticizers, 1,4 butanediol dibenzoate (BDB) and dioctyl succinate (DOS), have been proposed as potential replacements. Both have desirable properties as plasticizers and minimal *in vitro* biological effects. Herein, we present an *in utero* and lactational exposure study comparing DEHP with BDB, DOS, and 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH), a commercial alternative. Timed-pregnant Sprague-Dawley rats were gavaged with vehicle or one of these chemicals at 30 or 300 mg/kg/day from gestational day (GD) 8 until postnatal day (PND) 21. The offspring were examined for effects on developmental and endocrine markers until PND 46. DEHP treatment (300 mg/kg) decreased heart weight in dams and induced a significant decrease in anogenital index and an increase in hemorrhagic testes and multinucleated gonocytes in PND 3 male pups. An increase in the incidence of hemorrhagic testes was also observed on PND 8 after exposure to DINCH (30 and 300 mg/kg). The only other effects observed were decreases in serum alanine transaminase and magnesium in BDB 30 exposed dams. These data suggest that both BDB and DOS are viable alternative plasticizers.

Introduction

Phthalates are used as emollients, matrices, solvents, and excipients in industrial applications[1]. Among these, di(2-ethylhexyl) phthalate (DEHP) is commonly used as an additive to provide flexibility to polyvinyl chloride (PVC). DEHP is found in a wide range of consumer products, including construction materials, toys, packaging films and sheets, medical tubing, and blood storage bags[2,3]. Since phthalates are not covalently bound to PVC, they leach from their matrices[4] and become ubiquitous environmental contaminants[5]. Numerous studies have reported human exposure to these compounds[2,6]. Despite their relatively rapid metabolism and excretion[7], individuals are continuously exposed to these chemicals orally and, less commonly, by dermal, inhalational, and parenteral routes[8]. Early-life exposure to phthalates and their metabolites during pregnancy and early infancy also occurs as analytes are routinely detected in amniotic fluid[9], umbilical cord blood[10], and breast milk[11].

Maternal exposure to phthalates during pregnancy is of concern based on data from numerous animal studies that have investigated effects on offspring[12,13]. Decreased testosterone production[14,15], hemorrhagic testes[16], multinucleated gonocytes in the seminiferous tubules[14,16], decreased anogenital indices[14,16], and nipple retention[16] have been reported in the male offspring of dams exposed to DEHP during gestation. In females, phthalates have been shown to alter ovarian and oocyte development, folliculogenesis, the functionality of ovarian follicles and corpora lutea, and ovarian steroidogenesis (reviewed in[13]). Some studies have noted that the effects of DEHP and its metabolites extend beyond the reproductive system[17]. Gestational exposure has been reported to impair pancreatic β-cell function[18] and fetal lung maturation[19], and to decrease blood pressure and locomotor activity[20]. In many cases, these effects have been observed many months after treatment was completed, suggesting that early exposures may have life-long effects; even when the chemical or its metabolites are no longer detectable in biological samples.

Ethical restrictions, the combinatorial effects of other chemicals, and retrospective assessments of DEHP exposure[21] have made human studies on the effects of phthalates particularly challenging. Despite these limitations, epidemiological studies indicate that phthalate exposure in humans is positively correlated with effects on genital development, including

decreases in anogenital index and semen quality, steroidogenic defects[22-24], impaired cognitive and thyroid function, and respiratory problems (reviewed in [25]); many of these perturbations are associated with gestational exposure.

The growing body of evidence in support of the deleterious effects of phthalates has prompted legislation to protect consumers and limit daily exposure to these chemicals, particularly at an early age. The implementation of these measures has motivated the search for safer replacements. Several commercial alternatives for DEHP have entered the market; 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH) is one such alternative, but little is known about its potential toxicity. Previous studies have proposed 1,4 butanediol dibenzoate (BDB) and dioctyl succinate (DOS) as potential alternatives for DEHP; both compounds have desirable plasticizer properties, are relatively inexpensive to synthesize, and are biodegradable[26-28]. Both BDB and DOS have been shown to be devoid of toxicity in *ex vivo* organ cultures and *in vitro* functional and toxicogenomic assays[29,30]. A 28-day acute toxicity study in post-pubescent male Sprague-Dawley rats demonstrated that neither of these plasticizers showed acute toxicity at doses of 15 or 150 mg/kg[31].

The identification and characterization of toxicity in susceptible populations during critical windows of development is key to finding safe alternatives for phthalate plasticizers. The gestational and lactational windows of exposure represent a time when cellular identity and function are established via a myriad of signalling cascades, thereby making them vulnerable to xenobiotics[32]. The potential burden of early life exposure may be further amplified by the immature metabolic and excretion pathways of neonates[33]. These inherent vulnerabilities make fetuses and newborns an important population to examine when assessing the toxicity of a replacement compound for phthalate plasticizers.

Herein, we hypothesized that *in utero* and lactational exposure of three replacements for DEHP (BDB, DOS, and DINCH) would have fewer or no deleterious effects on the general health of offspring exposed during gestation and lactation, and would not have the endocrine disruptive effects that target the reproductive system and are characteristic of DEHP. The goal of these studies was to identify suitable alternative plasticizers that could be used for the manufacturing of PVC based plastics.

Materials and Methods

Chemicals and Reagents

Di-2-ethylhexyl phthalate (DEHP) was purchased from Sigma-Aldrich Corporation (CAS #117-81-7; Cat#80030, St. Louis, MO). 1,2-Cyclohexane dicarboxylic acid diisononyl ester (DINCH) (CAS # 474919-59-0 and 166412-78-8) was supplied by BASF Canada (Mississauga, ON). 1,4 butanediol dibenzoate (BDB) and dioctyl succinate (DOS) were synthesized as previously described[26,28,56]. Chemical structures are shown in **Figure 23a**. The purity of BDB and DOS was determined to be 99% by NMR analysis (unpublished data). Chemicals were stored in a vacuum chamber with desiccant at room temperature until mixed with corn oil (Catalogue #C8267; Lot#MKBN5383V, Sigma-Aldrich).

Animal Husbandry

All manipulations and terminal procedures were in accordance with protocol #7317, approved by the McGill University Animal Care Committee. This experiment was divided into three cohorts. Virgin female and proven-breeder male Sprague-Dawley rats were purchased from Charles River Laboratories (St-Constant, Quebec). Animals were kept on a 12-hour light/dark cycle and provided food and water *ad libitum*. Females in proestrus were placed in mating cages that contained two females and one male rat. Sperm positive females were placed in individual cages the next morning; this was considered gestational day (GD) 0. Animals were randomly assigned to vehicle (corn oil, Sigma-Aldrich), DEHP, DINCH, BDB, or DOS treatment groups on GD 8, when treatment began; each treatment was prepared daily. Animals were weighed and administered doses of 30 or 300 mg/kg by gavage. The lower dose (30 mg/kg) is representative of high human exposure to DEHP[57] with an adjustment for interspecies metabolism[58]. The higher dose (300 mg/kg) was selected based on previous literature that reported deleterious reproductive outcomes following exposure to DEHP during gestation and lactation[37]. Thus, the treatment doses were chosen based on studies with DEHP; since DOS and BDB readily biodegrade and have slower leaching rates than DEHP, we expect that if these chemicals were released into the environment human exposures would be lower than for DEHP. Control animals were administered 1 ml of corn oil; this was the maximum volume administered for any treatment. Dams were treated daily, with the exception of post-natal day (PND) 0 when the litters were not

disturbed. Treatment continued until weaning, which occurred on PND 21. Terminal and developmental endpoints are summarized in **Figure 23b**.

Inclusion Criteria

All animals that produced a litter were included in the study until PND 3 regardless of litter size. At PND 3, litters were culled to eight pups each. Litters with less than six pups at this time were excluded from future endpoints (**Table 7**).

Terminal Endpoints

Pups were euthanized at PND 3 and PND 8 by inducing hypothermia followed by decapitation. Dams and pups from PND 21 onwards were euthanized by CO_2 asphyxiation followed by cardiac puncture. Organs were weighed and collected for histology and RNA isolation.

Developmental Endpoints

Anogenital Index (AGI)

Average pup body weight (BW) over 10 seconds was determined using a NewClassic MF balance (Mettler Toledo, Mississauga, ON). The anogenital distance (AGD) was measured using a pair of Vernier digital calipers, as previously described[59]. AGI is defined as $AGD/(BW)^{1/3}$. AGI was calculated for each pup; the litter was considered as one unit and litter data were averaged for each treatment group.

External Malformations and Developmental Endpoints

At PND 3, each pup was closely examined for external malformations. Examiners were blinded as to the treatment group. The inspection included the head, digits, body, and tail. Several hallmarks of development were recorded over the study period; these included crown-rump length, pinnae detachment (PND 3), incisor eruption (PND 8), eye opening (PND 13), nipple retention (PND 13), vaginal opening (PND 25-43) and preputial separation (PND 35-46), as previously described[60]. Litter index represents the average percentage of littermates that completed either vaginal opening or preputial separation by PND 38 or 47 for females and males, respectively.

Tissue Processing

Histology

Organs were collected and fixed in Modified Davidson's fixative (30% of a 37–40% solution of formaldehyde, 15% ethanol, 5% glacial acetic acid, and 50% distilled water) for 24 hours at room temperature. Larger organs were cut into smaller pieces for better fixation. The next day, the fixative was removed and replaced with 70% ethanol for storage at 4°C.

RNA Isolation

Organs were collected, placed in cryogenic tubes, and immediately immersed in liquid nitrogen. Entire frozen testes from PND 21 rats were mechanically disrupted using a Polytron PT 10-35 GT homogenizer (Kinematica, Bohemia, NY) in a 5 ml conical tube containing 4 ml of RTL buffer (Qiagen, Toronto, ON). Based on the weight of the testes prior to freezing, an aliquot of the homogenate was collected and re-suspended in additional RLT buffer so that the amount of homogenized tissue did not surpass 30 mg/ml of RLT buffer. Entire frozen ovaries from PND 21 were processed similarly to testicular samples, except that the entire ovary was homogenized in 1 ml RLT buffer.

To ensure proper homogenization, tissue samples were further processed with a QIAshredder column (Qiagen) prior to RNA isolation with an RNeasy Mini Kit (Qiagen). RNA isolation was done as per the manufacturer's instructions with the optional on-column gDNA elimination step. All RNA samples had RIN values greater than 9.8 measured using the Bioanalyzer 6000 (Agilent Technologies, Santa Clara, CA) and 260/280 and 260/230 ratios greater than 1.95 and 2.0 respectively, measured by NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific, Wilmington, DE).

Blood Collection and Processing

Whole blood was collected by cardiac puncture using a 1 ml syringe and 25G 5/8 inch needle at PND 21 and a 10 ml syringe with 21G 1-1/2 inch needle at PND 46. Whole blood was collected in a BD Vacutainer SST tube (Becton, Dickinson and Company, Mississauga, ON) by negative pressure. The tubes were inverted several times and allowed to clot at room temperature for 30 minutes. To isolate serum, tubes were spun at 1000 x g in an Allegra-X 15R benchtop

centrifuge (Beckman Coulter, Pasadena CA) at 4°C with a SX4750 swinging bucket rotor. Serum was aliquoted and kept at -80°C until further use.

Serum Gonadotropin and Hormone Levels

Serum estradiol, progesterone and testosterone levels were assessed using enzyme-linked immunosorbent assay kits (reference numbers IB79103, IB79105 and IB79106, respectively; IBL America, Minneapolis, MN) according to the manufacturer's instructions. LH and FSH levels were assessed by the Ligand Assay & Analysis Core of the University of Virginia School of Medicine Center for Research in Reproduction using Millipore Pituitary Panel Multiplex kits (EMD Millipore, Saint Charles, MO).

Serum Biochemistry

Analyte concentrations were determined with a Vitros 250 (Ortho-Clinical Diagnostics, Markham, ON) with dry-slide matter.

***Ex-vivo* Organ Culture and Radioimmunoassay**

Testes from PND 3 pups were collected and cut into 5-10 fragments to maintain tissue integrity during short-term culture. Fragments were placed on a 60µm nylon mesh, which in turn was placed in a 24-well plate with a 12mm Millicell Cell Culture Insert (Catalogue #PICM01250, EMD Millipore, Etobicoke, ON). Each well contained 300µl of DMEM/Ham's F12 (1:1) supplemented with GlutaMax (Gibco) and 80µg/ml of gentamycin (basal media) (Sigma-Aldrich). Samples were incubated at 37°C with 3.5% CO_2. Fragments were kept at the air-liquid interface.

All testicular fragments were maintained in culture with basal media for the initial 24 hours. The left testis from each animal provided fragments for the basal condition, while the right testis fragments were stimulated with 50ng/ml human chorionic gonadotropin (hCG) on days two and three of culture. On the third day, fragments were fixed in a 4% paraformaldehyde solution (PFA) (Biolynx, Affymetrix Inc., Cleveland, OH) and sectioned for histology/immunohistochemistry. The medium was collected to quantify testosterone with an in-house radioimmunoassay kit, as previously described [30].

Morphometric Analysis of Gonocytes

PND 3 testes were paraffin embedded. Serial sections of 4µm were rehydrated and stained with H&E histological stain. Slides were digitally scanned with an Aperio AT Turbo (Leica Microsystems Inc., Concord, Ontario) at 40x magnification. To quantify the number of multinucleated gonocytes, the outlines of tubules were traced using ImageJ[61]. Only perpendicular cross-sections were quantified. To determine if a tubule was perpendicular, the major and minor axis had to be within 10% of each other. Gonocytes were considered within the focal plane if they had clearly defined cytoplasm and borders.

Quantitative qPCR

Using the RT^2 First Strand Kit (Qiagen) 400ng of total RNA was cleaned-up of potential genomic DNA contamination, and converted to cDNA as per manufacturer's instructions. A portion of cDNA was mixed thoroughly with the 2x RT2 SYBR Green Mastermix to prepare 75 reactions as per kit proportions. The mixture was pipetted into a frosted 384-well Custom RT^2 PCR Primer Array (Supplemental **Table 13**, Custom Array #CLAR21524; Qiagen) using a Janus Standard Workstation (Perkin Elmer, Woodbridge, ON) equipped with Varispan Arm and 500 µL dispensing syringe. Gene transcripts were selected based on previous findings. Several steroidogenic enzymes were selected for their role in cholesterol transport (Star, Tspo, Scarb1) or in the biotransformation of steroid hormones (Cyp11a1, Hsd3b1, Cyp17a1, Hsd17b3, Cyp19a1, Srd5a1, Srd5a2). Transcripts involved in feedback mechanisms expressed in the testis and ovary (Fshr, Lhcgr), and secreted signalling molecules (Inhba, Inhbb) were quantified to assess the integrity of the hypothalamic-pituitary-gonadal axis. Many of these transcripts have been reported to be differentially modulated following phthalate exposure[15,36,62,63]. In addition to steroidogenesis, receptors for steroid (Ar, Esr1, Esr2) or xenobiotic signalling (Ahr), transcription factors important for gonadal function and development (Kitl, Sf1, Rhox5, Nr5a2), and genes of reproductive interest modulated by phthalates[64,65] (Insl3, Gja1) were also assessed.

The plates were sealed and immediately run on with a Bio-Rad CFX384 (Bio-Rad, Saint-Laurent, QC). PCR was initiated by holding the temperature at 95°C for 10 minutes. This was followed by 40 cycles of 95°C for 15 seconds with a ramp speed of 1°C/sec and 60°C for 1 minute. A melt curve was generated to ensure the specificity of the PCR reaction. All targets had

a single melt curve peak. Threshold cycle values were determined using CFX Manager v3.1 (Bio-Rad Laboratories, Hercules, CA) software by a baseline subtracted curve fit followed by regression analysis. All samples were pipetted as technical duplicates, and each treatment had six biological replicates. Samples with a standard deviation greater that 0.35 (or Ct difference of more than 0.5) or a Ct greater than 35 were not included in the analysis due to high variation. The housekeeping genes (*Ppia*, *Hprt*) were averaged to form a single housekeeping entity for normalization purposes. Relative quantities of each target were determined by dividing the ΔCt values for each target by the geometric mean of the control biological replicates[66].

Statistical Analyses

The biological replicates reported are at the level of the litter. Significance was determined by ANOVA followed by Dunnett's post hoc test for continuous variables. For categorical variables, Fisher's exact test was used. For qPCR data, relative quantities were log transformed prior to ANOVA followed by Dunnett's multiple range test to account for different numerical ranges of downregulated versus upregulated genes. Outliers due to biological differences have not been removed from any of these data. Statistical calculations and visual representations were generated using GraphPad Prism 6.07 (GraphPad Software, La Jolla, CA). The data are presented without a family-wise error correction.

Results

The effects of DINCH, BDB and DOS **(Figure 23a)**, three plasticizers that are currently in use or have potential as alternatives to DEHP, were investigated in timed-pregnant Sprague Dawley rats exposed during gestation and lactation **(Figure 23b)**.

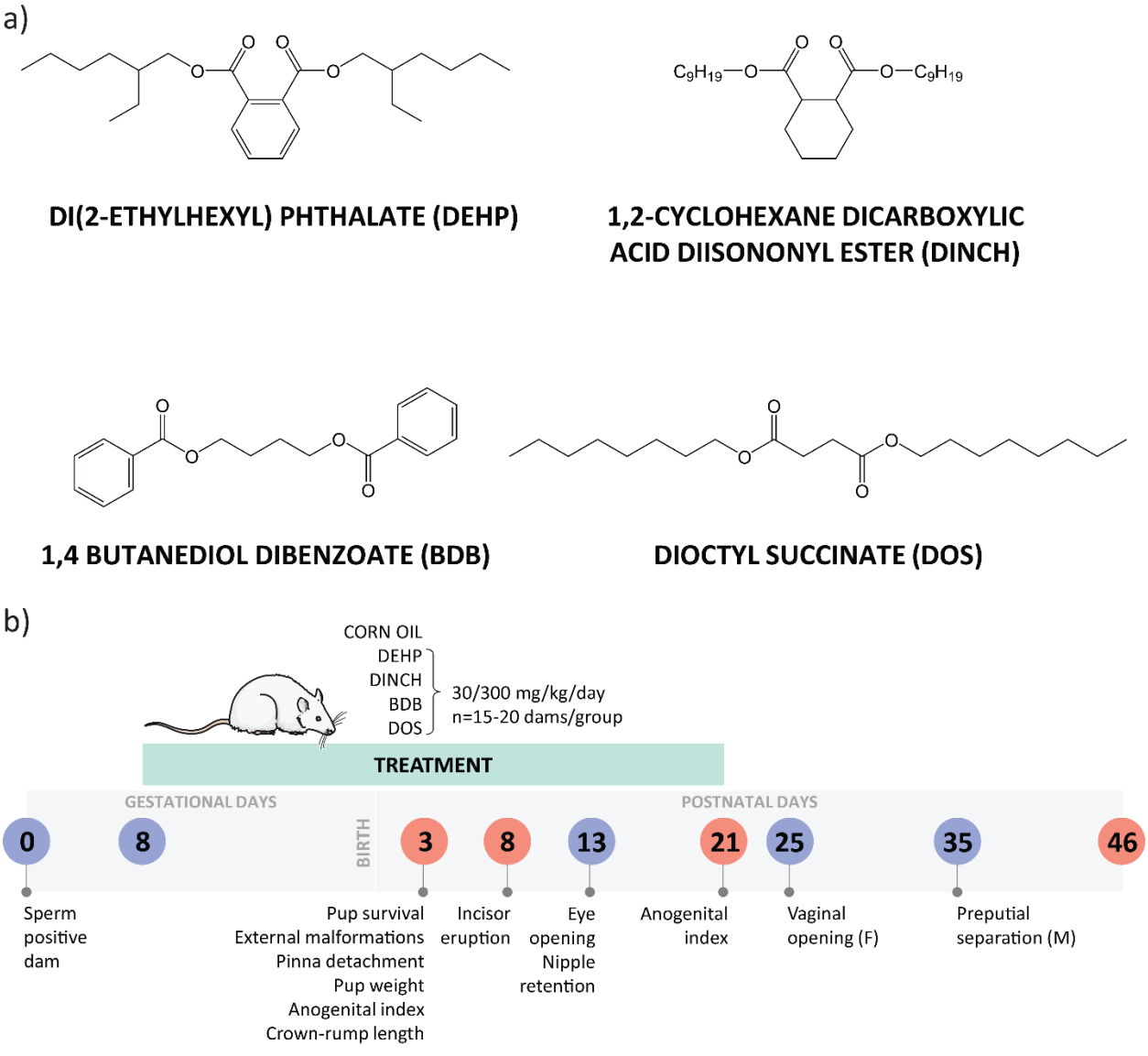

Figure 23: Chemical Structures and Experimental Design. *(a) Chemical structures of each plasticizer. DEHP is the current and most commonly used phthalate plasticizer. DINCH is a commercial alternative that is being marketed as a safer replacement. BDB and DOS are two*

alternatives that have been developed and tested, but are not commercially available for use. (b) Experimental schematic showing key endpoints. Timed pregnant dams were treated from GD8 until weaning with one of nine treatments (including control). Pups were observed for reproductive and developmental endpoints; on the specified days one pup from each litter was selected at random for necropsy. The rat illustration (http://www.servier.com/slidekit/?item=13) is from Servier Medical Arts Powerpoint Image Bank, Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

Maternal Health

The overall health of dams was assessed during the course of treatment and at necropsy on postnatal day (PND) 21. Five dams died spontaneously over the course of the study but these animals were not restricted to any particular treatment group; one rat was from the control group, one from DINCH 30 (30 mg/kg/day), two from DINCH 300 (300 mg/kg/day), and one from the BDB 30 (30 mg/kg/day) treatment group. Necropsies indicated that the cause of death for one rat (BDB 30) may have been gavage error, but did not reveal any cause of death for the others. The remaining dams all appeared healthy, gained weight and did not show physical symptoms of distress. At the time of necropsy there was no significant effect of treatment on body weight **(Figure 24a).** While dam liver, kidney, lung, spleen, ovary, and uterus weights were unaffected by treatment **(Figure 24a and Supplemental Table 8)**; a decrease in heart weight was observed in the DEHP 300 group **(Figure 24a)**.

Serum was collected to identify changes in biomarkers associated with pathogenesis. Among the serum biomarkers analyzed, only alanine transaminase and magnesium were affected and only in the serum of BDB 30 dams (**Figure 24b, c and Supplemental Table 9**). While an increase in alanine transaminase typically is associated with liver pathogenesis, a decrease in this enzyme is not considered to have untoward effects. The small (~10%) but significant decrease in serum magnesium may represent mild kidney dysfunction. In either case, complementary markers of kidney and liver function were unchanged.

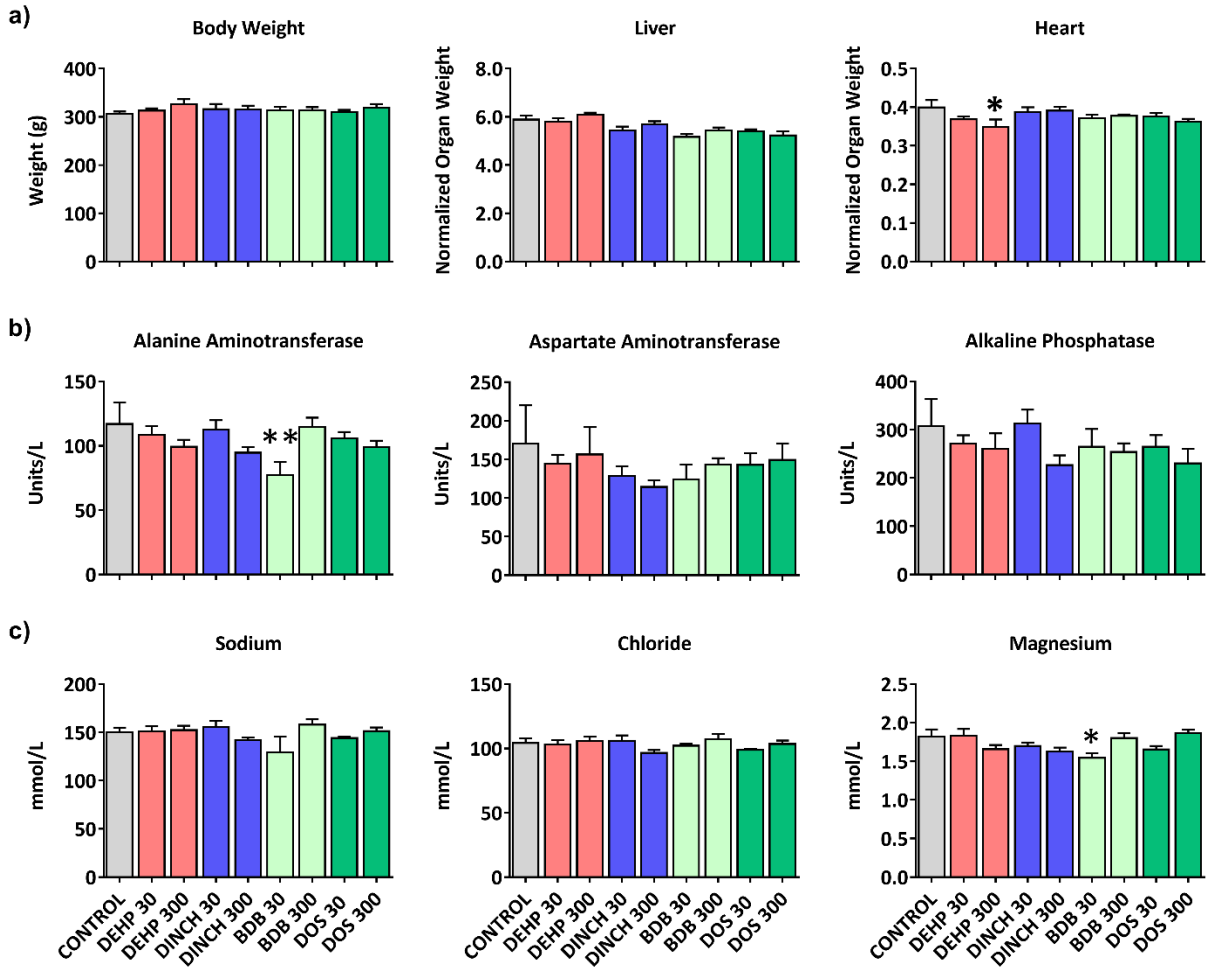

Figure 24: Organ Weight and Serum Parameters of Dams at Weaning. *(a) Animal weight and organ weights normalized to body weight are reported. n=11-16. Bars represent means ± SEM. One-way ANOVA, post-hoc Dunnett's, *p≤0.05. (b) Selection of serum biomarkers related to liver function (alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase) function. (c) Electrolytes measured from serum samples. n=10. Bars represent means ± SEM. One-way ANOVA, post-hoc Dunnett's, *p≤0.05, **≤0.01.*

Pregnancy Outcome

Several endpoints were used to determine whether exposure during pregnancy affected pregnancy outcome. Uteri from postpartum dams were collected and implantation scars within the uterine horns were counted to determine the number of implantation sites. The numbers of

viable pups were counted on PND 3 and their sex was determined. There were no significant effects of treatment on post-implantation loss, pup survival, or sex ratio, suggesting that litter characteristics were not affected (**Table 7**).

Table 7: Dam and Litter Parameters[3]

Treatment Group	Treated Animals	Animals Included	Implantation Scars/Litter	Pups/Litter (PND 3)
CORN OIL	19	14	15.4 ± 0.8	12 ± 3.7
DEHP 30	17	14	14.7 ± 0.8	13 ± 3.4
DEHP 300	18	12	13.8 ± 1.1	11 ± 4.1
DINCH 30	19	11	13.4 ± 1.1	10 ± 4.7
DINCH 300	16	13	16.4 ± 0.4	13 ± 3.0
BDB 30	16	12	16.0 ± 0.5	13 ± 3.5
BDB 300	17	16	15.2 ± 0.4	13 ± 3.2
DOS 30	17	14	14.5 ± 0.9	13 ± 3.2
DOS 300	15	14	14.5 ± 1.3	13 ± 4.6

There were no effects of treatment on the numbers of external malformations observed on PND 3 in any treatment group, but one pup was missing a tail (DEHP 300) and two pups appeared to have hydrocephalus (DOS 30, DOS 300). Pup weights and crown-rump lengths on PND 3 were not affected by treatment **(Supplemental Table 10)**. Developmental markers, such as incisor eruption and pinnae detachment, were not affected by treatment. At PND 21 and 46, there were

[3] Dam and litter parameters: All animals were sperm positive on GD 0 and were assigned to a group on GD 8 when treatment began. Only litters with greater than six pups met the inclusion criteria for the study's endpoints from GD 8-46. To ensure that the effect of small litters was not due to treatment, implantation scars were counted by transillumination of uteri dissected from dams at the time of weaning (PND 21). Pups that survived to PND 3 were also counted. There was no significant effect of treatment on litter size or pup survival. Values reported are the means ± SEM.

no effects of treatment on organ weights (liver, spleen, kidney, heart, lung, ovary, uterus, epididymis or testis; **Supplemental Table 11** and

Table 12).

Endocrine and Reproductive Effects on Female Offspring

Functional markers of reproductive development and endocrine function were monitored in female pups. There were no significant effects of treatment on anogenital index (AGI) in female pups at PND 3 and 21 **(Figure 25a)**. Vaginal opening was monitored daily as a marker of pubertal onset. While the age and weight of vaginal opening averaged within a litter did not change, the litter index at PND 38 (defined as the mean percentage of pups in a litter with completed vaginal opening at PND 38) was significantly decreased in the DEHP 300 group **(Figure 25b)**.

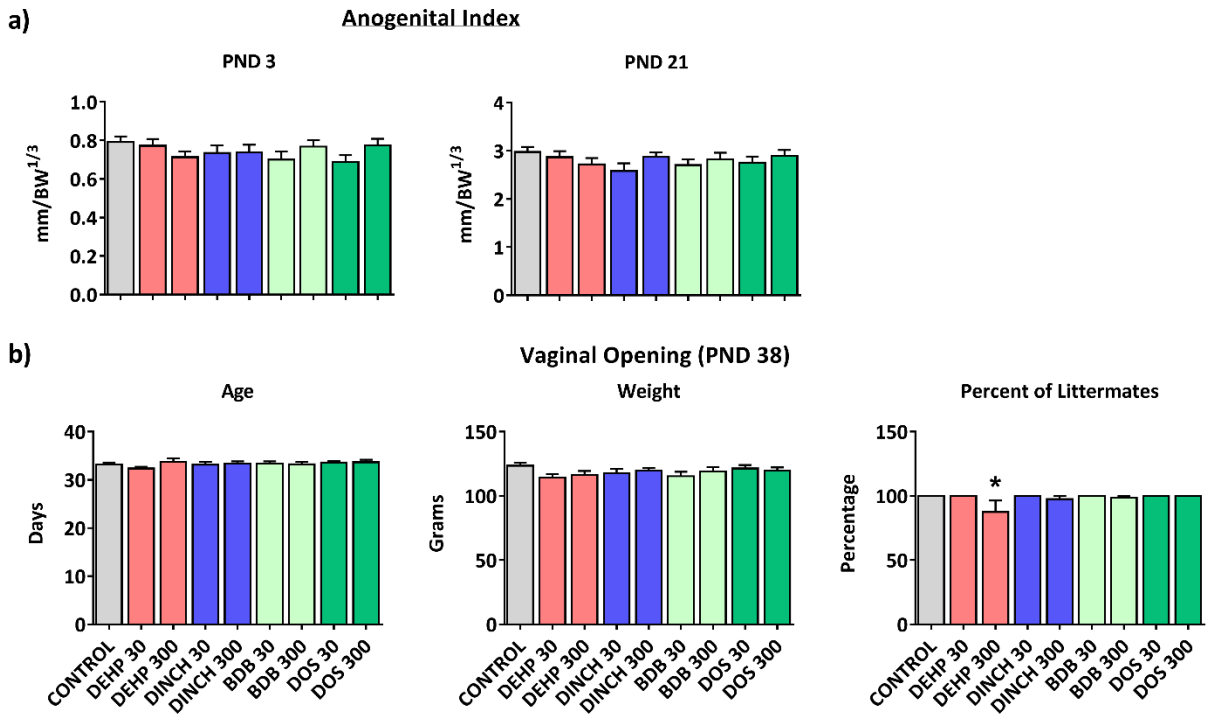

Figure 25: Female Markers of Endocrine Function. (a) Anogenital index of female pups at PND 3 and 21. (b) Average age and weight at the time of vaginal opening. This panel also

includes the average percent of littermates that completed vaginal opening by PND 38. Bars represent means ± SEM; n=11-16. One-way ANOVA, post-hoc Dunnett's, *p≤0.*05*

Serum hormone levels were measured at PND 21 to determine whether exposure affected steroid hormone production. While LH and FSH concentrations were not affected by any treatment, there was a significant increase in progesterone following treatment with BDB 30 at PND 21; however, this observation should be interpreted with caution as two values that were included in the analysis differed greatly from the mean and thus may be considered as outliers **(Figure 26)**. Estradiol levels were below the detection limit of the assay on PND 21.

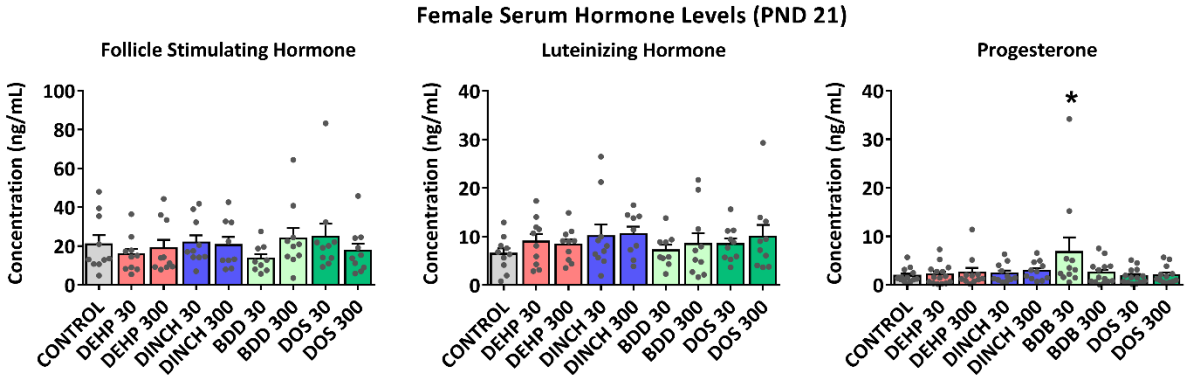

Figure 26: Serum Hormone Levels in Female Offspring at PND 21. *Each grey point represents an animal selected at this time-point for necropsy. Bars represent means ± SEM; n=10. One-way ANOVA, post-hoc Dunnett's, *p≤0.05*

A quantitative PCR gene panel was used to investigate the potential impact of DEHP and the alternative plasticizers on the female reproductive system. The expression of steroidogenic enzymes remained unchanged in all treatment groups **(Supplemental Figure 31)**. Furthermore, there were no significant changes in the expression of genes important for reproductive function **(Supplemental Figure 32)**.

<u>Endocrine and Reproductive Effects on Male Offspring</u>

Functional markers of reproductive development and androgen action were monitored in male pups. AGI was significantly decreased at PND 3 in the DEHP 300 mg/kg treatment group, but was unchanged at PND 21 **(Figure 27a)**. The presence of retained nipples at PND 13, a

marker of impaired androgen action, was not significantly altered **(Figure 27b)**. Preputial separation was also monitored as a marker of the onset of puberty **(Figure 27c)**. There was no significant change in preputial separation measured at PND 46.

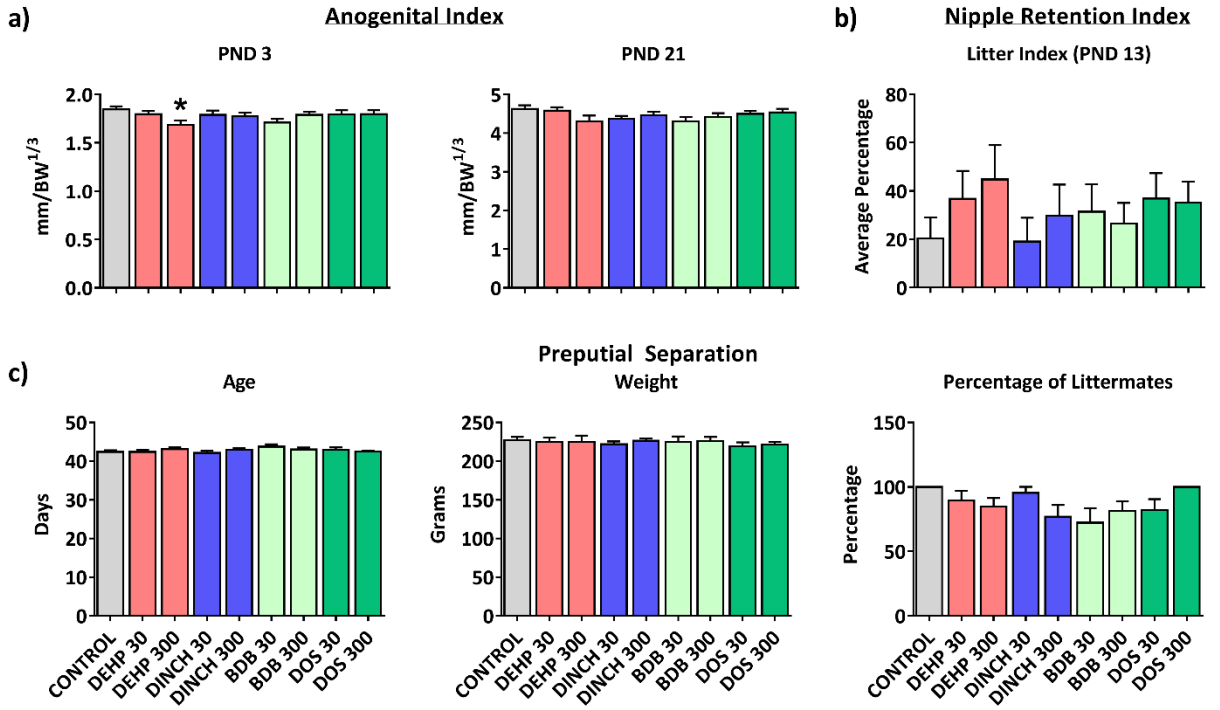

Figure 27: Markers of Male Endocrine Function. *(a) AGI at PND 3 in male pups. (b) Nipple retention in male pups at PND 13. (c) Average age and weight at the time of preputial separation. This panel also includes the average percent of littermates that completed preputial separation on or before PND 46. Bars represent means ± SEM; n=11-16. One-way ANOVA, post-hoc Dunnett's, *p≤0.05*

As development of the perineum is dependent on androgen action, basal and hormone-stimulated testosterone production were measured in PND 3 testes using an *ex vivo* testicular culture system. There was no significant change in testosterone production under either stimulated or basal conditions **(Figure 28a)**. Each animal provided material for the basal and stimulated conditions, therefore average fold change per animal was also calculated, but was not significant. Serum was also collected from male offspring to determine whether there was an

effect of treatment on steroidogenesis. There were no significant changes in serum FSH, LH, or testosterone at PND 21 **(Figure 28b)** in any treatment group.

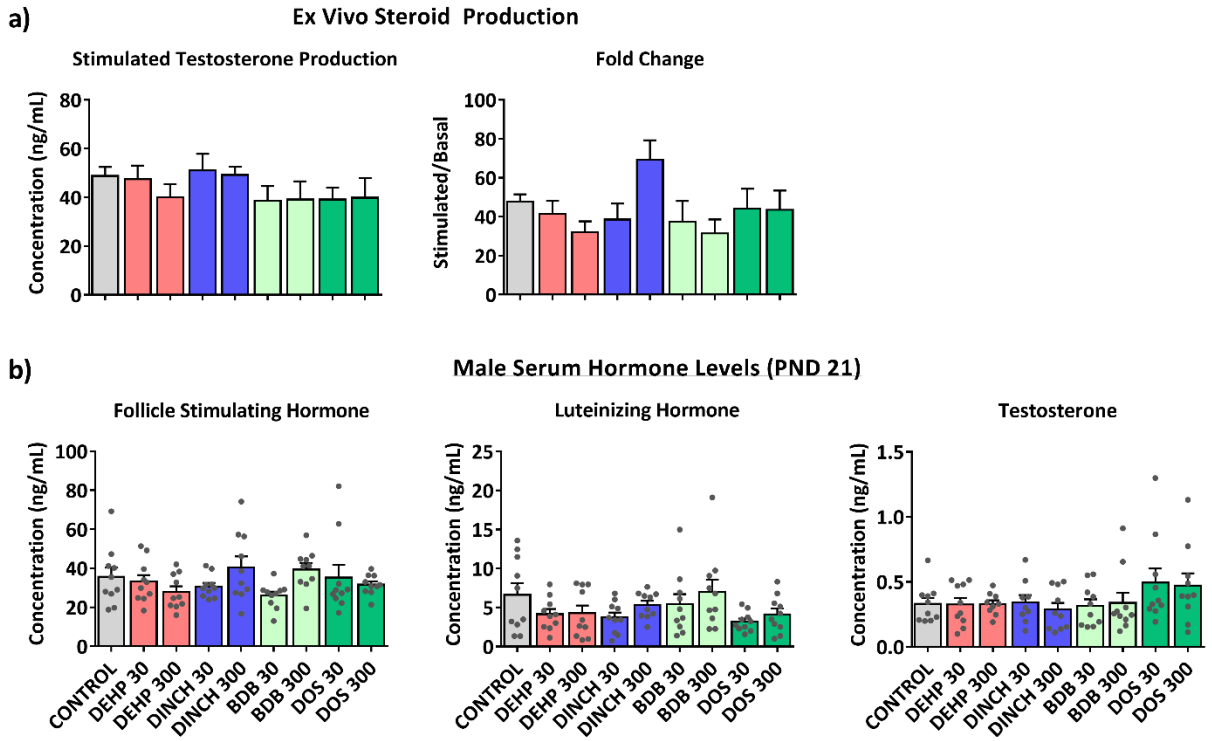

Figure 28: Assessment of Steroidogenesis. *(a) Ex vivo testosterone production assay. PND 3 testes from each animal were tested in both the basal and stimulated conditions. Fold changes (stimulated/basal) were determined for each animal and averaged across the treatment group. Bars represent means ± SEM; n= 7-11. One-way ANOVA, post-hoc Dunnett's (b) Serum hormone levels in male pups at PND 21. Bars represent means ± SEM; n= 10. One-way ANOVA, post-hoc Dunnett's. Grey points represent an individual animal.*

The potential impact of plasticizers on testicular gene expression was examined using a quantitative PCR gene panel. On PND 21 an increase in Hsd17b3 expression was observed in the BDB 30 group **(Supplemental Figure 33)**. There were no significant effects on the expression of other steroidogenic enzymes or other genes of reproductive relevance **(Supplemental Figure 34)**.

We also investigated the effects of plasticizers on testicular histology. The presence of multinucleated gonocytes in seminiferous tubules has been associated with exposure to DEHP in several studies; this has been observed in the absence of measurable decreases in testicular testosterone[34]. **Figure 29** shows representative histological sections from PND 3 testes from: (a) control; (b) DEHP 300, with an insert highlighting a multinucleated gonocyte; (c) DINCH 300; (d) BDB 300; and (e) DOS 300 offspring. The number of normal gonocytes was not affected by treatment **(Figure 29f)**. However, we observed a significant increase in the number of multinucleated gonocytes in the testes of offspring treated with 300 mg/kg/day of DEHP (14-fold compared to control; $p \leq 0.0001$) **(Figure 29g)**. No increase in multinucleated gonocytes was observed in any of the other treatment groups. Hemorrhagic spots or patches were observed in testes on PND 3 and 8. Testes were considered hemorrhagic if they had red spots, patches, or were fully hemorrhagic upon dissection. A significant increase in the incidence of hemorrhagic testes was observed at PND 8 in the DEHP 300 treatment group **(Figure 30c)**. Interestingly, the DINCH 30 and DINCH 300 treatment groups also showed a significant increase in the incidence of hemorrhagic testes; this was not significant in other treatment groups. At PND21, the presence of hemorrhagic testis was not observed, and testicular histology did not show any major abnormalities in any treatment group (not shown).

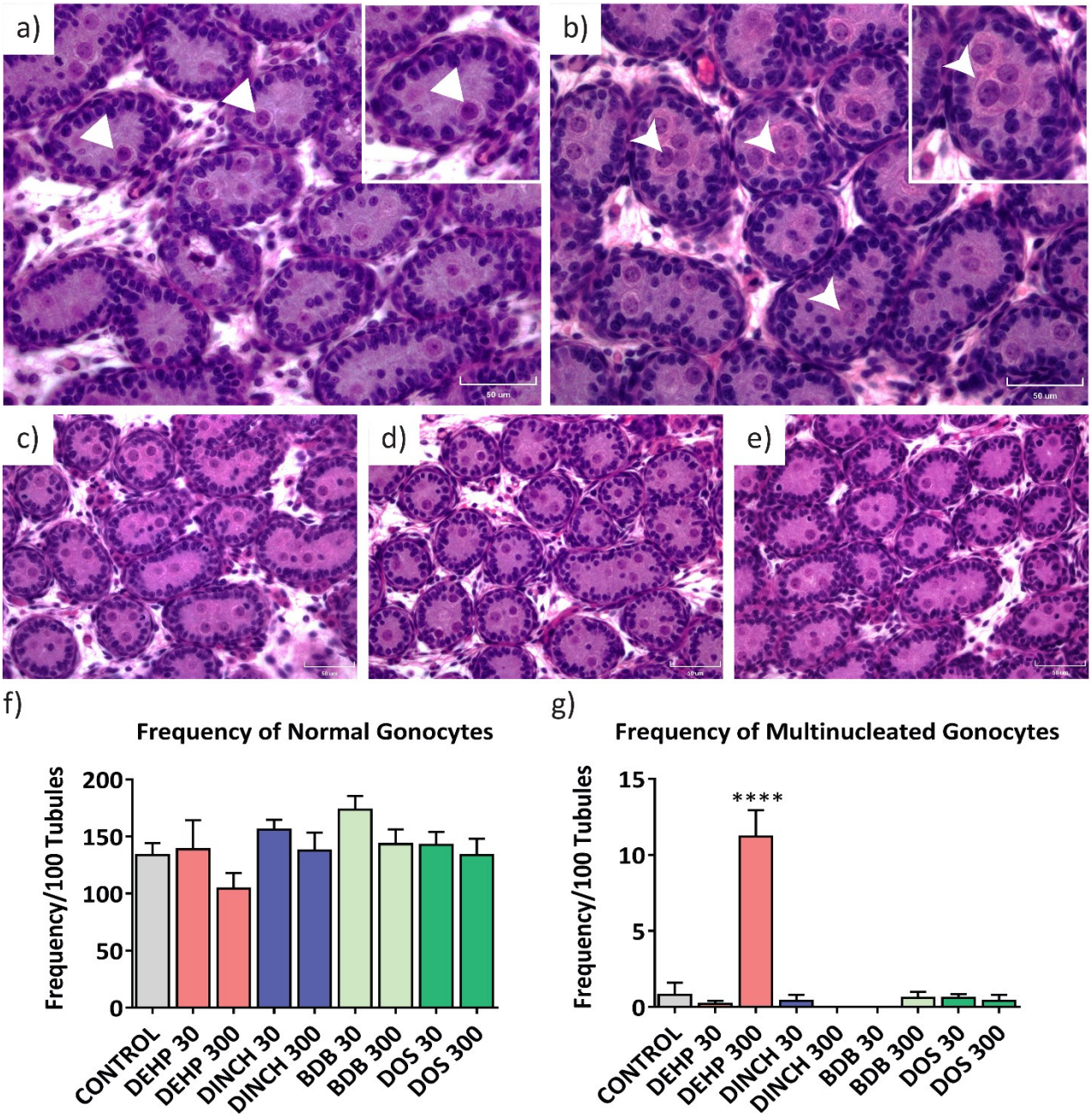

Figure 29: Histological Examination of Testes at PND 3. *High quality photomicrographs of PND 3 testes following exposure to (a) corn oil, or 300 mg/kg: (b) DEHP, (c) DINCH, (d) BDB, and (e) DOS (e). Normal gonocytes are indicated with an asterisk; multinucleated gonocytes are indicated with white arrowheads. (f) Multinucleated and (g) normal gonocytes were quantified from 100 perpendicular cross sections of seminiferous tubules. Bars represent means ± SEM, n=5. Scale bar represents 50µm. One-way ANOVA, post-hoc Dunnett's, ****p≤0.0001.*

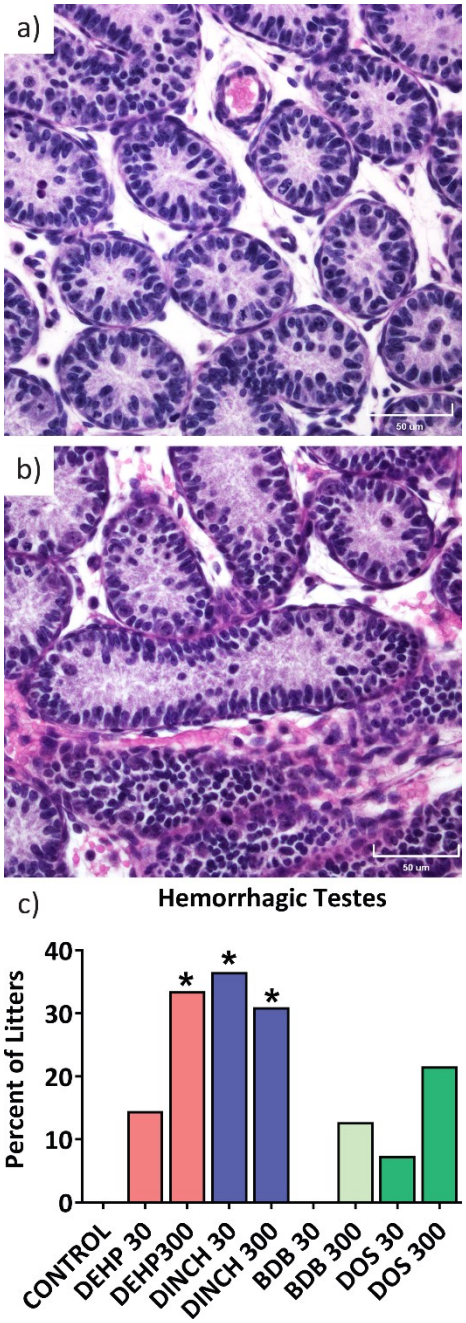

Figure 30 Histological analysis of testes at PND 8. *High quality photomicrographs of PND 8 testes displaying (a) normal histology or (b) partial hemorrhage. The occurrence of testicular hemorrhage at PND 8 was quantified (c). Values are percent of litters with partial or full hemorrhage. Scale bar represents 50µm. n=11-16. Fisher's exact test, *p≤0.05.*

Discussion

Herein, we present an in utero and lactational exposure study comparing DEHP and 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH) with two candidate alternative plasticizers, BDB and DOS, chosen on the basis of their plasticizing, leaching and biodegradability properties[26-28], and minimal biological activities in immortalized cell lines derived from male reproductive tissues[29,30]. We report that *in utero* and lactational exposure to BDB and DOS did not produce the endocrine disruptive phenotype classically described after exposure to DEHP. While DINCH had no effects on many of the endpoints associated with DEHP exposure, it did significantly increase the incidence of hemorrhagic testes in exposed offspring.

Treatment with 300 mg/kg/day DEHP significantly decreased the anogenital index and increased the number of multinucleated gonocytes in PND 3 male rat offspring; on PND 8, this exposure significantly increased the incidence of hemorrhagic testes. Our experimental model is clearly responsive to the effects of DEHP since we observed an effect on the anogenital index at a lower dose (300 mg/kg/day) than that used in a number of previous studies in the literature (750 mg/kg/day)[14,16]. Previous studies reported a decrease in testosterone production after *in vitro*[35,36] and *in utero*[15] exposure to DEHP or its monoester metabolite, MEHP. We did not note any significant alteration of testosterone production by *ex vivo* testicular explants, in serum testosterone levels, or in measures of biologically active testosterone such as seminal vesicle weights on PND 21 and 46 **(Supplemental Table 11 and Table 12)**. Differences between our study and previous studies may be due to the dose, window of exposure, and/or strain of rats used; all of these parameters are potential sources of discrepancy in the study of anti-androgenic compounds[12]. While profound effects on male reproductive function have been reported after treatment with DEHP at doses upwards of 750 mg/kg, it is also clear that deleterious effects on physiology that extend beyond the reproductive system have been observed after treatment with lower doses of DEHP and other phthalates[37,38]. Exposures in this study started as early as GD8 to target a wide window during embryo and fetal development that includes a period of susceptibility during the development of multiple organ systems. Many studies characterizing the anti-androgenic effects of *in utero* phthalate exposures start treatment at GD14, a time that is critical for androgen action on gonadal differentiation[39,40]. Finally, Sprague-Dawley rats were

selected as an outbred model that is commonly used in toxicology studies. While there are differences in response to phthalates between rat strains, steroidogenesis in Sprague-Dawley rats is perturbed following DEHP exposure, resulting in abnormalities in androgen dependent organs[41]. These perturbations in steroidogenesis are similar to those observed in studies with human testicular explants[42], making the Sprague-Dawley rat an appropriate model to assess perturbances in steroidogenic functions caused by phthalates and their potential replacements.

The effects of phthalates as endocrine disruptors in female animals have been largely unexplored. This is of particular concern as women at all ages are exposed to phthalates[9]. Here, DEHP (300 mg/kg/day) delayed the completion of vaginal opening, a marker of sexual maturation, in female offspring. Such a delay was described previously in Wistar rats after *in utero* and lactational exposure to DEHP[43]. These findings highlight the need for additional research on mechanisms by which phthalate plasticizers act as endocrine disruptors of female reproductive function.

Interestingly, DEHP also caused a decrease in heart weight in dams. The literature on phthalates and cardiac function is limited. Isolated cardiomyocytes treated with DEHP *in vitro* have been reported to have increased levels of reactive oxygen species from fatty acid metabolism, which can possibly result in an increased susceptibility for ischemic heart injury and ventricular dysfunction[44]. DEHP has also been reported to dysregulate electrical conduction and mechanical contraction in isolated neonatal cardiomyocytes by altering gene expression[45,46]. These findings suggest that further work is needed to understand the impact of DEHP on cardiomyocytes and heart function.

We report a significant increase in the occurrence of testicular hemorrhage in male offspring at PND 8 after exposure to high dose DEHP and to both the low and high doses of DINCH. DINCH was first introduced to the European market in 2002, received final approval from the European Food Safety Authority in 2006, and has been marketed since as a safe phthalate replacement. Nevertheless, there is limited knowledge about its safety or potential adverse health effects although widespread exposure, as assessed by increasing urinary levels of DINCH metabolites, has been documented[47,48]. Recent *in vitro* studies suggest that DINCH is a bioactive compound[29], a potent metabolic disruptor[49], and an endocrine disruptor[30]. The increase

in hemorrhaging testes that we observed after DINCH exposure occurred at doses that were below or equal to the no observed adverse effect level (NOAEL) for parenteral exposure[50]. Together, these data suggest a need for a deeper exploration of the possible endocrine disruptive properties of DINCH.

Importantly, BDB and DOS, our candidate compounds, did not significantly increase the incidence of classically described endocrine disruptive phenotypes. Among the significant effects produced by our compounds, the decrease in alanine transaminase and magnesium in pregnant dams treated with 30 mg/kg/day BDB is an unusual finding. An increase in serum alanine transaminase is commonly used as a biomarker of liver damage[51]. The finding of a decrease in alanine transaminase, accompanied by a lack of significant change in other biomarkers of liver damage, is inconsistent with the possibility of liver toxicity. Hypomagnesemia may cause perturbations in most organ systems as magnesium is a key activator for enzymatic reactions involving phosphorus[52]. Magnesium homeostasis is regulated in the gastrointestinal tract, where it is absorbed, and in the kidneys, which ultimately determine whether it is eliminated or reabsorbed in the proximal tubules[52]. Again, this finding of a small but significant change in serum magnesium is difficult to interpret as there is no indication from other biomarkers or electrolytes that there is kidney impairment and the animals were otherwise healthy and gained weight. In both cases, further studies may be warranted.

There were negligible changes in gene expression at PND 21 in the testes or ovaries of offspring. Hsd17b3 was the only gene to be significantly upregulated in this study, following treatment with BDB 30 in male rats. While there are several isoforms of this enzyme, Hsd17b3 is expressed primarily in the testes and is a marker of adult Leydig cells[53]. Furthermore, this enzyme preferentially converts androstenedione to testosterone[53]. This change in Hsd17b3 gene expression was not associated with any effects on serum testosterone, *ex vivo* testosterone production, or androgen-sensitive markers, suggesting that testosterone biosynthesis is unaffected in this model.

With an annual economic burden estimated at $340 billion in the United States[54] and more than €150 billion in Europe[55], endocrine disruptors have become one of the most prominent public health issues in modern society. In previous studies, we identified two novel candidate

plasticizers, BDB and DOS, with desirable plasticizing properties and biodegradability, minimal leaching, and an absence of toxicity in several immortalized cell lines. Here we demonstrate that *in utero* and lactational exposure to these compounds produced fewer or no significant adverse effects compared to DEHP. Both plasticizers have been subject to more extensive screening than most new chemicals, thereby promoting the use of responsible replacements for future generations.

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Supplemental Figures & Tables

Table 8: Dam Organ Weights at Necropsy[4]

Weight (g)	CORN OIL	DEHP30	DEHP300	DINCH30	DINCH300	BDB30	BDB300	DOS30	DOS300
Spleen	0.19 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.20 ± 0.01
Kidney (Paired)	0.77 ± 0.01	0.78 ± 0.01	0.81 ± 0.02	0.79 ± 0.02	0.78 ± 0.02	0.79 ± 0.01	0.77 ± 0.01	0.75 ± 0.01	0.80 ± 0.02
Ovaries (Paired)	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
Uterus	0.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.14 ± 0.02	0.12 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
Lungs	0.53 ± 0.04	0.46 ± 0.02	0.48 ± 0.02	0.47 ± 0.01	0.52 ± 0.03	0.50 ± 0.02	0.47 ± 0.02	0.50 ± 0.02	0.49 ± 0.03

[4] Table of organ weights normalized per every 100g of body weight. None of the treatments significantly affected these organ weights in the dams necropsied at the end of lactation. Values are means ± SEM. One-way ANOVA with Dunnett's post-hoc test; n=11-16.

Table 9: Dam Serum Parameters [5]

	CORN OIL	DEHP30	DEHP300	DINCH30	DINCH300	BDB30	BDB300	DOS30	DOS300
Total Protein (g/L)	59 ± 1	61 ± 2	61 ± 2	62 ± 2	59 ± 2	57 ± 2	65 ± 2	59 ± 2	62 ± 2
Albumin (g/L)	35 ± 1	36 ± 2	35 ± 1	36 ± 2	34 ± 2	32 ± 1	37 ± 1	34 ± 2	35 ± 1
Albumin/Globulin Ratio	1.4 ± 0.0	1.4 ± 0.0	1.3 ± 0.0	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.0	1.4 ± 0.0	1.3 ± 0.0
Glucose (mmol/L)	12.7 ± 1.1	12.6 ± 1.0	11.3 ± 0.7	12.2 ± 0.8	12.6 ± 0.8	11.6 ± 0.4	13.4 ± 1.0	11.4 ± 0.7	13.3 ± 1.1
Blood Urea Nitrogen (mmol/L)	9.5 ± 0.8	9.6 ± 0.4	10.2 ± 0.7	10.3 ± 0.4	8.7 ± 0.6	8.1 ± 0.8	10.3 ± 0.8	9.7 ± 0.3	9.5 ± 0.3
Creatinine (µmol/L)	33 ± 2	32 ± 2	33 ± 2	33 ± 2	31 ± 1	29 ± 2	33 ± 2	31 ± 2	36 ± 4
Creatine Kinase (U/L)	248 ± 73	142 ± 12	188 ± 56	139 ± 17	171 ± 38	796 ± 611	170 ± 29	159 ± 21	262 ± 83
Cholesterol (mmol/L)	2.61 ± 0.10	2.73 ± 0.13	2.30 ± 0.10	2.64 ± 0.15	2.44 ± 0.17	2.88 ± 0.22	2.81 ± 0.17	2.30 ± 0.08	2.46 ± 0.09
Potassium (mmol/L)	7.3 ± 0.7	6.4 ± 0.5	6.7 ± 0.4	6.5 ± 0.4	6.5 ± 0.3	6.8 ± 0.4	6.8 ± 0.5	6.1 ± 0.3	6.3 ± 0.4
Calcium (mmol/L)	3.05 ± 0.08	3.01 ± 0.17	3.00 ± 0.14	3.13 ± 0.16	2.86 ± 0.06	2.89 ± 0.07	3.15 ± 0.16	2.82 ± 0.04	2.92 ± 0.05
Phosphorus (mmol/L)	2.93 ± 0.34	2.56 ± 0.39	2.68 ± 0.17	2.67 ± 0.17	2.47 ± 0.17	3.10 ± 0.45	2.59 ± 0.18	2.14 ± 0.16	2.77 ± 0.25

[5] None of the treatments significantly affected these serum parameters in the blood collected by cardiac puncture from dams necropsied at the end of lactation. Values are means ± SEM. One-way ANOVA with Dunnett's post-hoc test; n=10.

Table 10: Markers of Pup Growth at Postnatal Day (PND) 3[6]

	CORN OIL	DEHP30	DEHP300	DINCH30	DINCH300	BDB30	BDB300	DOS30	DOS300
MALE									
Weight (g)	8.75 ± 0.36	8.93 ± 0.27	9.12 ± 0.41	9.45 ± 0.4	8.22 ± 0.19	8.14 ± 0.28	8.31 ± 0.34	8.65 ± 0.31	8.34 ± 0.29
CRL (mm)	51.24 ± 0.69	51.44 ± 0.59	51.7 ± 0.79	52.02 ± 0.62	50.31 ± 0.28	49.94 ± 0.48	50.19 ± 0.62	50.82 ± 0.47	50.48 ± 0.53
FEMALE									
Weight (g)	8.45 ± 0.37	8.49 ± 0.25	8.54 ± 0.42	8.96 ± 0.36	7.72 ± 0.2	7.86 ± 0.3	7.89 ± 0.28	8.3 ± 0.31	8.05 ± 0.32
CRL (mm)	50.05 ± 0.7	50.13 ± 0.54	50.35 ± 0.87	51.14 ± 0.58	48.97 ± 0.35	48.82 ± 0.62	48.98 ± 0.54	49.81 ± 0.52	49.46 ± 0.6

[6] Average weight and crown-rump length (CRL) of male and female pups at PND3. All pups within a litter were measured and averaged prior to averaging across litters within the same treatment (i.e. the biological replicate is the litter). No significant changes were observed in either parameter. Values are means ± SEM. One-way ANOVA with Dunnett's post-hoc test; n=11-16.

Table 11: Organ Weights of Male and Female Pups at PND 21[7]

	CORN OIL	DEHP30	DEHP300	DINCH30	DINCH300	BDB30	BDB300	DOS30	DOS300
MALES									
Weight (g)	59.9 ± 1.6	59.3 ± 1.5	57.7 ± 2.7	58.5 ± 1.6	57.5 ± 1.2	52.2 ± 2.1	57.0 ± 1.6	59.1 ± 1.1	55.8 ± 2.1
Liver	4.41 ± 0.07	4.46 ± 0.12	4.36 ± 0.11	4.34 ± 0.10	4.35 ± 0.09	4.13 ± 0.10	4.43 ± 0.06	4.45 ± 0.07	4.07 ± 0.23
Spleen	0.29 ± 0.01	0.28 ± 0.02	0.30 ± 0.03	0.29 ± 0.02	0.28 ± 0.01	0.23 ± 0.02	0.27 ± 0.02	0.28 ± 0.01	0.27 ± 0.02
Kidneys(Paired)	1.25 ± 0.03	1.24 ± 0.02	1.25 ± 0.02	1.25 ± 0.02	1.23 ± 0.02	1.29 ± 0.04	1.24 ± 0.03	1.23 ± 0.02	1.27 ± 0.03
Heart	0.66 ± 0.01	0.66 ± 0.02	0.67 ± 0.02	0.69 ± 0.02	0.67 ± 0.02	0.72 ± 0.03	0.68 ± 0.04	0.66 ± 0.01	0.67 ± 0.02
Lungs(Paired)	1.32 ± 0.07	1.24 ± 0.04	1.47 ± 0.14	1.28 ± 0.0	1.40 ± 0.10	1.33 ± 0.044	1.42 ± 0.08	1.38 ± 0.07	1.30 ± 0.06
Testes (Paired)	0.26 ± 0.01	0.26 ± 0.01	0.25 ± 0.02	0.25 ± 0.01	0.25 ± 0.01	0.23 ± 0.01	0.24 ± 0.01	0.26 ± 0.01	0.24 ± 0.01
Epididymides (Paired)	0.13 ± 0.02	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
Seminal Vesicles(Paired)	0.04 ± 0.02	0.02 ± 0.00	0.03 ±v0.01	0.02 ± 0.00	0.02 ± 0.00	0.04 ± 0.02	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
FEMALES									
Weight (g)	56.3 ± 1.5	57.9 ± 1.6	53.9 ± 2.4	55.8 ± 1.6	55.7 ± 1.4	53.4 ± 1.5	54.3 ± 1.5	57.2 ± 1.4	54.9 ± 2.2
Liver	4.29 ± 0.11	4.37 ± 0.09	4.3 ± 0.08	4.3 ± 0.08	4.26 ± 0.09	4.14 ± 0.08	4.35 ± 0.09	4.46 ± 0.07	4.27 ± 0.08
Spleen	0.27 ± 0.01	0.29 ± 0.01	0.27 ± 0.02	0.28 ± 0.02	0.26 ± 0.02	0.24 ± 0.02	0.26 ± 0.02	0.30 ± 0.01	0.27 ± 0.02
Kidneys(Paired)	1.26 ± 0.02	1.28 ± 0.01	1.24 ± 0.03	1.27 ± 0.03	1.24 ± 0.02	1.28 ± 0.03	1.23 ± 0.03	1.21 ± 0.02	1.31 ± 0.02
Heart	0.61 ± 0.04	0.65 ± 0.01	0.67 ± 0.02	0.66 ± 0.02	0.65 ± 0.02	0.62 ± 0.02	0.67 ± 0.03	0.62 ± 0.01	0.64 ± 0.02
Lungs(Paired)	1.13 ± 0.07	1.30 ± 0.07	1.35 ± 0.12	1.31 ± 0.06	1.37 ± 0.06	1.20 ± 0.05	1.29 ± 0.14	1.25 ± 0.09	1.27 ± 0.07
Ovaries(Paired)	1.13 ± 0.07	1.30 ± 0.07	1.35 ± 0.12	1.31 ± 0.06	1.37 ± 0.06	1.20 ± 0.05	1.29 ± 0.14	1.25 ± 0.09	1.27 ± 0.07
Uteri	0.06 ± 0.00	0.12 ± 0.05	0.07 ± 0.00	0.07 ± 0.00	0.06 ± 0.01	0.11 ± 0.05	0.06 ± 0.00	0.11 ± 0.04	0.11 ± 0.05

[7] Table of organ weights normalized per every 100g of body weight. One pup from each litter was selected at random and necropsied. No significant changes were observed in any organ weight parameters. Values are means ± SEM. One-way ANOVA with Dunnett's post-hoc test; n=11-16.

Table 12: Organ Weights of Male and Female Pups at PND46 [8]

	CORN OIL	DEHP30	DEHP300	DINCH30	DINCH300	BDB30	BDB300	DOS30	DOS300
MALES									
Weight (g)	259.8 ± 5.1	255.6 ± 4.7	256.9 ± 8.3	258.9 ± 4.9	257.1 ± 5.5	238 ± 3.8	252.4 ± 6	244.9 ± 5.5	254.6 ± 5.7
Liver	5.59 ± 0.10	5.59 ± 0.11	5.50 ± 0.14	5.67 ± 0.17	5.73 ± 0.11	5.50 ± 0.12	5.72 ± 0.10	5.40 ± 0.09	5.59 ± 0.13
Spleen	0.30 ± 0.01	0.28 ± 0.01	0.31 ± 0.01	0.30 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.31 ± 0.01	0.35 ± 0.06	0.32 ± 0.02
Kidneys	2.71 ± 0.06	2.74 ± 0.10	2.69 ± 0.08	2.75 ± 0.06	2.70 ± 0.06	2.40 ± 0.06	2.57 ± 0.10	2.37 ± 0.16	2.73 ± 0.07
Heart	0.48 ± 0.01	0.52 ± 0.03	0.51 ± 0.02	0.50 ± 0.01	0.50 ± 0.01	0.50 ± 0.01	0.50 ± 0.02	0.48 ± 0.01	0.49 ± 0.01
Lungs	0.66 ± 0.02	0.70 ± 0.03	0.72 ± 0.03	0.69 ± 0.02	0.74 ± 0.03	0.72 ± 0.03	0.78 ± 0.04	0.73 ± 0.03	0.76 ± 0.04
Testes	2.45 ± 0.06	2.40 ± 0.10	2.48 ± 0.06	2.47 ± 0.06	2.43 ± 0.05	2.43 ± 0.05	2.26 ± 0.07	2.37 ± 0.06	2.41 ± 0.06
Epididymides	0.15 ± 0.00	0.16 ± 0.01	0.15 ± 0.01	0.15 ± 0.00	0.15 ± 0.00	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.00
Seminal Vesicles	0.15 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.12 ± 0.01	0.16 ± 0.01	0.14 ± 0.01
FEMALES									
Weight (g)	187.4 ± 3.6	185.4 ± 4.3	179.4 ± 5.1	188.7 ± 2.9	181.2 ± 4.5	179.4 ± 4.4	184.9 ± 6.1	183.1 ± 3.4	181.6 ± 4.4
Liver	5.23 ± 0.11	5.12 ± 0.15	5.23 ± 0.08	5.19 ± 0.14	5.27 ± 0.09	5.01 ± 0.10	5.21 ± 0.09	5.16 ± 0.07	5.19 ± 0.09
Spleen	0.28 ± 0.01	0.25 ± 0.01	0.29 ± 0.01	0.33 ± 0.07	0.27 ± 0.01	0.27 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.29 ± 0.01
Kidneys	1.94 ± 0.06	1.91 ± 0.05	1.86 ± 0.04	1.74 ± 0.13	1.82 ± 0.04	1.81 ± 0.06	1.90 ± 0.07	1.84 ± 0.04	1.86 ± 0.05
Heart	0.46 ± 0.01	0.46 ± 0.01	0.49 ± 0.01	0.48 ± 0.01	0.48 ± 0.01	0.48 ± 0.01	0.47 ± 0.01	0.46 ± 0.01	0.47 ± 0.01
Lungs	0.74 ± 0.04	0.72 ± 0.03	0.76 ± 0.05	0.72 ± 0.03	0.75 ± 0.03	0.69 ± 0.02	0.78 ± 0.06	0.75 ± 0.03	0.68 ± 0.04
Ovaries	0.05 ± 0.00	0.06 ± 0.01	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.07 ± 0.01	0.06 ± 0.00	0.06 ± 0.01	0.08 ± 0.02
Uteri	0.21 ± 0.02	0.17 ± 0.02	0.19 ± 0.01	0.19 ± 0.02	0.18 ± 0.01	0.18 ± 0.02	0.22 ± 0.02	0.16 ± 0.01	0.22 ± 0.02

[8] See footnote of previous table

Table 13: RT^2 Profiler Primers and Catalogue Numbers.

Gene Symbol	Catalogue Number	Gene Name	Refseq Accession
Ahr	PPR52899F	Aryl Hydrocarbon Receptor	NM_013149
Ar	PPR44497A	Androgen Receptor	NM_012502
Cyp11a1	PPR42479A	Cytochrome P450, Family 11, Subfamily A, Polypeptide 1	NM_017286
Cyp17a1	PPR44710A	Cytochrome P450, Family 17, Subfamily A, Polypeptide 1	NM_012753
Cyp19a1	PPR47164A	Cytochrome P450, Family 19, Subfamily A, Polypeptide 1	NM_017085
Eef2	PPR50864A	Eukaryotic Translation Elongation Factor 2	NM_017245
Esr1	PPR44939B	Estrogen Receptor 1	NM_012689
Esr2	PPR48980A	Estrogen Receptor 2	NM_012754
Fshr	PPR61699B	Follicle Stimulating Hormone Receptor	NM_199237
Gja1	PPR44801A	Gap Junction Protein, Alpha 1	NM_012567
Hprt1	PPR42247F	Hypoxanthine Phosphoribosyltransferase 1	NM_012583
Hsd17b3	PPR45110A	Hydroxysteroid (17-Beta) Dehydrogenase 3	NM_054007
Hsd3b1	PPR45361B	Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta- And Steroid Delta-Isomerase 1	NM_001007719
Inhba	PPR44530A	Inhibin Beta-A	NM_017128
Inhbb	PPR53036A	Inhibin Beta-B	NM_080771
Insl3	PPR50254C	Insulin-Like 3	NM_053680
Kitlg	PPR06678A	KIT ligand	NM_021843
Lhcgr	PPR45301B	Luteinizing hormone/choriogonadotropin receptor	NM_012978
Nr5a2	PPR49556A	Nuclear receptor subfamily 5, group A, member 2	NM_021742
Ppih	PPR57387B	Peptidylprolyl isomerase H	XM_001073803
Rhox5	PPR49786A		
Rpl13	PPR42351A	Ribosomal protein L13	NM_031101
Scarb1	PPR52707A	Scavenger receptor class B, member 1	NM_031541
Sf1	PPR42393A	Splicing factor 1	NM_058210
Srd5a1	PPR43427F	Steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	NM_017070
Srd5a2	PPR65848B	Steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2)	NM_022711
Star	PPR45414A	Steroidogenic acute regulatory protein	NM_031558
Tspo	PPR06787A	Translocator protein	NM_012515

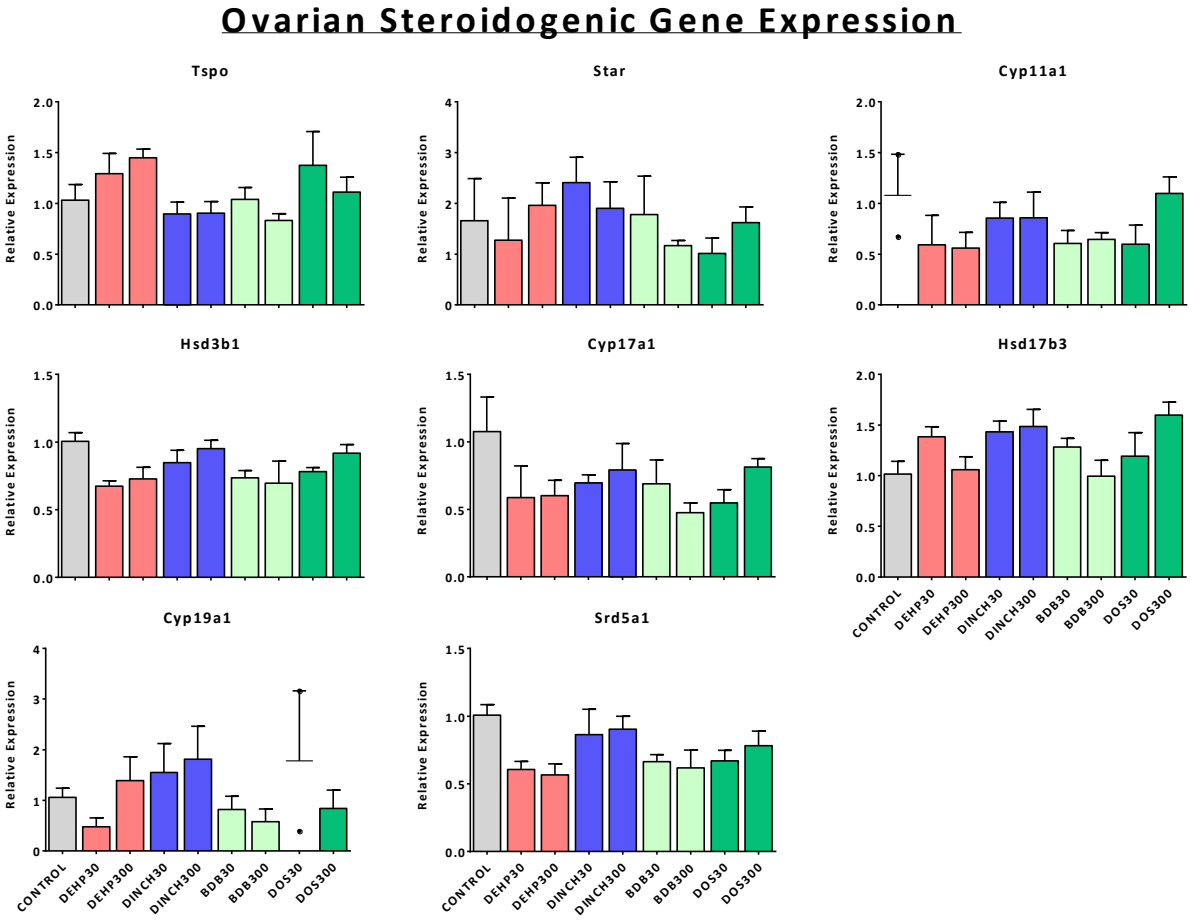

Figure 31: Steroidogenic Gene Transcripts (PND21 Ovary) *The data have not been shown if a transcript was not detected or the data are too variable (SD ≥ 0.35 between replicates). A full list of primers is provided as* ***Supplemental Table 13****. Bars represent means ± SEM; n=2-6. Single points show individual replicates when n<2. One-way ANOVA, post-hoc Dunnett's multiple range test.*

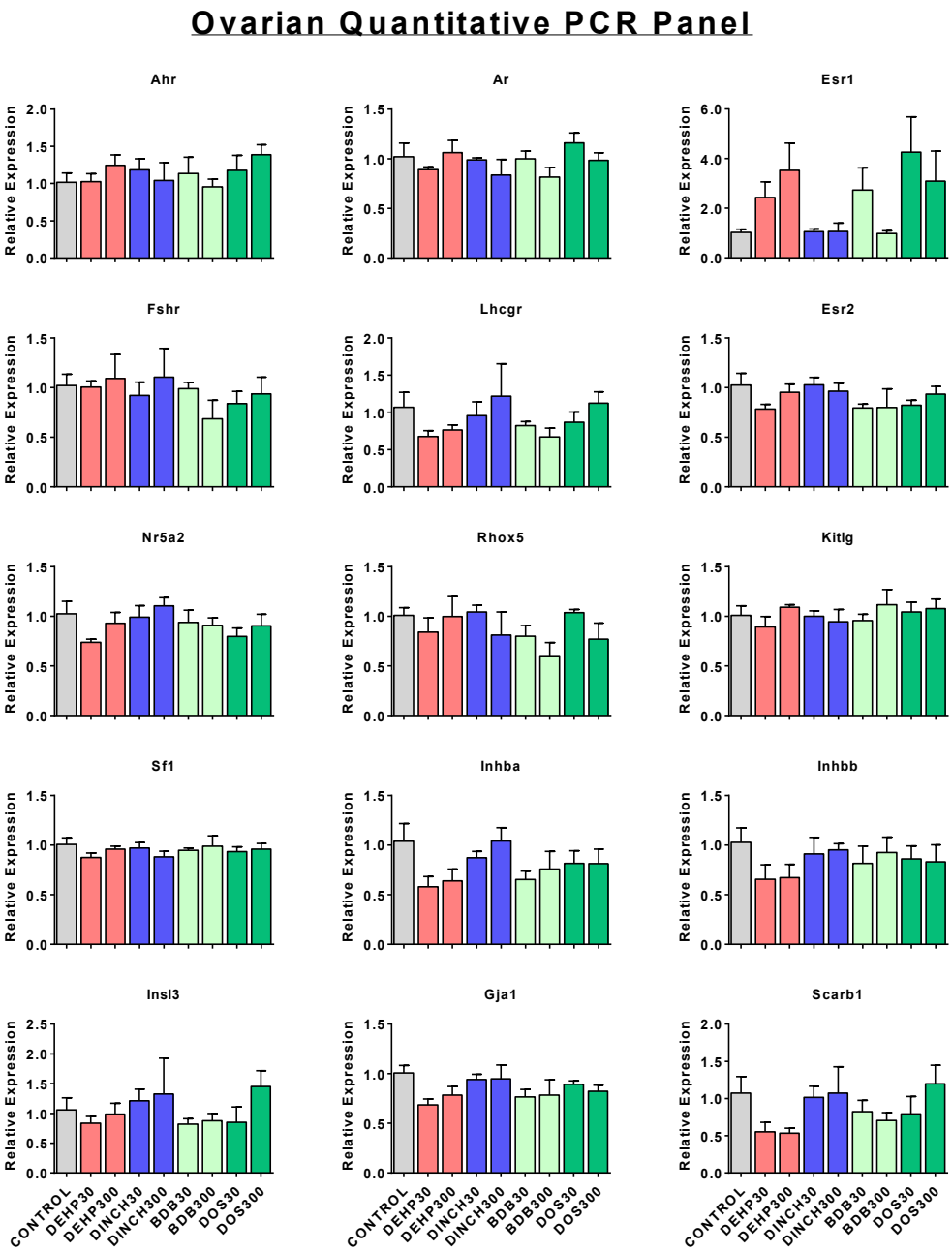

Figure 32: Gene Transcripts (PND21 Ovary) *The array includes gene transcripts that are important in reproduction and some targets that were previously reported to be affected by DEHP treatment. The data have not been shown if a transcript was not detected or the data are too variable (SD ≥ 0.35 between replicates). A full list of primers is provided as* ***Supplemental***

***Table 13**. Bars represent means ± SEM; n=2-6. Single points show individual replicates when n<2. One-way ANOVA, post-hoc Dunnett's multiple range test.*

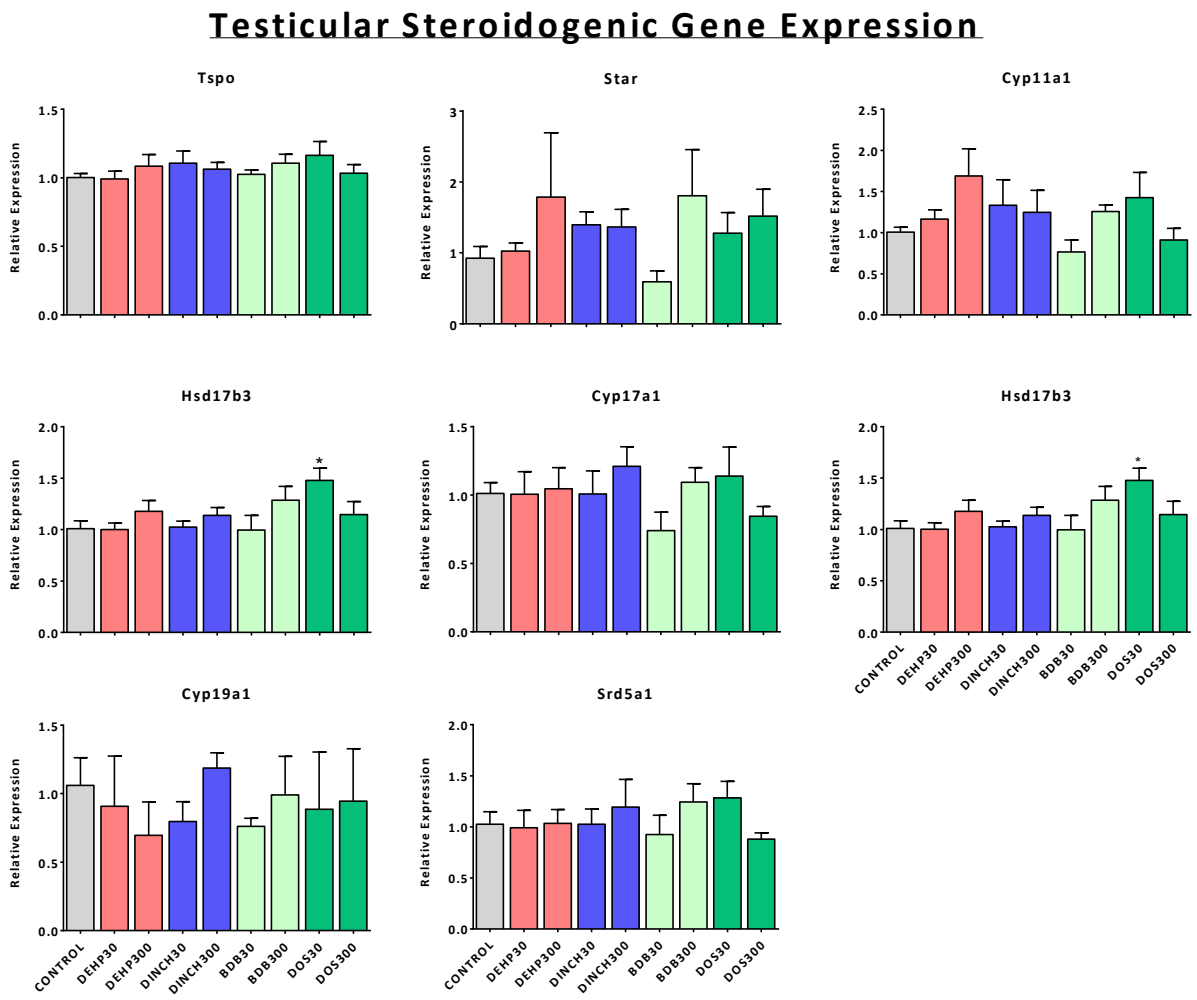

***Figure 33: Steroidogenic Gene Transcripts (PND21 Testis)** Quantification of gene transcripts involved in steroidogenesis in the PND 21 testes. The data have not been shown if a transcript was not detected or the data are too variable (SD ≥ 0.35 between replicates). A full list of primers is provided as **Supplemental Table 13**. Bars represent means ± SEM; n=2-6. Single points show individual replicates when n<2. One-way ANOVA, post-hoc Dunnett's multiple range test.*

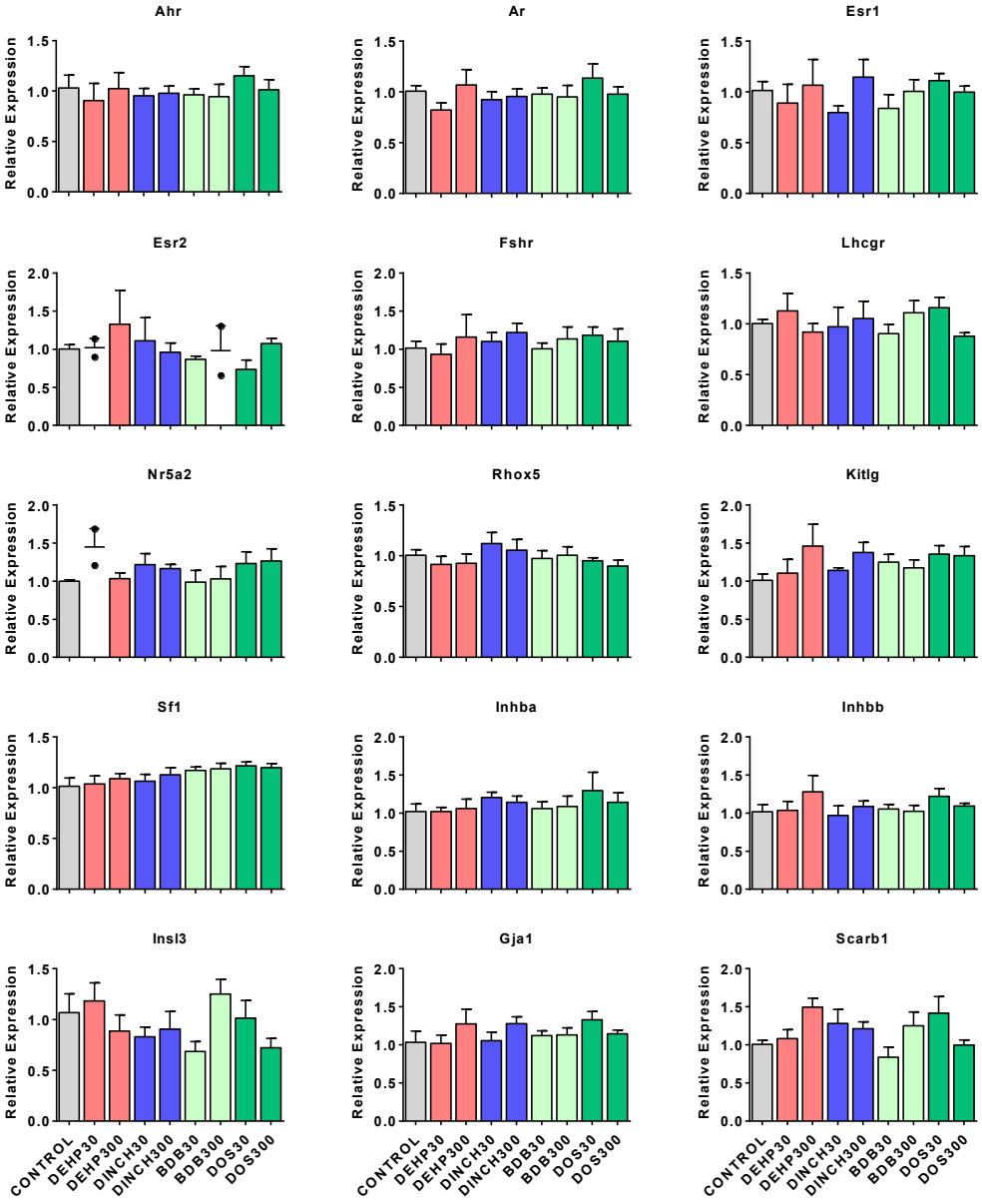

Figure 34: Gene Transcripts (PND21 Testis). *The array includes gene transcripts that are important in reproduction and some targets previously reported to be affected by DEHP treatment. The data have not been shown if a transcript was not detected or the data are too variable (SD ≥ 0.35 between replicates). A full list of primers is provided as* ***Supplemental Table 13****. Bars represent means ± SEM; n=2-6. Single points show individual replicates when n<2. One-way ANOVA, post-hoc Dunnett's multiple range test.*

Discussion

Endocrine disruptors are a burden to the health care system, and their continued use has environmental, financial, and social implications. As phthalate use is restricted or phased out by legislative measures and consumer demand, safer alternatives will be required to maintain product quality and standards expected by consumers. Identifying suitable replacements for phthalates is not a simple task as there are many factors to consider. Product development and innovation for a good part of the century has focused on optimizing phthalates for multiple applications, thereby making them a manufacturing staple and a baseline of comparison for plasticizing polymers. There is a consensus in the field that no one chemical or family of chemicals will replace all phthalates, therefore developing multiple plasticizers for different applications and plasticizer blends will allow manufacturers to make choices that benefit consumers in terms of cost and functionality of a product while maintaining reasonable margins on the products they sell.

1. Designing Green Chemicals

Chemical structure is a good predictor of a chemical's physical properties, but also provides valuable insight into its biological activity. Analyzing Quantitative Structure-Activity Relationships (QSAR) is a key strategy in toxicant screening and drug development, and there are several examples of its use in practice. Lipinski's Rule of Five is used by medicinal chemists to predict the oral absorption of drugs based on structural criteria[236]. In the field of endocrine active chemicals, QSAR successfully predicted estrogen receptor binding affinity of several chemicals[237].

One of the outcomes of the EPA ToxCast program has been the ability to predict chemical toxicity by matching chemical profiles with a dataset of thousands of chemicals that have been previously screened[238,239]. Using machine learning models and a library of empirically collected data, it is also possible to generate rules for designing new, safer chemicals[240].

Understanding how functional groups of a molecule influence its toxicological profile and functionality will be key towards identifying the next generation of safe "green" plasticizers. While the mechanism by which phthalates mediate their effects on steroidogenesis has yet to be

resolved, PPAR's have been proposed to play an important role in mediating these effects[184,186,188]. In an endogenous setting, PPAR's bind amphipathic carboxilates that closely resemble phthalate monoesters. Structural analysis of phthalate metabolites show that the presence of a carboxyl moiety with a long branched side-chain is more effective at activating PPAR than a shorter branched side-chain[241]. *In vivo* knockout studies show PPARα knockout animals do not have perturbed steroidogenesis, but still have a delayed "phthalate syndrome" phenotype[242]. While all three PPAR isoforms are expressed in the testes[185] there is only limited literature on the activity of the other two isoforms in mediating phthalate toxicity following PPARα knockout.

Realizing chemicals resembling phthalates are potent PPAR agonists, three series of chemicals containing ester groups (succinate, maleate, and fumarate based chemicals) which are important for plasticization[243], were selected for further studies. In order to optimize and understand how changes in molecular structure influences both toxicity and plasticizer properties, large amounts of empirical data were generated by our group to understand how the core molecular structure and varying alkyl chain length can influence these parameters. Overall, core-structure dictates toxicity of the chemical[224,244] while the length and branching of the alkyl chains conjugated to these core structures influences the efficiency of the plasticizer to lower the glass transition temperature of the polymer (and thereby function as an effective plasticizer)[226,245]. In the case of dibenzoates, the presence of an ether bond in diethylene glycol dibenzoate and dipropylene glycol dibenzoate resulted in the formation of toxic metabolites. By removing this group, biodegradability was enhanced without affecting plasticizer functionality[230–232].

While dioctyl succinate (DOS) and 1,4 butanediol dibenzoate (BDB) are good plasticizers in our opinion, with additional data on structure and toxicity one could theoretically further improve these chemicals by optimizing elements within their chemical structures.

2. Summary of Findings

These studies confirm previous findings and contribute to the literature on phthalate toxicity. PPAR signalling requires formation of a heterodimer with retinoid X receptor (RXR). RXR is also required for retinoic acid signalling. Following treatment with MEHP, RXR is

sequestered in the nucleus by PPAR, leaving RAR unable to translocate to the nucleus. Sertoli cells have an important role in Vitamin A regulation in the testes, and retinoic acid is an important signalling factor in gonocyte differentiation and spermatogonial recruitment. The upregulation of enzymes involved in the biosynthesis of retinoic acid from inactive precursors suggests a compensatory mechanism. Our study also reported an upregulation of genes involved in cholesterol biosynthesis, which is consistent with a previous study that reported the cholesterol levels in testicular tissue was increased in rats treated with DEHP[246]. From the gestational-lactational study, DEHP treated male animals had classically described markers of androgenic endocrine disruption (decrease in anogenital index in male pups) and testicular abnormalities (testicular hemorrhage and multinucleated gonocytes). While occurrences of testicular hemorrhage and multinucleated gonocytes have been previously described, their etiology is unknown. PPARs might have a role in mediating testicular hemorrhage as PPARs are expressed in testicular endothelial cells, and activation of PPARα and PPARγ can have differential effects on angiogenesis depending on the tissue[247]. Vascularization of the testes is an important aspect of testicular development, and many chemicals that cause male reproductive developmental toxicity are known to impair angiogenesis[248]. Similar to the abnormal vascularization of the testes, the occurrence of multinucleated gonocytes is not related to impaired androgen signalling[249]. It does however suggest abnormal gonocyte differentiation. This finding warrants further investigation because abnormal gonocyte differentiation may to be a key step in the progression of testicular cancer.

Dams treated with DEHP had decreased heart weights. While this is a novel finding, DEHP is known to alter cardiomyocyte metabolism and pulsatility in vitro. Further investigations of cardiac histology and gene expression might help elucidate how phthalates alter cardiac function.

Our studies also highlight the potential biological activity associated with DINCH exposure. From our microarray experiment, DINCH upregulated the expression of genes involved in important signal transduction pathways, reactive oxygen species adaptation, and cholesterol biosynthesis. While we did not do mechanistic studies, these pathways are important for Sertoli cell function, and are assessed as part of the ToxCast screening program which aims to identify chemicals with deleterious effects on human health[250]. FSH mediates its effects on

Sertoli cells by the cAMP-PKA, MAPK, PLA2, and PI3K signalling pathways[38]. Activation of these pathways by DINCH may result in aberrant FSH stimulation or FSH desensitization, but this remains to be explored as there are no data to suggest DINCH affects Sertoli cell number or spermatogenesis. Similar to DEHP, DINCH also increased the incidence of hemorrhagic testes in our gestational-lactational exposure study. Other peer-review studies suggest mono-isononyl-cyclohexane-1,2-dicarboxylate (a metabolite of DINCH) can also activate the PPAR receptor[223]. These findings suggest the need for additional studies on the biological activity of DINCH.

Finally, using *in vitro* high-throughput assays and toxicogenomic data, in addition to data about plasticizer efficiency and biodegradability, BDB and DOS were identified from over 20 potential candidates to replace DEHP in PVC based polymers. Both chemicals had no detrimental effects on the health of our animals.

Together, these studies provide support for BDB and DOS as "responsible replacements" for phthalate plasticizers, and emphasizes the need of more peer-reviewed studies on the effects of DINCH and DEHP. Along with previously published literature and personal communications, BDB and DOS are efficient plasticizers, cost competitive, and not environmentally persistent. These factors make them better choices than DEHP and other phthalates for the manufacturing of PVC.

3. Challenges in Marketing Responsible Replacements

The conflicting data from epidemiological studies on the effects of phthalates have made it difficult to impose widespread legislation to limit or ban the use of phthalates. While these studies are limited, new prospective, properly powered studies with accurate quantification of phthalate exposure will help clarify the bioactivity of phthalates in humans and guide regulatory policy appropriately.

Due to the lack of external pressures, implementation of safer alternatives will be slow unless manufacturers are incentivized to remove phthalates from consumer products. While this practice would be socially responsible, there are expenses involved with transitioning away from phthalates. New manufacturing equipment and processes need to be developed, and polymer formulations optimized to achieve the same physical properties as products containing phthalates. While BDB and DOS are either comparably priced or slightly more expensive than

DEHP, there is little incentive to invest in these chemicals. If these chemicals were to we adapted, the prices are expected to decrease as competition and production scale increase.

Ideally, these "responsible replacements" would replace phthalates all together, but for some applications, chemical blends (with much lower quantities of phthalates or mixtures of many "responsible replacements") might be more appropriate. In either case, one could expect phthalate dependency and exposure to decrease, which would be an acceptable outcome of these studies.

4. Functional Substitution of PVC

Another aspect that could be explored is whether more innocuous materials for certain applications can replace PVC by functional substitution[251]. The production, use, and disposal of PVC is associated with health hazards from exposure to vinyl chloride monomers during production[252], the leaching of additives from PVC-based products, and the potential to form dioxins once these products are incinerated[253]. Utilizing alternatives would not only reduce our dependence on phthalate plasticizers, but would also reduce some of the negative consequences associated with PVC.

Several alternatives for PVC have already been proposed: linoleum and wood for flooring, woven glass fibre and paper for wall coverings, and glass for medical applications could all be used instead of PVC[254]. Other polymers may also serve as suitable replacements for PVC[254]. These substitutions would be marginally more expensive initially, but cheaper over the lifespan of the product as these materials are generally more resilient or recyclable[253].

5. Implications for New Chemical Requirements

According to the New Substances Notification Regulations of the Canadian Environmental Protect Act all new chemicals not on the Non-Domestic Substances List that are produced or imported in quantities greater than 1,000kg are subject to an acute toxicity study[234]. This is the extent of mammalian testing that is required in Canada for the introduction of a new chemical. As research on endocrine disruptors and the origins of chronic disease advances, it is apparent that a standard acute toxicity test may not provide enough information to make informed policy decisions that benefit all Canadians.

Acute toxicity studies focus on high-dose exposure, but it is now clear that low-dose exposures must also be considered when assessing the safety of a chemical[255]. Many endocrine disruptors do not have classical, linear dose-response curves. Most *in vivo* literature on phthalates has focused on doses greater than 500mg/kg. Only a handful of studies have noted the importance of low, environmentally relevant exposures and the deleterious effects associated with such exposure[256–259].

Mixtures of chemicals with additive or synergistic effects can also complicate risk assessment and regulatory policy. Most individuals are exposed to more than one type of ortho-phthalate[118,147,161,260], and these chemicals have additive effects on steroidogenesis[168,192]. Combinations with other endocrine disruptors may further potentiate or antagonize these effects. Most regulatory guidelines establish limits for single-chemical exposures, and therefore do not take into account the potential effects of these environmentally relevant exposures.

The window of exposure used for acute toxicity studies is another factor that should be re-evaluated. Phthalates have differential effects depending on the window of exposure; with the most vulnerable time being gestational exposure[261]. As acute toxicity studies generally use young adult rats for screening, they may fail to recognize effects that are established during more sensitive times during development. The fetus is very receptive to the uterine environment provided by the mother, and erroneous cues to the fetus may increase susceptibility to chronic diseases in adulthood including obesity, diabetes, and cardiovascular diseases[262]. Phthalates inappropriately alter androgen action in the fetus during critical developmental windows during gestation[170,171]. Impaired androgen signaling during the male differentiation window is believed to predispose individuals to reproductive abnormalities that are apparent at birth (cryptorchidism, hypospadias), and in adulthood (testicular cancer and infertility)[69].

The rodent model may also be a limitation of acute toxicity studies. Animal models have been key to many advances in the biomedical sciences as a source of biological materials. In toxicology, rodent models are used to predict the pharmacokinetics and pharmacodynamics of chemicals in humans. There are many cases where animal models show good concordance with effects in humans[263], but there is a great deal of controversy as to whether non-human models can accurately predict human responses. In a statement by the FDA, “nine out of ten

experimental drugs fail in clinical studies because we cannot accurately predict how they will behave in people based on laboratory and animal studies"[264]. Careful consideration in choosing a model and interpretation of data is essential to predict how humans will respond to a therapeutic or toxicant. The rodent model had erroneously predicted phthalates to cause hepatic carcinoma by activating the hepatocyte PPARα receptor. It has since been shown that the human liver does not respond to PPARα stimulation by the same mechanisms, and that this finding was irrelevant to humans[265,266].

Despite these limitations, properly interpreted acute toxicity studies can still provide information that is nearly impossible to obtain by other methods. The future of chemical screening will require additional models to replace or complement these data in order to account for relevant exposure windows and dose. Ideally, this should be accomplished without significantly increasing use of animals, cost, or resources.

6. The Use of Alternative Models in Toxicology

The implementation of additional endpoints with traditional methods will increase the cost, labor, and use of animals. The chemical industry pays between $119,000-148,000 for a complete new chemical notification in Canada[267]. Adding reproductive and developmental toxicity studies would be particularly expensive and laborious because they generally require data from more than one generation of animals. To put this in perspective, of all the animals and resources needed to fill data gaps and develop a database of toxicants under REACH, reproductive and developmental studies account for about one third of the total allocation[268].

Alternative models will have an important role in the future of toxicological screening and safety assessment as they greatly reduce the time, resources, and use of animals for these type of studies. While they are a simplified system, many newly developed high-throughput assays are based on cells from human origins; thereby removing the need for cross-species extrapolation typical of animal models[269]. At the very least, they can serve as a guide for further screening as chemicals that are cytotoxic at low concentrations tend to be more toxic to animals than chemicals that are less cytotoxic[240]. Some of the most promising strategies are based on data from toxicogenomic and high-throughput functional assays. Toxicogenomic models have the added advantage in that they are more sensitive to milder phenotypes observed in animal models

as changes in gene expression generally precede an observable phenotype[269]. Both methods work to generate libraries of "chemical-fingerprints" based on physiological responses or differential gene expression. Once these libraries are generated, new chemicals can be screened against them to make predictions about their safety.

High-throughput and transcriptomic assays generate large amounts of data. One issue has been the interpretation and management of these large datasets. Developments in computational toxicology and machine-learning algorithms are expected to help the analysis of complex high-order data. The EPA's Virtual Embryo Project is an example of an *in silico* model meant to predict toxicity based on complex datasets[270]. Similarly, The EPA ToxCast program is already using alternative models as a strategy to prioritize chemicals for further studies[271].

7. Future Directions and Final Remarks

The testis is a complex organ comprised of multiple types of cells that work together to ensure the continuation of the species. Classically, the study of interactions between the various cell types have been limited to those within the seminiferous tubules (Sertoli-germ) and those in the interstitial space (myoid, Leydig, endothelial, immune). As our understanding of reproductive and developmental biology progresses, it is apparent that this notion is too simplistic. This is particularly true for testicular development, where multiple signalling factors and feedback mechanisms work in harmony to establish the identity of the bipotential gonad.

As we learn more about phthalate toxicity, it is apparent this class of chemicals has widespread effects on human health. Furthermore, within the context of the testes, phthalates may be acting on multiple cell types. While phthalates have been extensively studied in Sertoli and Leydig cells, there is much to be discovered on the role of phthalates in other testicular cell types. Testicular macrophages are known to form tight interactions with Leydig cells and can influence steroidogenic capacity[17]. Macrophages are also important for angiogenesis[272] and testicular cord formation[273], and studying the effects of phthalates on these cells might help to elucidate the developmental origins of hemorrhagic testes following exposure to DEHP.

DINCH is a chemical with a structure that has a great resemblance to DEHP. From our *in vitro* studies both DINCH and MEHP targeted genes involved in cholesterol biosynthesis and nuclear receptor signalling. From the gestational-lactational exposure study, both chemicals

increased the incidence of hemorrhagic testes. At the moment, it is not known how DINCH mediates its effects, but a study was able to block the adipogenic effects of its metabolite, cyclohexane-1,2-dicarboxylic acid mono-isononyl ester (MINCH) using a PPAR antagonist[223]. Given the similarities to DEHP, it would be interesting to characterize the interactions of this chemical with the PPAR receptor and further investigate its effects on Leydig cells as it has already been shown *in vitro* to have biphasic effects on steroidogenesis[224]. Furthermore, given that both chemicals had a similar effect on the occurrence of hemorrhagic testes, analyzing commonalities in gene expression between both treatment groups may provide some insights into the origins of this poorly understood finding.

Finally, in vitro models are a rapidly growing tool in the field of toxicology and show great promise for chemical screening. When used effectively, this can reduce the costs, time, and use of animals associated with these types of studies. In some cases, they are even more senstive[269] than traditional animal models at detecting potential effects. One major hindrance in the implementation of *in vitro* models has interpreting their predictive value for *in vivo* effects. Our first objective describes several genes that are differentially expressed in immortalized Sertoli cell lines following treatment with MEHP and other plasticizers. One potential avenue would be to explore whether similar pathways or genes are altered in Sertoli cells treatment *in vivo*. As alternative models are developed and validated, they will become a more prominent aspect of chemical screening and regulatory decisions. In the meantime, understanding the readouts of these assays and their biological meaning should be a top priority for guiding regulatory policy and chemical development.

This thesis is the first to propose alternatives to phthalate plasticizers that have been extensively tested using classical and alternative *in vitro* models, and provides a conceptual framework by which new compounds should be screened prior to their introduction in the marketplace. From these data, we have identified BDB and DOS as plasticizers that appear to be safe and ready to be developed for commercial use in the manufacturing of consumer products. In order to gain some traction in the manufacturing sector, the next steps would be to do a cost analysis to assess the expense associated with using these chemicals (raw materials, development of polymer blends, changes to manufacturing equipment), and provide a report highlighting the benefits of these environmentally friendly, socially responsible alternatives. In doing so, one

could highlight the advantages of implementing alternative “responsible replacements” thereby ensuring the health of generations to come.

List of Original Contributions

- First characterization of the potential effects of alternative plasticizers on cell viability in immortalized Sertoli cell lines.
- Assessed how "families" of chemicals based on the core-structures of maleates, fumarates, and succinates can influence toxicity by read-across.
- Using a toxicogenomic approach, identified pathways that are activated following exposure to MEHP, DINCH, and DOM:
    - Treatment with MEHP affected pathways involved in cholesterol biosynthesis and nuclear receptor signalling.
    - Treatment with DOM, a molecule resembling diethyl maleate (which is a potent glutathione-depleting molecule) upregulated gene expression for genes involved in neutralizing cellular oxidative stress. Cells treated with DOM also initiated many cell-cycle arrest and DNA repair pathways, a finding that is consistent with high levels of reactive oxygen species.
    - While there is some literature to suggest DINCH is bioactive, this is the first account of DINCH targeting major signal transduction pathways. Similar to MEHP, DINCH also upregulated genes involved in cholesterol biosynthesis.
- Using a toxicogenomic approach, we showed BDB and DOS do not affect Sertoli cell function or major pathways shared between cell types and are potential replacement plasticizers for DEHP.
- First study to characterize the lack of effects of both BDB and DOS on animal health. This was assessed using classical endpoints including animal weight, food intake, organ weight, and analysis of serum analytes and hematology. Several histological preparations including brain, heart, lungs, kidney, liver, and spleen were analysed.
- Successfully identified the lack of deleterious effects of treatment with BDD or DOS following gestational and lactational exposure. Animals were followed into young adulthood with no deleterious effects on health, developmental markers, or markers of endocrine disruption.
- Demonstrated that treatment with DEHP caused changes in biomarkers related to androgen activity (anogenital index). DEHP also increased the incidence of testicular

abnormalities (multinucleated gonocytes and testicular hemorrhage) at lower doses than previously reported in the literature.

- DEHP was found to increase the age of vaginal opening.
- DEHP caused a decrease in heart weight in dams. This is the first report of an effect on heart weight, but previous literature suggests the heart may be a target of phthalates based on in vitro data.
- First report of DINCH causing testicular abnormalities (hemorrhagic testes).
- Together, these data provide support for BDB and DOS as potential alternatives to DEHP and other phthalate based plasticizers. They also provide support for the need of additional studies regarding the safety of current commercial alternative plasticizers (DINCH).

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