The Impact of Realistic Environmental Chemical Exposure on Male Gonadal Development and Reproductive Health

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“We should be taught not to wait for inspiration to start a thing. Action always generates inspiration. Inspiration seldom generates action.”

-- Frank Tibolt

“I am not young enough to know everything.”

-- Oscar Wilde

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Abstract

Continuing declines in human male reproductive health are of increasing concern. Many believe low-dose exposure to vast numbers of chemicals through the environment, particularly during fetal development, are a contributary factor in this decline. To address limitations with traditional, component-based methodologies of assessing chemical mixtures, this research utilised a unique, ovine based, whole-mixture exposure model. This model was used to investigate the impact of gestational exposure to realistic numbers of chemicals, at appropriately low doses, on male reproductive development. The research detailed herein characterises exposure-induced changes to the testes of neonatal, pre-pubertal, and adult male offspring of mothers exposed to an environmental chemical mixture prior to and during pregnancy. A testicular dysgenesis syndrome (TDS)-like phenotype was described in neonatal and pre-pubertal testes. This TDS-like phenotype was complemented by transcriptomic analyses which showed an extremely high degree of similarity between the testicular transcriptome of the affected pre-pubertal male offspring and those of human TDS patients. While this phenotype was not apparent in the same manner by adulthood, morphological and transcriptomic alterations were still apparent. This both exemplifies the potential for xenobiotic exposure during fetal development to impact reproductive health in later life, despite the cessation of exposure at birth, and indicates periods of post-partum vulnerability to xenobiotic exposure crucial to the persistence of or recovery from the TDS-like phenotype. Further investigations following transcriptomic analyses identified perturbations in the transcription, activation, and/or nuclear localisation of various transcription factors. Of these, there is supporting evidence that one (HIF1α) may have an important role in the pathogenesis of the TDS-like phenotype, while another (CREB1) may facilitate an amount of post-exposure recovery and might also be important in determining susceptibility or resistance to developing the TDS-phenotype. Overall, these findings strengthen the increasing evidence that gestational exposure to realistic levels and mixtures of environmental chemicals can have a negative impact on male reproductive health and provides leads for future investigations into the pathogenesis of TDS.

Table of Contents

List of Tables

List of Figures

Abbreviations

A(SP)A	Animal (Scientific Procedures) Act
AC	adenylyl cyclase
AChE	acetylcholinesterase
AGD	anogenital distances
Ahr	arylhydrocarbon receptor
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AMPK	adenosine monophosphate-activated protein kinase
AOP	adverse outcome pathways
AST	aspartate aminotransferase
B	biosolids exposed
BADGE	bisphenol A diglycidyl ether
BBP	benzyl butyl phthalate
BDP	bisphenol A bis(diphenyl phosphate)
BPA	bisphenol A
BPAF	bisphenol AF
BPE	bisphenol E
BPF	bisphenol F
BPS	bisphenol S
BTB	blood-testes barrier
BTP	biosolids treated pasture
BzBP	benzylbutyl phthalate
bZIP	basic leucine zipper
C	control
cAMP	cyclic adenosine monophosphate
ChE	cholinesterase
CREB	cAMP-response element binding protein
CYP	cytochrome p450
DA	dose addition
DAB	3, 3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DAR	draft assessment report
DBP	disinfection by-product
DBP	dibutyl phthalate
DCHP	dicyclohexyl phthalate
DDD	dichlorodiphenyldichloroethane
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DEG	differentially expressed gene
DEHP	diethylhexyl phthalate
DENTP	di-2-ethylhexyl terephthalate
DEP	diethyl phthalate
DGE	differential gene expression
DiBP	diisobutyl phthalate
DiDP	diisodecyl phthalate
DiHP	diisoheptyl phthalate
DINCH	di(isononyl) cyclohexane-1,2-dicarboxylate

DINP	diisononyl phthalate
DMP	dimethyl phthalate
DOP / DnOP	di-n-octylphthalate
DPP	dipentyl phthalate
DPX	dibutyl phthalate plus xylene
EC	environmental chemical
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ED_{50}	median effective dose
EDC	endocrine disrupting chemical
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ES	effect summation
FDR	false discovery rate
FFPE	formalin-fixed paraffin embedded
FSH	follicle-stimulating hormone
GD	gestation day
GnRH	gonadotropin releasing hormone
GnRHR	gonadotropin releasing hormone receptor
GO	gene ontology
GPCR	G protein coupled receptor
H&E	haematoxylin and eosin
HFHS	high fat high sucrose
HIF1α	hypoxia Inducible Factor 1 alpha
HK	hexokinase
HPG	hypothalamic–pituitary–gonadal
HRP	horseradish peroxidase
IA	integrated addition
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	knockout
LABC	levator ani plus bulbocavernosus
LOAEL	lowest observable adverse effect level
MCL	maximum contaminant level
MCPB	4-(4-chloro-o-tolyloxy)butyric acid
Me-PFOSA-AcOH	2-(N-Methyl-perfluorooctane sulfonamido) acetic acid
MRL	maximum residue limit
mTOR	mechanistic target of rapamycin
mTORC	mTOR complex
NBF	neutral buffered formalin
NGF	nerve growth factor
NOAEL	no observable adverse effect level
NP / 4NP	4-nonylphenol
NPY	neuropeptide Y
NPYR	NPY receptor
NR	nipple retention
OECD	Organisation for Economic Co-operation and Development
PAH	polycyclic aromatic hydrocarbon
PBDE	polybrominated diphenyl ether
PCB	polychlorinated biphenyl

PDPK	3-phosphoinositide dependent protein kinase
PFAS	perfluoroalkyl and polyfluoroalkyl substances
PFBA	perfluorobutanoic acid
PFDA	perfluorodecanoic acid
PFHxS	perfluorohexanesulfonic acid
PFNA	perfluorononanoic acid
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonate
PFOSA	perfluorooctanesulfonamide
PFPeA	perfluoropentanoic acid
PI3K	phosphoinositide 3-kinase
PND	post-natal day
POD	point of departure
PP	pancreatic polypeptide
Pparα	peroxisome proliferator-activated receptor α
PPCP	pharmaceuticals and personal care products
PPCP	pharmaceuticals and personal care products
PYY	peptide YY
QSAR	quantitative structure-activity relationship
RA	response addition
RfD	reference dose
RLRS	real-life risk simulation
SCO	Sertoli cell-only
SSC	spermatogonial stem cells
STAR	steroidogenic acute regulatory protein
TBS	tris-buffered saline
TCDD	tetrachlorodibenzo-p-dioxin
TDI	tolerable daily intake
TDS	testicular dysgenesis syndrome
TEF	toxic equivalency factor
TF	transcription factor
TGF-α	transforming growth factor alpha
TSH	thyroid stimulating hormone
VEGFA	vascular endothelial growth factor A
VOC	volatile organic compounds
WT	wild type

Authors Declaration

I declare that, except where explicit reference is made to the contribution of others, this represents my own work. I was responsible for obtaining and analysing data, the interpretation of results, and writing-up this thesis.

The work contained herein has not been previously submitted to the University of Glasgow, or any other institution, for any degree.

_____ 16th December 2022

Christopher S Elcombe Date

Chapter 1.

Introduction

1.1. Overview

Since the industrial revolution, the quantity and diversity of chemicals being used, and subsequently released into the environment, have been steadily increasing. As a result, humans and wildlife are continuously exposed to an increasingly vast array of chemicals found in the environment. Worryingly, concurrent to this increase in the numbers of environmental chemicals (ECs), is the increasing prevalence of many diseases and disorders (Figure 1-1).

Although ECs can produce adverse effects through a variety of mechanisms, recently there has been a focus on the potential health effects of exposure to endocrine disrupting chemicals (EDCs), especially when exposure occurs during fetal development, a known window of vulnerability to xenobiotic toxicity (Diamanti-Kandarakis et al., 2009; Skakkebaek et al., 2011). As such, evidence has been presented which indicates human and wildlife exposure to environmental EDCs may be a contributory factor in declining male and female reproductive health and increasing rates of hormone-related cancers, thyroid diseases, and disorders of neurodevelopment, adrenal function, bone formation, metabolism, and the immune system (Annamalai and Namasivayam, 2015; WHO,

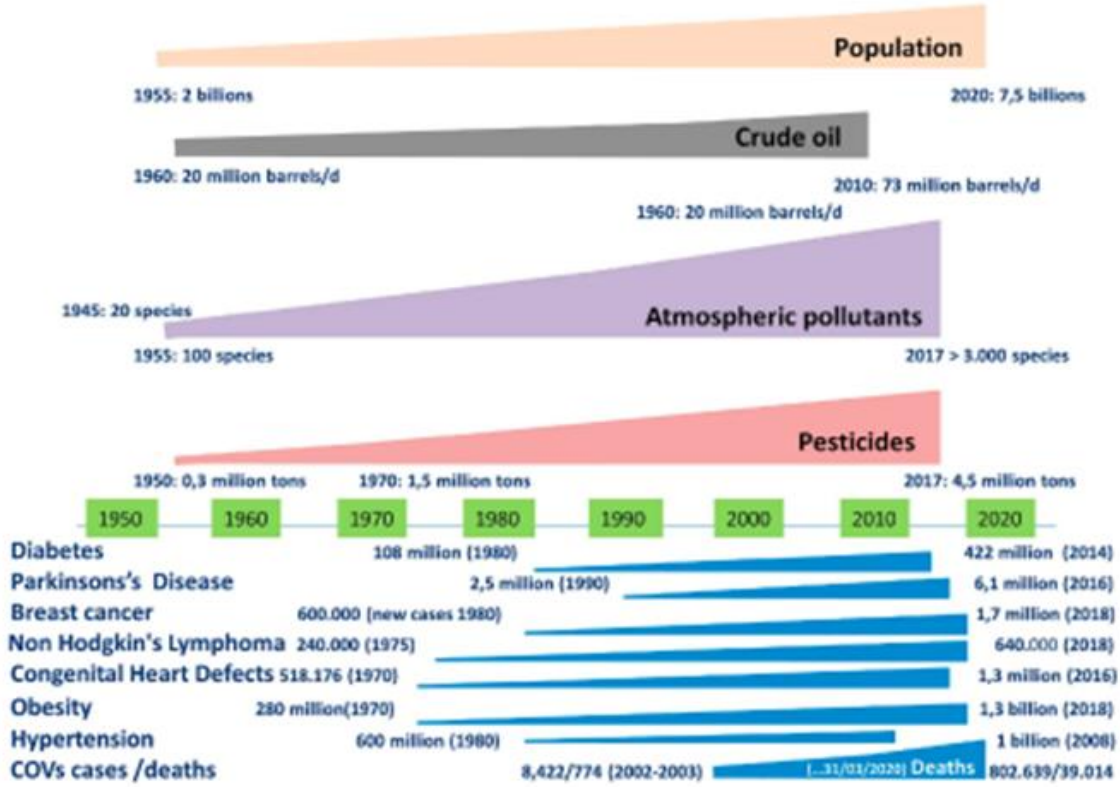


Figure 1-1. Illustration of increasing prevalence of various diseases and increased quantities and/or diversity of chemical production, use, and pollution. Taken from Tsatsakis et al. (2020).

2012). Although the use of many well-known EDCs (e.g., dioxins, DDT, PCBs) have been heavily restricted or banned in most parts of the world for many years, other chemicals with very useful properties and diverse applications are still widely produced (pesticides, flame retardants, plastic additives, cosmetics, etc.) and are also known to have endocrine disrupting properties. As such, it is estimated that 800 – 1000 currently used chemicals may cause endocrine disruption, however this is likely a gross underestimate, as a lot of testing for endocrine disruption in intact organisms is lacking or incomplete (Endocrine Society, 2022; WHO, 2012). Additionally, EDCs have not been officially codified as a regulatory hazard category (Kassotis et al., 2020). Therefore, more novel EDCs, such as perfluoroalkyl and polyfluoroalkyl substances (PFAS) and phthalates, are only recently having restrictions applied (European Commission, 2021, 2018).

In addition to risks posed by exposure to chemicals individually, is the risk posed by exposure to complex and varied mixtures of chemicals, as is encountered in real life. As toxicological testing to determine safety limits are predominantly performed using single compounds, there is concern about synergistic actions occurring between chemical components when exposure is to a chemical mixture (Boobis et al., 2011; Kortenkamp, 2008; Kortenkamp et al., 2009). This increases the risk of adverse effects occurring from co-exposure to multiple chemicals, even at doses of individual chemicals which would not produce an adverse effect if exposed to the chemicals individually. Consequentially, many have suggested that exposure to real-life chemical mixtures, even where all individual chemical components are below levels determined to be “safe”, is having an impact on human health. As such, to assure safety, there is not only a great need for closely monitoring chemical exposure, but also to ensure accurate predictions can be made regarding exposure to chemical mixtures. To assess the accuracy of predictions requires exposure to realistically low doses, and relevant models to test real-life chemical mixtures. Each of these aspects are discussed below.

1.2. Xenobiotic exposure

There are currently over 42,000 chemicals in commerce (U.S. EPA, 2022), with roughly 8,700 chemicals commonly used across various industries (U.S. EPA, 2016). Many chemicals find their way into the environment, leading to human

exposure through the air, food, and water. While the US Centre for Disease Control, in one of the largest chemical exposure monitoring programmes, routinely monitors levels of over 400 chemicals and metabolites in the American population (U.S. HHS, 2019), even this does not capture the full extent of chemical exposure. It does, however, give an insight into many chemicals to which humans are exposed, and an approximation of general exposure levels, which mostly remain around the pg-ng/L blood concentration level, with occasional instances where chemicals are reported as µg/L. The main groups of chemical classes routinely detected by the CDC in the American population are discussed below.

1.2.1. Bisphenols

Bisphenol A (BPA) is a widely used plasticiser to which humans are almost ubiquitously exposed. In the most recent CDC biomonitoring publication, over 50% of serum samples from the tested human population had detectable amounts of BPA, BPF, and BPS (U.S. HHS, 2019). BPA is a known EDC; indeed, it was originally developed as a synthetic oestrogen (Dodds and Lawson, 1936), but BPA not only has affinity for oestrogen receptors, but androgen and thyroid receptors also. As such, there is a large body of experimental evidence indicating reproductive toxicity from BPA exposure. In male rodents, BPA exposure has been shown to reduce sperm counts, increase the proportion of sperm with abnormal morphology, damage Sertoli cells, and decrease steroidogenesis, cumulating in impaired fertility *in vivo*, especially following gestational and/or neonatal exposure (Barbonetti et al., 2016; D'Cruz et al., 2012; Qi et al., 2014; Salian et al., 2011, 2009; Vom Saal et al., 1998). In female rodents, BPA exposure has been shown to inhibit oocyte maturation, inhibit germ cell nest breakdown, alter hormonal balance and response, and reduce the fertilisation ability of oocytes (Berger et al., 2016; Delclos et al., 2014; Ferris et al., 2016; Moore-Ambriz et al., 2015; Vigezzi et al., 2015; T. Wang et al., 2016). In addition to experimental evidence, there is also mounting epidemiological evidence of endocrinological disruption from BPA adversely affecting human reproductive and metabolic health (Rochester, 2013). Consequently, BPA is being phased out of consumer products in many regions and replaced by structural analogues such as bisphenol A bis(diphenyl phosphate) (BDP), bisphenol A diglycidyl ether (BADGE), bisphenol E (BPE), bisphenol F (BPF),

bisphenol AF (BPAF), and bisphenol S (BPS), among others (den Braver-Sewradj et al., 2020). However, it should be noted that these substitutes have similar EDC properties as BPA with potencies around the same order of magnitude (den Braver-Sewradj et al., 2020; Rochester and Bolden, 2015; Siracusa et al., 2018).

1.2.2. Phthalates

Phthalates are esters of phthalic acid, which represent a vast and diverse range of plasticising agents used extensively in the production of plastic products, including consumer products (including children's toys), food packaging, tubing for medical devices, vinyl flooring, lubricating oils, and personal-care products. As phthalates to not bond covalently to the plastic matrix, chemical leaching contaminates the external environment. Over 50% of the tested human population reported in the most recent CDC biomonitoring publication had detectable amounts of metabolites for BzBP, DBP, DiBP, DCHP, DEP, DEHP, DiDP, DMP, DOP, DINCH, and DEHTP in serum samples (U.S. HHS, 2019). Although precise mechanisms have not been fully elucidated, it is known that phthalates affect the hypothalamic–pituitary–gonadal axis and steroidogenesis (Hlisníková et al., 2021), and have been associated with reproductive toxicity experimentally. In male rodents, gestational and/or postnatal exposure has been long been known to cause severe damage to the testes, including seminiferous tubule atrophy, Leydig and Sertoli cell damage, and germ cell detachment and apoptosis (Gangolli, 1982; Gray and Gangolli, 1986; Jones et al., 1993; Richburg and Boekelheide, 1996). Although the female reproductive system is less sensitive to phthalate induced toxicity, experimental evidence in rodents has shown phthalate exposure to delay vaginal opening and oestrus cycling, suppress oestradiol production and ovulation, and induce mid-pregnancy abortions (Davis et al., 1994; Grande et al., 2006; Gray et al., 2006). Epidemiological data in humans supports these findings and indicates that public phthalate exposure may be negatively affecting sperm quality and quantity, reproductive hormone concentrations, and gestational age at birth, as well as impairing physical and mental development and immune function, especially when exposure occurs prenatally or prepubertally (Braun et al., 2013; Jurewicz and Hanke, 2011). While multiple expert panels have previously concluded minimal risk from exposure to single or multiple phthalates at realistic levels, the risk for DINP, DiDP, DOP, DEHP, DBP, and BBP to cause reproductive and developmental

toxicity was sufficient for many countries to restrict the proportion allowed in plastics (Kamrin, 2009).

1.2.3. Perfluoroalkyl and polyfluoroalkyl substances

Per- and polyfluoroalkyl substances (PFAS) have been in production since the 1940s, and now comprise of thousands of individual chemicals with uses in almost every industry (Glüge et al., 2020). Detectable amounts of PFDA, PFHxS, PFNA, PFOA, n-PFOA, PFOS, n-PFOS, Sm-PFOS, PFOSA, and Me-PFOSA-AcOH were found in over 50% of serum samples from the tested human population in the most recent CDC biomonitoring publication (U.S. HHS, 2019). Due to their wide use, physiochemical properties, and resistance to biodegradation, PFAS are globally distributed in every environment, including the poles (e.g., the Antarctic) (Cousins et al., 2019). Elimination half-lives for PFAS range from long to extremely long; in humans, the metabolic half-life of PFBS is estimated at 44 days, PFOA is estimated at 0.5-2.5 years, and some PFOS isomers are estimated at 2.5-5.5 years (Elcombe et al., 2013; Li et al., 2022; Xu et al., 2020). This metabolic resistance underpins PFAS bioaccumulation, and consequentially any remedial actions against PFAS environmental release and subsequent human and wildlife exposure will take many years to take effect. While only a small number of PFAS have toxicological data, experimental and epidemiological studies have shown adverse effects on the immune system, thyroid function, liver and kidney disease, metabolic dysregulation, and developmental and reproductive toxicity (Fenton et al., 2021).

1.2.4. Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are synthetic organic chlorinated hydrocarbons, derived from biphenyls, which were used in paints and printing, thermal and electrical insulation, agriculture pest control, window glazing, and as plasticisers for consumer products and food packaging (Reddy et al., 2019). Due to incredible chemical stability and bioaccumulation, despite being universally banned in the Stockholm treaty on persistent organic pollutants, PCBs remain ubiquitous environmental toxicants (Porta and Zumeta, 2002; Reddy et al., 2019). The most recent CDC biomonitoring publication reported detectable amounts of PCB 44, PCB 49, PCB 126, PCB 138, PCB 153, PCB 158, PCB 180, PCB 206, and PCB 209 in over 50% of serum samples from the tested human

population (U.S. HHS, 2019). PCBs have been identified experimentally as EDCs with thyroid disrupting properties, and a wide range of adverse effects, mostly developmental, reproductive, and metabolic toxicity (Buha Djordjevic et al., 2020). Epidemiological evidence also exists implicating PCBs in human health concerns, including abnormal mental and motor development in young children, hormonal imbalance in new-born and adolescent males, dysregulated immune responses, increased risk for type 2 diabetes, and fertilisation ability of oocytes (Eskenazi et al., 2017; Hofe et al., 2014; Jirsová et al., 2010; Kuwatsuka et al., 2014; Ruel et al., 2019; Tang et al., 2018). Additionally, many PCBs exhibit strong dioxin-like properties and may also cause pathogenic oxidative stress through methylsulfonyl and quinone metabolites (Liu et al., 2020; Van den Berg et al., 2006).

1.2.5. Polybrominated diphenyl ethers

Polybrominated diphenyl ethers (PBDEs) are a broad group of environmentally persistent, bioaccumulating, biphenyl-based brominated hydrocarbons used as flame retardants. Due to physiochemical properties, PBDE congeners are found globally in environmental samples taken from land, sea, and air (Ohoro, 2021). In the most recent CDC biomonitoring publication, detectable amounts of PBDE 28, PBDE 47, PBDE 99, PBDE 100, PBDE 153, and PBB 153 were found in over 50% of serum samples from the tested human population (U.S. HHS, 2019). Equally concerning is the variety of toxicological endpoints observed following exposure to one of more PDBEs, including thyroid and pituitary hormonal dysfunction (Meeker et al., 2009; Turyk et al., 2008), altered prenatal neurodevelopment (Herbstman et al., 2010), and abnormal male reproductive health (Abdelouahab et al., 2011).

1.2.6. Organochlorine and organophosphorus pesticides

Organochlorine pesticides are chlorinated hydrocarbon derivatives used extensively throughout the world. In over 50% of serum samples from the tested human population of the most recent CDC biomonitoring publication, detectable amounts of the organochloride compounds chlordane, DDE and hexachlorobenzene, and the chlordane metabolite oxychlordane were reported (U.S. HHS, 2019). Although many classical organochlorine pesticides (e.g., DDT, DDE, DDD and aldrin) are restricted or banned in more developed nations, their

use is still commonplace in less advanced countries, due to low production costs and local pest control needs (Jayaraj et al., 2016; Porta and Zumeta, 2002). Most organochlorine pesticides show swift neurotoxicity upon acute exposure, due to agonism of axon sodium gates or antagonism of GABA chloride ionophore complexes (Coats, 1990). While the most notorious organochloride pesticide, DDT, and its metabolite DDE, are commonly remembered for eggshell thinning of birds of prey (Grier, 1982; Porter and Wiemeyer, 1969), and reproductive dysfunction in alligators (Guillette et al., 1995, 1994; Guillette and Crain, 1996), mammalian studies have also shown effects on testosterone synthesis and metabolism, Leydig cell function, and degeneration of seminiferous tubules and spermatogonia (Bal, 1984; Gray et al., 1989; Rhouma et al., 2001).

Organophosphorus compounds are any organic derivatives of phosphorus, predominantly those with a phosphoryl or thiophosphoryl bond (Balali-Mood, 2014). In the most recent CDC biomonitoring publication, over 50% of serum samples from the tested human population had detectable amounts of para-nitrophenol and 3,5,6-trichloro-2-pyridinol, as well as dialkyl phosphates (diethylphosphate, dimethylphosphate, diethylthiophosphate, and dimethylthiophosphate), which are metabolites most associated with exposure to chlorpyrifos and parathion (U.S. HHS, 2019). Although many organophosphorus compounds have been heavily restricted, they are still widely used as fire retardants, solvents, plasticisers, and drugs, although most commonly as pesticides (e.g., dimefox, mipafox and dichlorovas), and, notably, also as toxic nerve agents in chemical warfare (e.g., sarin and novichok) (Soltaninejad and Shadnia, 2014). Toxicity from acute exposure to organophosphorus compounds is mostly explained by inhibition of acetylcholinesterase (AChE) and the accumulation of acetylcholine at nicotinic and muscarinic cholinergic synapses, resulting in hyperstimulation (Morris et al., 2014). Distinct from AChE inhibition, acute organophosphorus exposure can result in delayed polyneuropathy, and chronic exposure can lead to neuropsychiatric disorder (Satoh and Jokanović, 2014).

1.2.7. Parabens

Alkyl esters of p-hydroxybenzoic acid – parabens – are used extensively as preservatives in cosmetics, food products, personal hygiene products, and

pharmaceuticals. The most recent CDC biomonitoring publication reported detectable amounts of methyl paraben and n-propyl paraben were found in serum samples from over 50% of the tested human population (U.S. HHS, 2019). Parabens display weak oestrogenic activity, and as such have been regarded as safe for use based on extrapolations to endogenous oestrogenic ligands and naturally occurring phytoestrogens (Golden et al., 2005). However, due to the extent that parabens are used within cosmetics, it is recommended to avoid excessive quantities of cosmetic products, or simultaneous use of multiple cosmetic products, which contain parabens (Matwiejczuk et al., 2020).

1.2.8. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are a group of widely distributed gaseous and particulate persistent organic pollutants, which contain two or more fused aromatic rings. PAHs are predominately anthropogenic in origin, produced from the combustion of coal, petrochemicals, wood, organic polymers, and burnt meat, and through leakage from crude oil and coal products, such as tar and asphalt, but are also produced naturally during forest fires and volcanic eruptions (Patel et al., 2020). Over 50% of serum samples from the tested human population in the most recent CDC biomonitoring publication had detectable amounts of 2-hydroxyfluorene, 3-hydroxyfluorene, 9-hydroxyfluorene, 1-hydroxyphenanthrene, 2-hydroxyphenanthrene, 3-hydroxyphenanthrene, 4-hydroxyphenanthrene, 1-hydroxypyrene, 1-Naphthol, 2-Naphthol, which are metabolites of the PAHs fluorene, phenanthrene, pyrene, and nephthalene (U.S. HHS, 2019). Due to very low aqueous solubility, PAHs persist on particulates and in soil media for extended periods of time (Sun et al., 2021). As the definition of PAHs covers a magnitude of very different chemicals, toxicity profiles are varied. However, many PAHs have been shown to be EDCs, genotoxic carcinogens, immunotoxic, neurotoxic, and teratogenic, and can cause reproductive and developmental toxicity (Patel et al., 2020; Sun et al., 2021).

1.2.9. Phytoestrogens

Botanically derived oestrogen-like compounds – phytoestrogens – are common in nature and cover several chemical families, with most categorised as isoflavonoids or lignans (Sirotkin and Harrath, 2014). Phytoestrogens are found in many foods routinely eaten by people, including but not limited to apples,

carrots, celery, chickpeas, coffee, garlic, kidney beans, pomegranates, potatoes, red clover, rice, soybeans, sweet potatoes, and wheat (Dixon, 2004; Sirotkin and Harrath, 2014). Detectable amounts of the phytoestrogens daidzein and genistein, and metabolites of matairesinol, secoisolariciresinol, and daidzein (enterodiol, enterolactone, equol, and O-desmethylangolensin) were reported in over 50% of serum samples from the tested human population in the most recent CDC biomonitoring publication (U.S. HHS, 2019). While many phytoestrogens have been proposed as beneficial to health, especially with regards to androgen-driven cancers and menopausal women (Dixon, 2004; Sirotkin and Harrath, 2014), there are concerns that phytoestrogens may be contributing to declining reproductive health in humans. Studies in male rodents have shown phytoestrogens alter Leydig cell steroidogenesis, cause atrophy of the testes and prostate gland, reduce sperm counts and litter sizes, impair penile erection, and reduce fertility and fecundity (Adnan et al., 2022; Akingbemi et al., 2007; Assinder et al., 2007; Cederroth et al., 2010; Eustache et al., 2009; Tarragoó-Castellanos et al., 2006). In female rodents, phytoestrogens inhibit germ cell nest breakdown, alter steroidal biogenesis, extend oestrous cycles, produce ovarian cysts and multi-oocyte follicles, and decrease fertility (Burroughs et al., 1990; Jefferson et al., 2006, 2005; Kouki et al., 2003; Newbold et al., 2001; Patel et al., 2016). In addition to these animal studies, secoisolariciresinol and genistein have been associated with lower sperm counts and reduced sperm motility in humans (Yuan et al., 2019).

1.2.10. Metals and metalloids

While environmental exposure to metals and metalloids is not completely anthropogenic in nature, industrialised actions have increased human exposure. In the most recent CDC biomonitoring publication, over 50% of serum samples from the tested human population had detectable amounts of antimony, arsenic, barium, cadmium, caesium, cobalt, copper, lead, manganese, mercury, molybdenum, selenium, strontium, thallium, tin, tungsten, uranium, and zinc in (U.S. HHS, 2019). It is noteworthy that some of these are involved in biological processes (e.g., cobalt, manganese, zinc), and a detectable amount is not unexpected. Although precise mechanisms of toxicity for many metals and metalloids is not completely understood, there are many ways in which these interact with biological systems. Examples are inhibition of critical enzymes

(lead on heme biosynthesis), mimicry and displacement (thallium mimics potassium), catalytic action towards oxidative damage (nickel and chromium), or the creation of DNA and/or protein adducts (chromium) (Liu et al., 2008).

1.3. Low dose mixture toxicity models

There are two approaches to assess the impact of exposure to chemical mixtures for hazard identification and cumulative risk assessment (Hernández et al., 2017). The most common approach, and exclusively used in regulatory assessments, are component-based methodologies combined with mathematical models. In this process, exposure is to a defined mixture of chemicals at precise doses. Toxicological outcomes are compared to predictions by various mixture toxicity models, based on defined rules regarding the mode of action for toxicity. The mathematical models are then used in cumulative risk assessment of EC mixture exposure. However, the constraints to which chemicals are grouped for cumulative risk assessment may be too restrictive (Kortenkamp et al., 2009), and there is evidence of non-conformance to mixture toxicity models as the number of chemicals within a mixture increases (Christiansen et al., 2009; Rider et al., 2010, 2008). It is also impossible to test every combination, exposure pattern, and dose of chemicals to which exposure may happen by component-based methodologies, especially when considering the numbers of chemicals and low dose levels experienced in real-life scenarios. The lesser used approaches are whole-mixture methodologies, whereby exposure is to a mixture of chemicals already present in a sample. The subject of low dose exposure to chemical mixtures and mixture toxicity modelling is the focus of Chapter 2. As shown in Chapter 2, the only whole-mixture model which is currently used to test real-life chemical mixture exposure, at realistically low levels, is the biosolids treated pasture (BTP) sheep model.

1.3.1. Biosolids treated pasture sheep model

As biosolids originate from sewage wastewater treatment, an encompassing range of chemicals are contained within, which reflect the human exposome of the general population (Rigby et al., 2020). In pastures fertilised with biosolids (a common agricultural practise), these chemicals are present in the herbage grown, which provides a route of exposure to grazing sheep. It is particularly worthy of mention that biosolids contain a wide array of chemicals encompassing

those monitored by the CDC (and discussed above) and more (Rhind et al., 2010, 2002, 2013; Venkatesan and Halden, 2014a, 2014b), which can be detected in tissue samples from sheep grazed on BTP and their offspring (Rhind et al., 2002, 2013; Venkatesan and Halden, 2014a; Zhang et al., 2015). These include alkylated phenols, dioxin-like compounds, pharmaceuticals and personal care products (PPCP), phthalates, plasticising agents (e.g., bisphenols), PAHs, PBDEs, PCBs, PFAS, and metabolites thereof. While empirical determinations of oral dosage are limited to three chemicals (dioctyl phthalate, octyl phenol, and nonyl phenol), which were each concluded to be below tolerable daily intake levels (Rhind et al., 2002), differences in organ chemical loads between BTP exposed sheep and sheep reared on pastures fertilised with inorganic fertilisers are very small and did not reach statistical significance (Evans et al., 2014; Rhind et al., 2010, 2002, 2013). Despite this, the BTP sheep model has shown effects from exposure on behaviour, bone development, cellular processes, hormonal systems, liver function, and male and female gonadal development (Bellingham et al., 2016, 2013, 2012, 2009; Erhard and Rhind, 2004; Filis et al., 2019; Fowler et al., 2008; Hombach-Klonisch et al., 2013; Lea et al., 2022, 2016; Lind et al., 2009; Paul et al., 2005).

In addition to the translatability of BTP sheep exposure to humans, the use of sheep as a model system is also very translatable to humans, especially when compared to traditional (murine) species. Sheep are outbred, longer lived, and have longer gestation periods for fetal developmental than rodents. These are important factors to consider, as genetic variation can create differential susceptibility within a population, and longer fetal development allows a greater period for xenobiotic toxicity. There are also many physiological similarities between sheep and humans, where there is disparity with rodents, including similarities in reproductive cycle, degree of organ development at birth, steroidal biosynthesis pathways, and puberty start and duration. Particularly relevant to the present research are similarities in testicular development between sheep and humans; the timing and duration of many events is key for proper testicular development. Where rodents exhibit large differences to humans in terms of functional onset of the hypothalamic–pituitary–gonadal axis, plasma androgen and anti-Mullerian hormone levels, genital tubercle formation and external genitalia differentiation, sheep are very similar to humans

(O'Shaughnessy and Fowler, 2011). These factors show that the BTP sheep model is unique in its translatability to investigate real-life EC exposure induced changes to testicular development.

Separate to the research contained herein, research on the effects of gestational BTP exposure on male gonadal development and reproductive health is limited to two studies in the fetus, and one in the adult offspring. These previous studies indicated that at gestation day (GD) 110, following continuous exposure, fetal body weights and testes weights were lower than controls. Within the testes of biosolids rams, there were fewer Sertoli cells, Leydig cells, and gonocytes than in controls. Blood concentrations of testosterone and inhibin A were also lower in exposed offspring than controls (Paul et al., 2005). In a separate study, earlier and later time points in gestation were studied, as well as the timing of exposure during gestation. No significant differences were observed between exposed and control fetuses at GD 80; however, a variety of changes were observed in continuously or transiently exposed fetuses by GD 140. In those exposed from GD 60 to GD 140, fetal body weights, testis weights, and adrenal weights were lower, and anogenital distances reduced – hallmarks of anti-androgenic exposure. In all transiently exposed fetuses (GD 0 to GD 80, GD 30 to GD 110, and GD 60 to GD 140) there were fewer Sertoli cells per gram tissue than in controls. All exposure groups had lower staining area for CYP17A1, and continuously exposed and GD 0 to GD 80 exposed fetuses had lower staining area for CYP11A1; both examined cytochrome p450 enzymes are crucial for steroidal biosynthesis. However, only the GD 60 – GD 140 exposed fetuses had lower blood serum testosterone levels. Transcriptomic analysis on continuously and GD 0 to GD 80 exposed fetuses against controls identified many differentially expressed genes and enriched pathways, and suggested that transcription was perturbed more by early transient exposure than by continuous exposure (Lea et al., 2022). The remaining study investigated effects evident in 19-month-old adult rams following gestational exposure plus lactational and direct exposure during the first 7 months after parturition. No significant differences were observed for any examined body metrics, blood hormone concentration, or in testes histopathology when overall group means were compared between exposed and control animals. However, 5 of the 12 exposed animals had markedly different testicular morphology, with fewer counts for

total germ cells, spermatocytes and round spermatids, lower germ cell absolute volumes and smaller ratios for germ cell to Sertoli cell counts and volumes. In addition to these findings, the subgroup of exposed males also had a higher incidence of Sertoli-cell-only (SCO) seminiferous tubules (Bellingham et al., 2012).

These findings in the testes of fetal and adult male offspring exposed to BTP during development indicate that exposure to ECs may be having a negative effect on male reproductive health. Reduced germ cell numbers would be suggestive of a lower capacity for sperm production. Although no analysis was performed on semen from the adult offspring, in sheep lower sperm production may not present as lowered sperm counts due to gonadal and epididymal sperm reserves. However, in species without this physiology, lowered sperm counts could be expected, which has been observed in the human population. Changes in testes morphology, especially the increase in SCO seminiferous tubules, is similar phenotypically to testicular dysgenesis syndrome (TDS), an increasingly prevalent condition in human males. Therefore, the BTP sheep model may be an appropriate model for examining the role of gestational exposure to ECs in the decline in human male reproductive health.

1.4. Decline in human male reproductive health

Male reproductive health has been reported to have been in decline for the past 85 years, most prominently since the 1960s. Historically, the gold-standard reference for semen quality and sperm quantity was that produced by MacLeod and Gold (1951). In the mid-70s, reduced sperm counts and ejaculate volumes were noted by Nelson and Bunge (1974) and Rehan et al. (1975). These findings were criticised by Macleod and Wang (1979) based on their own historical data (1951 – 1977) and the lack of motility or morphology data to complement sperm count data, as they are better gauges of male fertility and fecundity. The authors further stated their opinion that there was no decline in sperm counts, if a decline was present then it was so small as not to have a clinical outcome, and that differences are likely to be merely a factor of geographical variability. In the following years additional evidence was presented, however, there were major differences between studies impeding direct comparisons, most significantly the population sampled (randomly selected volunteers, fertile or

infertile married men, young sperm donors, and pre-vasectomy patients), analysis methods, and metrics examined (just sperm counts, or also including motility and morphology). It wasn't until Carlsen et al. (1992) published the largest meta-analysis to date that the subject of declining sperm counts re-entered mainstream debate. The analysis of 61 studies examining men with no history of infertility from 1938 – 1991 showed a decrease in mean sperm counts of 40% over 50 years (Carlsen et al., 1992), however, the study reignited debate and controversy; the meta-analysis was criticised for its inclusion criteria, accessibility of study data, sample sizes of some included studies, not accounting for publication bias, and statistical analysis (Farrow, 1994). What followed were many large, retrospective, longitudinal studies with spatial variation, with a mixture of results showing decreases, increases, or no changes in sperm counts in various countries. Again, there were major differences between studies including populations sampled, analysis methods, and metrics examined (Jouannet et al., 2001). A much-expanded meta-analysis, however, which incorporated new studies and accounted for age, duration of abstinence, fertility status, and methods of collection and analysis, confirmed a decline in sperm counts of 1.5% per year in the population of the USA from 1938-1988, and 3.1% per year in the European population from 1971 – 1990 (Swan et al., 2000). This proved to be enough to give a general consensus that the reported declines in sperm counts are "real", and evidence has been presented that these may be having an impact on low fertility rates (Jensen et al., 2008; Paasch et al., 2008). However, despite a continual flow of analyses showing reduced sperm counts and deterioration of sperm morphology (Levine et al., 2017; Rolland et al., 2013), with the most recent and largest meta-analysis showing accelerating rates of declining sperm counts globally (Levine et al., 2022), the subject of declining sperm counts remains controversial (Tong et al., 2022).

Concurrent to the debate over declining sperm counts and semen quality were observations of increasing rates of other male reproductive health issues. These include testicular cancer (Adami et al., 1994; Møller, 1998) and anomalies of the male external genitalia, most noticeably cryptorchidism and hypospadias (Campbell et al., 1987; Chilvers et al., 1984; Matlai and Beral, 1985; Paulozzi et al., 1999). Due to the similarities with gestational exposure to diethylstilbestrol, these trends were hypothesised to be a result of gestational xenoestrogen

exposure (Sharpe and Skakkebaek, 1993). This hypothesis was later refined by (Skakkebæk et al., 2001), who proposed that these trends, including reduced sperm counts, were different facets of one underlying condition of fetal origin: TDS. Later, the working hypothesis for TDS pathogenesis was modified to include a direct involvement of gestational EDC exposure, particularly to anti-androgenic and oestrogenic chemicals, in the disruption of fetal programming and testicular development (Skakkebæk, 2002). Since the proposal of TDS as a common link between the various adverse trends in male reproductive health, other factors have been shown to have influence, including improper nutrition, sedentary lifestyle, and stress (Crean and Senior, 2019; Ilacqua et al., 2018). Some, however, have questioned the existence of TDS altogether (Akre and Richiardi, 2009). Yet, while TDS may not be involved in all cases of hypospadias and impaired spermatogenesis (Jørgensen et al., 2010), increased rates of individuals with multiple clinical aspects of TDS in conjunction with histopathological commonalities strongly supports the existence of TDS, along with an increasing body of epidemiological evidence supporting the role of gestational EDC exposure in the pathogenesis of TDS (Nistal et al., 2017; Toppari et al., 2010; Wohlfahrt-Veje et al., 2009). Indeed, while no precise mechanism has been discovered for TDS, it is likely that the pathogenesis is multifaceted, with contributing factors including EDC exposure during development, genetic predisposition, intrauterine growth disorders, and lifestyle influences (Xing and Bai, 2018).

1.5. Aims

The discussed experimental evidence gained from fetal and adult testes indicates a repeatable and sustained testicular phenotype can result from gestational exposure to BTP. Mechanistic insights into how gestational exposure to real-life chemical mixtures alter male reproductive development in BTP sheep offspring would provide greater understanding of the effects current exposure is having on living organisms. Due to the unique translatability of the BTP sheep model to humans, this could also provide information on the pathogenic mechanisms underlying declining sperm counts and increasing rates of TDS. However, there is a large data gap from late gestation to 19 months of age,

which limits knowledge of the pathogenesis and progression of adverse effects on gonadal development.

The aims of this body of work were to characterise the progression the testicular phenotype previously observed in male offspring gestationally exposed to BTP through maternal grazing, and to gain mechanistic understanding of influencing factors. It was hypothesised that pathogenic alterations to gene transcription may be evident from birth to early adulthood. Therefore, transcriptome analysis might identify pathways causative to the testicular phenotype. To this end, three crucial timepoints for male gonadal development were selected for investigation: neo-natal (1-day-old), pre-pubertal (8-week-old), and maturation (11-month-old).

What follows is a published critical review of recent publications on mammalian low-dose exposure to chemical mixtures (Chapter 2). The subsequent chapters describe primary research using the BTP model: published primary research detailing morphological and transcriptomic investigations in neonatal male offspring (Chapter 3), published primary research detailing morphological and transcriptomic investigations in pre-pubertal male offspring (Chapter 4), and primary research submitted for publication detailing morphological and transcriptomic investigations in adult male offspring, and transcription factor analysis of all three ages (Chapter 5).

Chapter 2.

Critical review and analysis of literature on low dose exposure to chemical mixtures in mammalian *in vivo* systems

2.1. Abstract

Anthropogenic chemicals are ubiquitous throughout the environment. Consequentially, humans are exposed to hundreds of anthropogenic chemicals daily. Current chemical risk assessments are primarily based on testing individual chemicals in rodents at doses which are orders of magnitude higher than that of human exposure. The potential risk from exposure to mixtures of chemicals is calculated using mathematical models of mixture toxicity based on these analyses. These calculations, however, do not account for synergistic or antagonistic interactions between co-exposed chemicals. While proven examples of chemical synergy in mixtures at low doses are rare, there is increasing evidence which, through non-conformance to current mixture toxicity models, suggests synergy. This review examined the published studies that have investigated exposure to mixtures of chemicals at low doses in mammalian *in vivo* systems. Only seven identified studies were sufficient in design to directly examine the appropriateness of current mixture toxicity models, of which three showed responses significantly greater than additivity model predictions. While the remaining identified studies were unable to provide evidence of synergistic toxicity, it became apparent that many results of such studies were not always explicable by current mixture toxicity models. Additionally, two data gaps were identified. Firstly, there is a lack of studies where individual chemical components of a complex mixture (>10 components) are tested in parallel to the chemical mixture. Secondly, there is a lack of dose-response data for mixtures of chemicals at low doses. Such data is essential to address the appropriateness and validity of future chemical mixture toxicity models.

2.2. Introduction

A wide variety of natural and anthropogenic chemicals are found throughout the environment in air, water, food, soil, and dust. Sources of such environmental chemicals (ECs) include agrochemical food residues, consumer products, industrial chemical effluent, occupational use, and pharmaceutical or recreational drugs, to name but a few. Biomonitoring of EC exposure is routinely performed by various agencies across the world, which commonly see a range of ECs in blood, plasma, and urine samples from across the general population. These include heterocyclic amines, organochlorides, polychlorinated biphenyls

(PCBs), polychlorinated dibenzofurans, polycyclic aromatic hydrocarbons (PAHs), metals and metalloids, and volatile organic compounds (VOC) (U.S. HHS, 2019). These components, at high doses, have a diverse range of toxicological profiles; many are known to be carcinogenic, hormonally active, hepatotoxic, nephrotoxic, and/or neurotoxic. Co-exposure to ECs from multiple sources, or accumulation of EC exposure over time, is of increasing concern to both regulators and the public, however the exposome (the totality of EC exposure over a lifespan) is particularly difficult to risk assess (Rappaport, 2011; Sarigiannis and Karakitsios, 2018) and may be having an impact on increasingly prevalent chronic diseases and viral susceptibility (Tsatsakis et al., 2020).

Within traditional chemical risk-assessment, points of departure (PODs) such as the no observable adverse effect level (NOAEL), the highest administered dose which did not produce a statistically significant toxicological effect, are crucial values to determine for every chemical. PODs are defined relative to toxicological dose-response curves for individual chemicals and have been cornerstones of toxicology for decades (reviewed by Dorato and Engelhardt, 2005). Indeed, the idea of a NOAEL was first conceived in the 16th century by the "father of toxicology" – Paracelsus (Temkin, 1941). PODs are used to determine the tolerable daily intake (TDI) for a chemical, by dividing PODs by safety factors for inter- and intra-species variance (usually around 100 – 1000). While invaluable in the context of individual chemical toxicological assessments, the toxicological profile of a chemical mixture is not just the sum of the effects of the component chemicals but may be altered by additive and/or synergistic interactions between chemicals in the mixture, even where each component chemical is below accepted individual PODs (here described as "low dose" mixtures). This has led to calls for an urgent assessment of the effects of chemical mixtures on human and environmental health (Drakvik et al., 2020; Ribeiro et al., 2017; WHO, 2012).

Assessing risk from exposure to chemical mixtures poses many unique problems, including the near infinite number of possible chemical combinations, interactions between chemicals within mixtures, and the attribution of responses to component chemicals (Bopp et al., 2018). There are two main approaches for evaluating risk from exposure to chemical mixtures: whole-

mixture and component-based. Whole mixture methodologies provide a comprehensive assessment of a specific mixture where unidentified chemical components and inter component reactions are maintained. This "top-down" approach is analogous to the assessment methods used for individual chemicals (Hernández et al., 2017). Whole-mixture methodologies, however, are not always feasible, due to variation between chemical mixtures, changes due to chemical degradation or metabolism, and factors that affect environmental load, over time. In addition, whole-mixture methodologies do not identify which chemicals within the mixture are responsible for a response(s), nor any interactions between chemicals within the mixture. Component-based methodologies use a "bottom up" approach, where a limited number of chemicals with defined concentrations are mixed and tested, or toxicological information for defined chemical components within a mixture are analysed with additivity models, to assess the risk from mixed chemical exposure (Hernández et al., 2017; Heys et al., 2016). Additivity models, however, assume no compounding toxicodynamics or toxicokinetics, and the required toxicological information for such models can be inconsistent, or absent (Boobis et al., 2011) in particular regarding *in vivo* data pertaining to chemical interactions (synergy/antagonism) (Heys et al., 2016).

Current models to define and predict mixture toxicity fall into four categories: dose addition (DA), response addition (RA, also known as independent action), effect summation (ES), and integrated addition (IA) (Rider et al., 2018). Appropriate choice of model is critical for accurate predictions of mixture toxicity, however equally important is which chemicals within a mixture are grouped together for mixture risk assessment – a subject deeply debated (Kortenkamp, 2020). Indeed, these two considerations are connected, and while similarities exist between toxicological models for chemical mixtures, the model which a chemical mixture is thought to follow most closely depends on the individual mixture components. Toxicological similarities between mixture components may occur at one or more levels (Rider et al., 2018). The highest form of similarity is chemicals which share a common active metabolite, as is the case of benzyl butyl phthalate and dibutyl phthalate (which assumes an inactive parent molecule). Next similar are compounds which share a molecular initiating event, and whose toxic events therefore occur *via* identical pathways,

as with parathion and chlorpyrifos which are both acetylcholinesterase inhibitors. At the next level of similarity, which is less defined, chemicals share adverse outcome pathways (AOPs), but convergence of different initiating events may occur at several key events in that pathway. For example, perchlorates and dioxin both reduce circulating thyroid hormone concentrations, but they act to reduce the production of, and increase the elimination of, thyroid hormones, respectively (Boas et al., 2006). Less similar still are chemicals which induce toxicity within the same organ system but *via* different mechanisms. For example, caffeine and ephedrine can individually produce cardiotoxicity through separate mechanisms, but when co-administered act synergistically to produce enhanced toxicity (Dunnick et al., 2007). Toxic additivity of chemicals at this level of similarity within a mixture is a topic of debate, as they may or may not compound each other. Finally, toxicologically least similar are chemicals which share a common disease outcome. For example, diethylstilbestrol and cyclophosphamide are both recognised carcinogens but act *via* different mechanisms of action and can affect different organ systems. Here, the nature of the disease is of greater importance.

Reports indicate advances in terms of hazard identification, exposure and risk assessment, and subsequent risk management relative to co-exposure to chemicals in mixtures (reviewed by Bopp et al., 2019) as well as harmonisation of methodologies for mixture toxicity assessment (EFSA Scientific Committee et al., 2019). However, much of the focus of this work has been towards intentional mixtures, such as formulations within pesticides or cosmetic products, and does not cover doses below PODs (Rotter et al., 2018). Furthermore, even less attention has been paid to 'real-life' chemical exposure, which is characterised as being to vastly more complex and varied mixtures, at doses far below PODs. This pattern of exposure is that to which the wider human and wildlife populations are exposed throughout their lifetimes. Regulatory agencies often review literature on mixture toxicity. Although varying conclusions by different bodies, the general approach is to separate chemicals into groups with distinct modes of action before individually applying additive models to each group for risk assessment, noting that in the case of knowledge gaps dose additivity should be assumed and that interactions between chemical components are rare, and generally only occur at mid to high doses (NAS, 2017a; OECD, 2018;

SCHER/SCCS/SCENIHR, 2013; U.S. EPA, 2007; WHO, 2009). Few publications, however, focus on EC mixtures around or below POD doses, or chemical interactions at these doses. A review of literature concerning low dose mixtures of pesticides from 1985-1998 concluded that exposure to such mixtures is not a source of concern to human health (Carpy et al., 2000). An EU commissioned report on the state of the art of mixture toxicity risk assessment concluded that generally mixture toxicity risk assessments by dose addition models were reliable. However, they also noted several important examples of synergy which highlights the need for clarity as to which chemicals to group in cumulative risk assessments (Kortenkamp et al., 2009). A review of low EC dose synergy noted synergy as rare and that the effects of synergy did not exceed additivity models by more than a factor of 4 (Boobis et al., 2011). The most recent and most extensive review of low dose mixtures was performed by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC). Assessing mixture toxicity studies where component concentrations were at or below NOAELs and examining toxicity reported as more than expected based on current models of mixture exposure (i.e., more than additivity in the case of similar chemicals, and more than independent action in the case of dissimilar chemicals), ECETOC concluded that there is no evidence of a risk to human health from exposure to complex mixtures of components where each is below regulated levels (ECETOC, 2012). However, to only consider effects provably greater than model predictions, rather than effects unsatisfactorily explained by model predictions, is tantamount to requiring proof of synergistic toxicity – a considerably larger endeavour. To place the burden of proof on the literature rather than the toxicity model might also be considered contrary to the traditionally conservative nature applied in toxicology. Still, despite this requirement, over 5% of studies reviewed by the ECETOC showed a deviation from additivity that suggested synergistic chemical effects (ECETOC, 2012).

Here we critically review the literature which has investigated the effects of exposure to chemical mixtures in mammalian *in vivo* systems, with each component at or below respective POD doses. The review covers the period of 2000 to 2020, inclusive, as a resource collating the most recent literature for those interested in mixtures research and to identify and highlight areas in need of additional research.

2.3. Methodology

A literature search was conducted across multiple journal databases (PubMed, Google Scholar, and SCIRUS) for peer reviewed studies published between 2000 and 2020 relating to low dose mixtures of chemicals in mammalian *in vivo* systems. Low dose mixtures were defined as those in which all components were at or below their POD. Identified literature with at least one experimental group fulfilling these criteria were included for discussion. Figure 2-1 illustrates the logic underpinning the literature search and subsequent evaluation for inclusion. To identify any missed literature a wider search of non-scientific sources (Google) was performed, and publication histories of relevant authors or research groups were interrogated. For each identified study which met all inclusion criteria, detailed experimental information was extracted (mixture components, dosing levels and regime, route, species, duration, and timing). Reported significant outcomes relevant to exposure were captured and

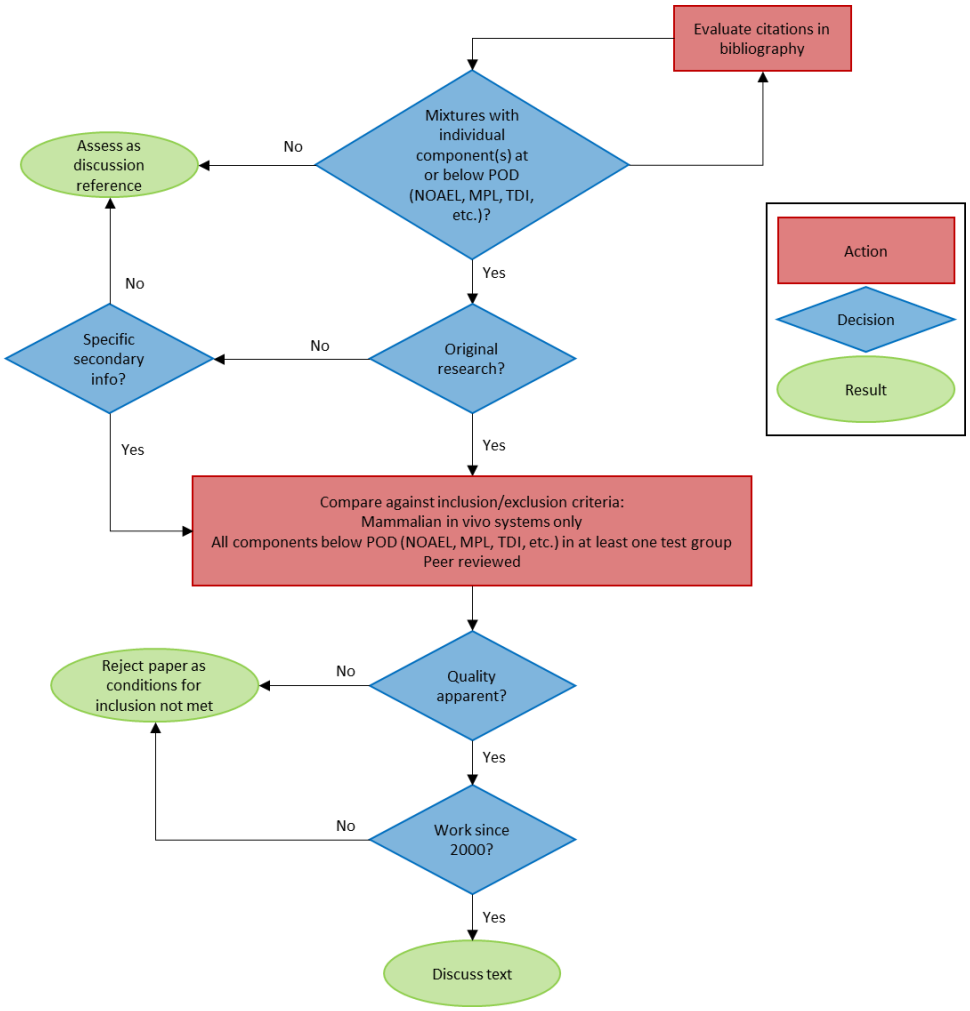


Figure 2-1. Decision logic tree for the inclusion of studies.

summarised. Results from authors' original analyses were used, and no statistical re-analysis was performed.

2.4. Results

2.4.1. General Findings

Literature searches identified sixty-eight research articles which met the inclusion criteria. Fifty-four reported findings from studies that used component-based methodologies, and fourteen from studies that used whole-mixture methodologies. Detailed experimental information can be found in Appendix 1.

Thirty research articles that used component-based methodologies tested mixtures with at least one component at or close to its NOAEL. In nineteen of these research articles this was the lowest dose tested. Twenty-one research articles that used component-based methodologies had at least one group exposed to a mixture with all components at or below TDI values. Research articles with component-based methodologies were divided into groups, firstly on the number of components (simple mixtures that contained ≤10 individual chemicals, complex mixtures that contained >10), and secondly simple mixtures were divided based on commonality between the characteristics of individual mixture components. These secondary groups were antiandrogen mixtures, antiandrogen plus xenoestrogen mixtures, mixed endocrine disrupting mixtures, neurotoxic mixtures, and mixed mode of action mixtures.

Identified research articles that used a whole-mixture methodology were from studies that used one of two experimental models: the biosolid treated pasture (BTP) sheep model, and a disinfection by-product model. As such, that literature was grouped by model type, and then for BTP articles also as a function of age; effects in the fetus, juveniles, and adult offspring.

2.4.2. Component-based methodologies

2.4.2.1. Antiandrogen mixtures

Six research articles tested mixtures of known antiandrogens and chemicals with antiandrogenic action. Phthalates were included as while not strictly antiandrogens, phthalates induce antiandrogenic effects through non-androgen

receptor mediated mechanisms. Effects on sexual development were reported in male rat offspring following *in utero* exposure *via* parental oral dosing with antiandrogen mixtures with individual chemicals at/below NOAEL. Exposure to a mixture of vinclozolin, procymidone, linuron, prochloraz, BBP, DBP, and DEHP at NOAEL resulted in increased areola numbers at post-natal day (PND) 13 (Rider et al., 2008). Nipple retention (NR) was observed following exposure to a mixture of vinclozolin, procymidone, linuron, prochloraz, BBP, DBP, and DEHP, DiBP, DiHP, and DPP at NOAEL (Rider et al., 2010). Exposure to a mixture of vinclozolin, finasteride, prochloraz, and DEHP at NOAEL resulted in reduced anogenital distances (AGD) at birth and increased nipple retention (NR) at PND13 (Christiansen et al., 2009). However, a study by Schneider et al. (2017) reported no effects of chemical exposure on male or female offspring at PND1, 12, 20, or 83, following gestational and lactational exposure to a mixture of flutamide, prochloraz, and vinclozolin at TDI and NOAEL (parental oral and direct oral dosing after weaning). The apparent lack of an effect of exposure to the chemical mixture was despite observation of multiple statistically significant physiological effects, both on the mothers, such as increased gestation length and lower oestradiol concentrations, and male offspring, which exhibited decreased progesterone concentrations and decreased absolute cauda, epididymis, and relative bulbourethral gland weights. These effects were not attributed to chemical exposure due to the absence of significant effects in other organs which are known to be more sensitive to androgen disruption, and the limited magnitude of the observed effects. In the same study, however, rats were exposed to flutamide, prochloraz, and vinclozolin at LOAEL, both individually and as a mixture (Schneider et al., 2017). In these groups they observed greater than additivity (i.e., potentially synergistic) in the effects of chemical mixture exposure with regards to increased NR and delayed sexual maturation in male offspring, and increased androstenedione in mothers (Schneider et al., 2017). This would indicate that the lack of effects seen at or below NOAEL may be due to the limited number of chemical components within the mixture, rather than a lack of synergy between mixture components.

As noted above, authors reported no effects from co-exposure to flutamide, prochloraz, and vinclozolin at NOAEL or TDI, but greater than additive effects when the chemicals were administered at LOAEL (Schneider et al., 2017). More

in-depth comparison of chemical effects to additivity models were reported in the previous three studies (Christiansen et al., 2009; Rider et al., 2010, 2008), which investigated more complex mixtures of antiandrogens. Rider et al. (2008) reported logistically regressed ED_{50} values for hypospadias and undescended testes from co-exposure to vinclozolin, procymidone, linuron, prochloraz, BBP, DBP, and DEHP, which differed with statistical significance to those from additivity models (DA, TEF, RA, IA), with each model predicating higher ED_{50} values than those observed, while models based on additivity (DA and/or TEF) were accurate for predicting AGD shortening, epididymal agenesis, ventral prostate weight reductions, epididymal and seminal vesicle weight, and gubernacular agenesis. On balance, Rider et al. (2008) reported that additive toxicity models were generally accurate despite mechanistically distinct chemicals examined, which would have been risk assessed by RA or IA. Similarly, with regards to vinclozolin, finasteride, prochloraz, and DEHP co-exposure, the differences between effective doses for producing AGD shortening, cleft phallus, and malformations in general derived from observations and from additivity models (DA, RA) were statistically significant, with models predicting higher effective doses than those observed (Christiansen et al., 2009). However, Rider et al. (2010) showed DA additivity model predictions to be accurate for all studied endpoints (AGD reduction, various malformations, NR, organ weights) except ventral prostate weight, where the effect magnitude was under-predicted. In Rider et al. (2010) non-phthalate components were identically dosed as in Rider et al. (2008), however, 6 phthalates were used instead of 3 while total phthalate dosage was maintained. That DA model predictions were accurate in Rider et al. (2010) yet inaccurate in Rider et al. (2008) suggests unknown interactions between phthalates and other mixture components, yet current guidelines would use an RA approach to these mixtures, which gave inaccurate predictions in both Rider et al. (2008) and Rider et al. (2010). Discrepancies between model predictions and observations despite dissimilarities between antiandrogenic mechanisms across mixture components supports concerns regarding compounding and/or synergistic effects of antiandrogens on the fetus, and of improper considerations of additivity/synergy between mechanistically distinct antiandrogens by currently employed mixture toxicity models.

Two studies were identified that investigated mixtures containing only phthalate plasticisers. Exposure of juvenile male rats to a mixture of six phthalates (DMP, DEP, DBP, BBP, DEHP, and DnOP), at doses equivalent to 1x or 0.1x NOAEL, *via* dietary exposure for 15 weeks, resulted in reduced body weights and altered the weight of several vital organs (Gao et al., 2017). The exposed mice had lower testosterone and higher luteinizing hormone concentrations compared to the controls and the testes showed increased deciduous spermatids (at both dose levels), and reduced concentrations of several proteins involved in testicular development and spermatogenesis (at 1x NOAEL) (Gao et al., 2017). Effects on body weight and the reproductive system were also seen when male mice were exposed to a mixture of 3 phthalates with 2 alkyl-aromatics (DEHP, DBP, BBP, NP, and 4-tert-octylphenol) at doses equivalent to individual TDI values from conception to PND60 *via* drinking water. In this instance, the mixture exposed mice exhibited increased body weights and reduced relative testes weights compared to the controls (Buñay et al., 2018). Within the testes of exposed mice, the seminiferous tubules showed multiple signs of damage: tubule widths were reduced, germ cell exfoliation was increased, a greater proportion of tubules were without lumen, and steroidogenic gene expression was altered. Germ cells also showed signs of disruption: an increase in germ cell death, apoptotic signalling, and a greater proportion at an undeterminable stage of spermatogenesis due to the high level of degeneration/atrophy (Buñay et al., 2018). Of these two studies, only that of Buñay et al. (2018) also tested for the effects of phthalates individually in parallel to the mixture (at TDI doses), where each effect observed in response to the mixture was also observed in response to an individual chemical, however the number of animals affected was, in general, greater when exposed to the mixture of chemicals.

2.4.2.2. Antiandrogen plus xenoestrogen mixtures

Seven research articles tested mixtures of known antiandrogens and/or xenoestrogens. Six of these investigated offspring effects following gestational and lactational exposure to a mixture of genistein and vinclozolin *via* parental oral dosing, in rats. Both chemicals were administered at 1 mg/kg/day, which is below the NOAEL for vinclozolin and at the levels of genistein expected for animals on a soya-bean diet, which is accepted as below the TDI for genistein. Following exposure to this mixture, litter sizes were significantly smaller, and

pups were significantly heavier than unexposed controls (Eustache et al., 2009). While litter sizes were also smaller, and with heavier pups, following exposure to genistein alone, the effect was greater when mothers were exposed to the mixture of genistein and vinclozolin. Mixture exposed male offspring exhibited reduced testosterone secretion upon ex vivo stimulation at PND3 (Lehraiki et al., 2011), AGD shortening and more frequent immature penile development at weaning, and decreased epididymis and seminal vesicle weights, sperm quality and quantity, and increased FSH and oestradiol at PND80 (Eustache et al., 2009). However, of these effects, all except reduced seminal vesicle weight and lower sperm quantity were also seen in animals dosed with either genistein or vinclozolin alone. Interestingly, microarray analysis of testicular gene expression in animals at PND80 also demonstrated a correlation between the effects of the low dose mixture and individual exposure to a 30x higher vinclozolin dose, which would suggest a synergistic action between the two chemicals when present in the mixture (Eustache et al., 2009). Mixture exposed female offspring exhibited earlier vaginal opening, and multiple histopathological changes in mammary gland structure at PND35, including increased duct sizes, thicknesses, and cellular proliferation, however some of these effects were also noted in animals dosed with either genistein or vinclozolin alone (Saad et al., 2011). Female rats exposed to genistein and vinclozolin from conception through weaning *via* parental oral dosing also exhibited fewer striated submandibular salivary ducts, an increased area of striated salivary ducts, and decreased proliferation with lower expression of growth factors EGF, NGF, and TGFα in submandibular salivary glands, however most of these findings were also seen in animals exposed to just vinclozolin, although the effect size was greater when exposed to the mixture (Kouidhi et al., 2012).

Direct and multigenerational effects on bone formation in the paw and spine following gestational exposure to genistein and vinclozolin alone or in combination with BPA have been reported. Specifically, alterations in forepaw digit lengths of F1 (exposed *in utero*) and F2 (not directly exposed) males towards a more feminine digit length ratio have been reported following F1 gestational exposure to mixtures of various combinations of genistein at sub-NOAEL, vinclozolin at NOAEL or TDI, and BPA at TDI (Auger et al., 2013). Histopathological alterations in the vertebral growth plates (intertransverse

apophysis width increases and v5 to v8 vertebral length shortening) has been reported in F1 females, but not males, at PND30-110 following similar exposure to genistein, vinclozolin and BPA, however these effects were also seen when F1 animals were exposed to individual chemicals and did not continue into the F2 generation (Auxietre et al., 2014). Interestingly, F1 males exposed to vinclozolin and/or genistein at sub-NOAEL, alone or as a mixture, were unaffected, while co-exposure of either vinclozolin at the lower TDI dose, or genistein, at sub-NOAEL, with BPA at TDI resulted in histopathological alterations in the vertebral growth plates, but these findings were not present following exposure to genistein, vinclozolin and BPA, at these levels (Auxietre et al., 2014). In all the above studies, where individual chemicals were tested in parallel to mixtures of the chemicals, some effects were also noted from exposure to the individual chemicals, in some cases effects were only noted from exposure to an individual chemical. However, mostly, effects were more numerous and/or greater in size when animals were exposed to the mixture.

While the above studies noted compounding effects from chemicals when administered as a mixture, compared to when administered individually, the remaining study concluded that co-exposure to certain chemicals could, through counteraction measures, result in fewer physiological effects. This conclusion was based on the observation that, compared to controls, mice exposed (*via* parental oral dosing during gestation and lactation) to sub-LOAEL doses of DEHP (daily) or BPA (daily), or TCDD (single treatment) had lower body weights (PND14 and PND21), increased relative brain weights at PND14, and lower absolute brain weights at PND42, with changes in midbrain dopaminergic nuclei at both PND14 and PND42. However, when animals were dosed with the chemicals as a mixture, body weight was unaffected, brain weight (relative and absolute) was increased at PND14, and there were no signs of changes in midbrain dopaminergic nuclei (Tanida et al., 2009).

2.4.2.3. Mixed endocrine disrupting chemical mixtures

Fourteen research articles tested mixtures of known EDCs. One study investigated the effects of drinking water disinfection by-products at higher levels than maximum contaminant levels (MCLs), but with two groups (500x and 1000x MCL) ≤NOAEL (0,5x and 1x, respectively). Pregnant rats and their offspring

were exposed from F1 GD0 to F2 PND6 *via* drinking water. Water consumption was reduced in treated dams intermittently during gestation and consistently throughout lactation at 1000x MCL, and in F1 females at 500x and 1000x MCL. F1 pup weights were reduced in males at PND26 and in females at PNDs 21 and 26, and the onset of puberty was delayed in both F1 sexes at 1000x. No effects were seen in the F2 generation (Narotsky et al., 2015).

Multiple research articles were identified that had investigated mixtures of endocrine disrupting pesticides at low doses. When juvenile rats were exposed, *via* a standard diet, to three azole fungicides (cyproconazole, epoxiconazole, and prochloraz) at individual doses equivalent to 1x NOAEL and 0.01x NOAEL (TDI) either no adverse effects were seen (Schmidt et al., 2016), or effects were noted on organ weights and circulating hormone levels, but these effects were not attributed to exposure to the chemical mixture as they were only seen at the 0.01x NOAEL dose and not at 1x NOAEL (Rieke et al., 2017). However, gestational, or gestational and lactational exposure to 5 fungicides of different classes (procymidone, mancozeb, epoxiconazole, tebuconazole, and prochloraz), *via* parental oral gavage, at individual doses equivalent to 0.083x – 1x NOAEL, did result in adverse reproductive effects. In fact, increased gestation duration, pup mortality and birthing complications were so severe at 0.75x and 1x NOAEL doses that this part of the experiment had to be discontinued (Hass et al., 2012; Jacobsen et al., 2010). Against the lower doses, the pups exhibited reduced birth weights, females had increased AGD, whereas males had decreased AGD, and increased incidence of genital dysgenesis and NR (Hass et al., 2012; Jacobsen et al., 2010). At weaning, relative liver weights were reduced in both sexes, and in males prostate and epididymis weights were reduced (Jacobsen et al., 2010). When allowed to mature, the relative weights of sexual organs were reduced (PND16 and 280), and reduced sperm counts, and increased prostate hyperplasia were observed (PND280) (Jacobsen et al., 2012). When the mixture dose response data was compared to mixture toxicity models (DA and IA) using dose response data from individual chemicals, a synergistic effect was reported for the mixture on gestation length and NR, and potentially also towards genital malformations however synergy could not be distinguished from a marked joint effect as no significant genital malformations were observed in response to chemicals individually (Hass et al., 2012). Mice that received, from conception

to PND98, dietary exposure to a mixture of the pesticides atrazine, chlorpyrifos, and endosulfan, at doses equivalent to individual TDI values, expressed increased circulating glucose concentrations at PND21 and PND98, and by PND98 a significant reduction in bodyweight was observed (Demur et al., 2013). At PND21, the EC exposed animals, of both sexes, were characterised by lower levels of many amino-acids and nutrients compared to the controls, however, by PND98 this profile was reversed in the males (Demur et al., 2013). Effects on cognition from gestational exposure to atrazine in combination with 3 different EDCs (PFOA, BPA, and TCDD), all at doses equivalent to TDI values or below NOAEL values, have also been reported. The results indicated reduced environmental habituation in exposed mice of both sexes, and males were found to display decreased exploratory behaviour, short-term memory, and attention to task, relative to the controls (Sobolewski et al., 2014). In both above cases which looked at the effects of chemical mixtures including atrazine, effects from exposure to individual chemicals were also noted, however the observations were of greater effect sizes when chemical exposure was to a mixture, which indicates at least additivity despite separate toxicological mechanisms.

Administration of a mixture of TCDD, PCB-153, DEHP, and BPA through dietary supplementation equivalent to TDI induced oestrogeno-mimetic and metabolic effects in mice, effects which were also shown to be influenced by diet. At 7-12 weeks old, various metabolic changes were observed in male and female offspring of parents exposed prior to mating, through gestation and lactation, and which received direct exposure after weaning, *via* supplementation of a standard (Labaronne et al., 2017) or high-fat-high-sucrose (HFHS) diet (Naville et al., 2015, 2013), with the EC mixture. These include changes in glucose tolerance, circulating and hepatic cholesterol, triglyceride, fatty acid levels, and adipose tissue mass. In the HFHS diet groups, changes in hepatic metabolic gene expression were reported in response to the mixed chemical exposure, which included increased arylhydrocarbon receptor (Ahr) and peroxisome proliferator-activated receptor α (Pparα) gene expression, and alterations in the expression patterns of various xenobiotic transforming and metabolic enzymes (Naville et al., 2015, 2013). In animals which received the chemical mixture in the standard diet, enriched hepatic gene expression was found relative to drug and xenobiotic

metabolism, steroid biosynthesis, and fatty acid metabolism (Labaronne et al., 2017). A follow up study in juvenile male mice compared the effect of standard or HFHS with and without EC supplementation (Naville et al., 2019). Metabolic changes like those previously recorded against a HFHS diet were reported in response to EC exposure, however, several effects reversed in direction between standard and HFHS diets or were only present when administered in one type of diet (Naville et al., 2019). Studies in ovariectomised female mice receiving the same chemical exposure (TCDD, PCB-153, DEHP, and BPA at TDI equivalent doses in HFHS diet) demonstrated oestrogeno-mimetic effects from exposure to the chemical mixture from prior to conception to a juvenile age, with additional metabolic alterations including insulin and glucose tolerance (worsened with EC exposure in sham operated but alleviated with EC exposure in ovariectomised mice) and increased hepatic triglycerides (Julien et al., 2019, 2018). EC mixture exposure after ovariectomy, with or without oestrogen replacement, also resulted in tissue and steroid replacement specific effects on steroid hormone production and several oestrogen pathways (Julien et al., 2019). Consistent across these studies was the observation of an increase in hepatic oestrogen receptor expression, which was also observed in male mice exposed to the chemical mixture *via* standard and HFHS diets (Julien et al., 2019, 2018; Naville et al., 2019). None of the above studies which tested mixtures of TCDD, PCD153, DEHP, and BPA concurrently investigated the physiological effects of individual chemical exposure.

2.4.2.4. Neurotoxic mixtures

Five research articles tested mixtures of chemicals which alone can produce neurotoxic effects. Although the primary toxicity of organophosphorus insecticides is that of neurotoxicity, the identified research which investigated the effects of organophosphorus insecticide mixtures demonstrated hepatic and nephrotoxic effects. Adult male rats, exposed to a mixture of diazinon, chlorpyrifos, malathion, and profenofos for 28 days at doses equivalent to individual NOAEL values, *via* oral gavage, exhibited reduced body weight and increased relative liver weight (Mossa et al., 2011). Upon closer examination, liver damage was evident, as indicated by histopathological changes (dilatation and congestion of central veins sinusoids, dilated cystic bile ducts, oedemas, and hepatocyte degradation) as well as serum clinical chemistry indicative of liver

damage (increased ALT, AST, ALP, and ChE) (Mossa et al., 2011). Whereas exposure of rats to a mixture of dichlorvos, dimethoate, acephate, and phorate, at doses equivalent to respective individual NOAEL values, for 24 weeks, *via* drinking water, found no effect on hepatic antioxidative defence mechanisms or lipid peroxidation (Yang et al., 2012). However, increased circulating triglycerides, lipoproteins, and cholesterol concentrations, and a greater incidence of renal tubular epithelial cell swelling and granular degeneration have been reported following the same EC mixture exposure (Du et al., 2014). Organophosphorus insecticides were tested only individually in parallel with the chemical mixtures in the study by Yang et al. (2012), where no effects were seen at NOAEL following exposure to individual chemicals or the mixture, and they did not compare observations to mixture toxicity models.

Despite pyrethroids being the most widely used commercial insecticide, only three research articles were identified that investigated low dose pyrethroid mixtures. Mild signs of neurotoxicity were seen in adult rats orally dosed with a mixture of 11 pyrethroid pesticides (S-bioallethrin, permethrin, cypermethrin, deltamethrin, esfenvalerate, ß-cyfluthrin, fenpropathrin, tefluthrin, λ-cyhalothrin, bifenthrin, resmethrin) at doses below individual NOAEL values. For 8 hours after exposure to the pyrethroid mixture, at both 43% and 85% individual NOAEL values, animals displayed mild whole-body tremors and reduced motor activity (Wolansky et al., 2009). When rats were dosed with all pesticides simultaneously, the mixture produced effects which conformed well to DA model predictions based on the effects of each individual pesticide, but when the chemicals were dosed in three sets to align the effect maxima of individual chemicals (at 1, 2 or 4 hours) the threshold was 3.7x lower than dose addition model predictions. As this reduction in threshold was not statistically significant ($p=0.07$) the study concluded that the data supported the suitability of DA models for predicting the effects of this pesticide mixture (Wolansky et al., 2009) as opposed to there being a synergistic mixture effect. A similar conclusion was reached when it was shown that a mixture of tefluthrin, bifenthrin, cypermethrin, and deltamethrin dosed to juvenile rats at doses determined in a preliminary study to be below that which individually cause a lowering of body temperature, did not influence body temperature as a mixture (Ortega et al., 2018). Similarly, no significant effects were found on ventilation

efficiency, pulmonary perfusion, cardiac output, or metabolic rate in mice following inhalation of prallethrin and phenothrin, each at NOAEL, individually or as a mixture (Santiasih et al., 2020).

2.4.2.5. Mixed mode of action mixtures

Ten research articles studied mixtures of chemicals which alone can produce adverse effects through various modes of action. Nine of these investigated the physiological effects of exposure to mixtures of pesticides with mechanistically distinct modes of action. Two pesticide mixtures induced haematological changes. Dietary exposure to alphacypermethrin, bromopropylate, carbendazim and mancozeb (neurotoxic, hepatotoxic, EDC) at NOAEL equivalent doses, and chlorpyrifos ranging from 0.04x NOAEL to 1x NOAEL, lowered haematocrit, haemoglobin, and red blood counts, with liver and thyroid weights increased, and thymus weight reduced (Jacobsen et al., 2004). While exposure to a mixture of alachlor, captan, diazinon, endosulfan, maneb, and mancozeb (neurotoxic, hepatotoxic, EDC), at TDI doses *via* thrice weekly oral gavage, altered bone marrow colony compositions in terms of cell type proportions and haematopoietic protein levels, reduced liver weight and increased spleen weight, with distinct and distinguishable metabolic profiles for both exposure and sex (Merhi et al., 2010). Neither study investigated exposure to individual chemicals in parallel to the mixture.

Reproductive effects were reported in male and female rats following dietary exposure to a mixture of dicofol, dichlorvos, permethrin, endosulfan, and dieldrin (hepatotoxic, neurotoxic, EDC) at NOAEL equivalent doses. Specifically, male rats had reduced sperm motility and increased numbers of immotile sperm (Perobelli et al., 2010), while various effects were seen in female rats which appeared to be dependent on strain: decreased oestrous cycle and diestrus period lengths in Lewis rats, decreased ovarian primordial and primary follicles, antral follicles, and corpora lutea in Sprague-Dawley rats, and interestingly, no statistically significant effects in Wistar rats, the most frequently used experimental strain (Pascotto et al., 2015). Individual chemicals were only tested in parallel to the mixture in males, where effects were also observed for some individual chemicals, but the effect size was greater when animals were exposed to the pesticide mixture (Pascotto et al., 2015; Perobelli et al., 2010).

This could indicate synergistic effects between chemicals within the mixture, however, the study design did not provide dose response relationships appropriate to compare observations to mixture toxicity models. Reproductive effects have also been reported following gestational exposure *via* maternal dietary supplementation with cyromazine, MCPB, pirimicarb, quinoclamine, thiram, and ziram (reprotoxic, nephrotoxic, neurotoxic, teratogenic), at doses related to 0.05x – 0.375x of the benchmark doses for a 5% birthweight reduction, which were derived from regulatory draft assessment reports (DARs) (Hass et al., 2017; Svingen et al., 2018). Body weight was reduced in both dams and pups through gestation and from birth to PND16 (Hass et al., 2017) following EC mixture exposure, comnpared to untreated controls. EC exposed male offspring had reduced relative liver and retroperitoneal fat pad weights at PND16, but not at 5-6 months of age (Svingen et al., 2018), relative to untreated controls. Overall, at 5-6 months old, no statistically significant differences were observed in the male offspring, whereas the females showed significantly elevated plasma leptin concentrations relative to the controls (Svingen et al., 2018). Although pesticides were not tested individually in these studies, the empirically derived chemical mixture LOAEL for birthweight was used to determine the dose level to compare to DAR derived LOAEL values. As each chemical was at between 0.2x – 0.09x respective LOAELs, a mixture of 6 chemicals producing the reported effects is in approximate agreement with DA model predictions, which supports the appropriateness of the model (Hass et al., 2017).

Hepatic effects were reported following 12 months dietary exposure to a mixture of ziram, chlorpyrifos, thiacloprid, boscalid, thiofanate, and captan (neurotoxic, EDC, hepatotoxic) at TDI equivalent doses in wild type (WT) and Car knockout (KO) C57Bl/6J mice (Lukowicz et al., 2018). Exposure to the chemical mixture was associated with increased body weight gain in both strains of mice. WT mice exhibited increased hepatic steatosis and triglycerides, with decreased fasting glucose and glucose tolerance, and altered glutathione redox states. KO mice did not exhibit the same hepatic responses, and predictably displayed differential hepatic expression of many detoxifying enzymes and circulating levels of metabolites. Interestingly, subsequent microarray analysis of WT livers identified over 500 genes were upregulated, and over 500 down regulated, in both sexes in response to chemical exposure but less than 10% of the gene

changes were common between the sexes. Pathway analysis also highlighted enrichment in differing pathways between sexes (Lukowicz et al., 2018). These results highlight that the effects of exposure to chemical mixtures can be sex specific or sexually differentiated - an important element when considering studies in which only one sex was examined or where sex is either unconsidered or unreported.

Neurobehavioral effects were reported in male and female rats exposed to diquat, imazamox, bentazone, imazethapyr, tepraloxydim, and acifluorfen (hepatotoxic, teratogenic, genotoxic) *via* drinking water (Sergievich et al., 2020; Tsatsakis et al., 2019c). Exposure to the mixture at 0.25x and 1x NOAEL doses resulted in decreased anxiety at 3, 6 and 12 months, with additional changes in researching activity and problem solving (Sergievich et al., 2020). Rats exposed to the same mixture but at TDI values for 9 months, either with 100% or 25% RDI of essential vitamins, saw reduced locomotor activity resulting from chemical mixture exposure or vitamin deficiency, but not both (i.e., in vitamin deficient controls and vitamin sufficient treated). Locomotor activity and spatial orientation activity were reported to be increased in exposed animals that received insufficient vitamins, which interestingly also did not present increased anxiety that was seen in control animals that received insufficient vitamins (Tsatsakis et al., 2019c). Neurobehavioral effects have also been observed in mice gestationally exposed to a mixture of the flame retardant DecaBDE and lead (EDC, neurotoxic) *via* subcutaneous osmotic pumps, at doses far below (<0.017x) TDI and reference doses, respectively (Chen et al., 2019). In this study, chemical exposure was associated with increased repetitive stereotyped behaviours and impaired spatial learning ability and these behavioural effects were accompanied by increased circulating levels of many cytokines and a reduction in the number of hippocampal neuronal cells. Chen et al. (2019) also noted effects from exposure to DecaBDE or lead alone, especially lead which resulted in equally lowered hippocampal neuronal cell numbers as the mixture exposure group. While the effect size with regards to serum levels of pro-inflammation cytokines was greater when animals were exposed to the mixture, which suggests a synergistic action, the study design did not permit comparison of observations to mixture toxicity model predictions. These results may be of concern as the levels of lead were considerably lower than the accepted TDI.

2.4.2.6. Complex chemical mixtures

Eleven research articles tested complex chemical mixtures with various toxicological mechanisms. These ten research articles reported findings from five separate experiments, none of which tested mixture chemicals individually.

Exposure to 16 organochloride pesticides and 2 heavy metals, for 70 consecutive days, *via* oral gavage, at the lowest investigated dose level (equivalent to individual TDI, reference dose (RfD), maximum residue limit (MRL), or NOAEL values) resulted in increased epididymal sperm counts and greater natural killer cell activity from ex vivo splenocytes from exposed male rats (Wade et al., 2002a). This was accompanied by multiple signs of thyroid toxicity: increased thyroid stimulating hormone (TSH), larger median thyroid follicle areas, and reduced hepatic thyroxine outer-ring deiodinase activity (Wade et al., 2002b). Hepatic effects have also been reported in rats following exposure to a mixture of 27 chemicals (8 Heavy metals, 4 pollutants, 3 pesticides, 2 food-derived carcinogens, 2 plasticisers, 2 preservatives, 2 surfactants, 1 disinfectant, 1 food additive, 1 photostabilizer, and 1 polycyclic musk) at values lower than TDI (Hadrup et al., 2016). In this study, chemical ratios were based on comparisons to measured concentrations in human samples, and certain chemical classes were grouped into one of that class (e.g., PCBs were represented by PCB153 at a level equal to the sum of all PCBs). As a result, the comparability to individual PODs was reduced. The results indicated that chemical mixture exposure was associated with increased liver weight, macrovesicular changes, and vacuolisation. Hepatic lipid metabolomics also indicated separate and distinguishable metabolic profiles for chemical mixture exposed and control animals, however, direct quantification indicated that only one, unidentified, lipid was significantly changed in response to chemical mixture exposure (Hadrup et al., 2016). While these studies support an effect of low dose chemical mixtures on hepatic function, a study that used a Solt-Farber model of hepatocellular carcinoma demonstrated that a mixture of 12 pesticides of various chemical classes at doses equivalent to TDI had no effect on hepatic carcinogenesis (Perez-Carreon et al., 2009).

Two studies investigated reproductive effects of gestational exposure to a complex mixture of chemicals with a diverse range of toxicological mechanisms.

Gestational exposure of rats to 13 chemicals (3 plasticisers, 6 various pesticides, 2 pollutants, 1 preservative, and 1 analgesic) at doses equivalent to individual NOAEL values for AGD or NR, or where NOAEL values were not available LOAEL values divided by a conservative value, indicated specific effects on the reproductive system suggestive of at least additive, potentially synergistic, chemical action. Despite exposure to such low concentrations of mechanistically dissimilar chemicals, male offspring exhibited an increased number of nipples at birth, and greater NR at PND13 (Christiansen et al., 2012). Administration of a mixture of 18 chemicals (5 pesticides, 3 pharmaceuticals, 9 phthalates, and 1 pollutant) to rats during gestation resulted in male offspring with multiple signs of developmental/reproductive toxicity. Reduced testes, epididymis, and levator ani plus bulbocavernosus muscles (LABC) weights (PND21) were seen in offspring exposed to the mixture from ≤0.125x individual NOAEL, reduced AGD (PND2) and glans penis weights (PND21) from ≤0.25x NOAEL, and increased NR (PND13), seminal vesicle weights, and total malformation rates (PND21) at ≤0.5x and ≤1x NOAEL, (Conley et al., 2018).

The remaining five research articles all reported findings from an 18-month rat study which exposed rats *via* drinking water to a mixture of 13 chemicals (4 preservatives, 4 insecticides, 1 herbicide, 1 fungicide, 1 plasticiser, 1 food additive, and 1 chelating agent) at doses equivalent to 0.25x, 1x and 5x TDI values. The reports detailed clinical observations and serum clinical chemistry at 6 and 12 months of exposure (Docea et al., 2019, 2018), behavioural tests (Tsatsakis et al., 2019a), oxidative stress findings in serum at 12 months of exposure and in organs at 18 months of exposure (Fountoucidou et al., 2019), and genotoxic, cytotoxic, and histopathological findings at 18 months of exposure (Tsatsakis et al., 2019b). Over the first 12 months, mixture treated animals had increased weight gain, yet reduced food and water consumption (Docea et al., 2019, 2018). By 12 months, animals that received the lower two doses of the chemical mixture exhibited increased exploratory behaviour (Tsatsakis et al., 2019a). ALT and ALP were increased in animals of each exposure groups at 6 and 12 months of exposure, which is indicative of liver damage (Docea et al., 2019, 2018). At 18 months of exposure deleterious histopathological findings were found in the liver and stomach of animals that received the chemical mixture at 1x and 5x TDI, and in testes, kidneys, lungs,

and brains at all dose levels (Tsatsakis et al., 2019b). At 12 months of exposure, signs of oxidative stress or adaptive redox capacity were mixed within the chemical mixture exposed groups, but these animals had generally lower protein carbonyl levels than controls (Fountoucidou et al., 2019). By 18 months of exposure to the chemical mixture at 0.25x and 1x TDI most tissues showed lower protein carbonyls and thiobarbituric acid reactive substances and higher redox capacity, which suggests that compensatory mechanisms may have become operative in the exposed relative to control animals. However, at 5x TDI, protein and/or lipid oxidation biomarkers were generally increased relative to controls (Fountoucidou et al., 2019), indicating sufficient oxidative stress to overcome compensatory responses, and suggesting greater than additivity (potential synergy), as 13 chemicals at 5x TDI (approximately 0.05x NOAEL) would not be expected to elicit a response even by conservative DA modelling.

2.4.3. Whole-mixture methodologies

Fourteen research articles used whole-mixtures methodologies, and these were all associated with one of two experimental models: the biosolid treated pasture (BTP) sheep model and a concentrated drinking water model.

2.4.3.1. BTP sheep model

Eleven research articles used sheep reared on pastures fertilised using biosolids, a by-product of wastewater treatment. Due to the origins of biosolids they contain a diverse range of anthropogenic chemicals which encompass the human exposome (Rigby et al., 2020), which result in organ chemical loads of 0.5 – 200 µg/kg dry matter (Bellingham et al., 2012; Filis et al., 2019; Rhind et al., 2010, 2009, 2005). Due to a lack of toxicological studies in sheep, empirically determined PODs are not available. A series of studies have quantified the chemicals in biosolids, soil and herbage from BTP and blood and tissue samples from animals grazed on BTP. Chemical levels are small and not significantly different from control pastures; however, it must be noted that due to their ubiquitous nature many chemicals are also detectable in control pastures (Evans et al., 2014; Rhind et al., 2010, 2002, 2013). Chemical quantification and oral dosage estimations are limited to dioctyl phthalate, octyl phenol, and nonyl phenol, which were concluded to be below TDI (Rhind et al., 2002). As where residual chemical levels are monitored in crops and animals grazed on BTPs,

they remain below human TDI values, the model was included in this review. Articles are presented further grouped as a function of age, i.e., effects in the fetus, juveniles, and adult offspring.

2.4.3.1.1. Effects in the fetus

Seven research articles which utilised the BTP sheep model investigated the effects on fetuses. Gestational exposure to BTPs was shown to cause lower body weights in exposed male (Paul et al., 2005) and female (Fowler et al., 2008) fetuses at gestation day (GD) 110. These studies also documented multiple reproductive effects of exposure, in both sexes. Exposed male fetuses showed lower circulating testosterone concentrations, and had testis with reduced weights, fewer Sertoli cells, Leydig cells, and gonocytes, and less androgen receptor expression (Paul et al., 2005). Exposed female fetuses showed decreased oocyte numbers and altered oocyte type ratios. Proteomic analysis identified differentially expressed ovarian proteins related to core cellular processes (Fowler et al., 2008). Alterations of the hypothalamic-pituitary axis have also been reported in both sexes at GD110, with reduced kisspeptin gene expression and protein levels in the hypothalamus and pituitary and altered pituitary cellular composition (Bellingham et al., 2009) in lambs exposed to the chemical mixture.

In two separate studies the timing of exposure to BTP and therefore gestational chemical exposure, prior to and/or during gestation, was investigated: prior to conception only (TC), gestation only (CT), and prior to conception and throughout gestation (TT), compared to controls (CC) where the mothers were never grazed on BTP. These studies reported that at GD110 foetal ovary weight was increased from TT exposure, yet effects on ovarian cell type counts and ratios were only seen in TC and CT (acute exposure) fetuses (Bellingham et al., 2013). All groups exposed to chemical mixtures, regardless of when exposure occurred, showed an increase in ovarian proteins involved in stress, oocyte maturation, and apoptosis, relative to the controls (CC). Ovarian proteomic pathway analysis identified enrichment in two functional networks: 1) cancer, gastrointestinal disease, and cellular movement, and 2) cancer, genetic disorder, and respiratory disease (Bellingham et al., 2013). The timing of exposure to the chemical mixture also alters the reported effects on the thyroid

and the hypothalamic-pituitary axis (Bellingham et al., 2016; Hombach-Klonisch et al., 2013). The most severe thyroid effects of exposure to a mixture of chemicals were seen in CT and TC (acute exposure) groups, and males (Hombach-Klonisch et al., 2013). Thyroid weight was significantly increased, displayed reduced blood vessel area, reduced follicle numbers and areas, and increased cell proliferation in these acute BTP exposure groups. In the hypothalamic-pituitary axis the most notable observations from BTP exposure were altered expression of gonadotropin releasing hormone (GnRH) and its receptor (GnRHR), KISS1, the gene that encodes kisspeptin, and its receptor (KISS1R), oestrogen receptor, and androgen receptor. These expression changes were seen in most BTP exposed groups, for both sexes, although effects of exposure were not consistent between exposure group or sex (Bellingham et al., 2016).

The timing of maternal exposure to BTPs in early, middle, late, or the entirety of gestation, has also been demonstrated to cause differential effects in female fetuses (GD140) (Lea et al., 2016), including body weight reductions, altered relative weights for the uterus, thyroid, and liver, increases in AGD, and changes to circulating testosterone, and free T3 and T4 concentrations (Lea et al., 2016). Despite differences between the effects of the timing of mixed chemical exposure, females in all the exposed groups had a lower proportion of healthy type 1a follicles relative to the controls, with a concordant increase in atretic type 1 and 1a follicles (Lea et al., 2016). Transcriptomic and proteomic analyses of ovaries identified many differentially expressed genes and proteins, but with very little overlap between groups. Pathway analysis of transcriptomic data identified enrichment within cellular growth and differentiation, cell cycle regulation, cell death, cellular development, and cell movement functions. Pathway analysis of proteomic data identified enrichment within free radical scavenging, cell-to-cell signalling and interaction, small molecule biochemistry, drug metabolism, and protein synthesis functions (Lea et al., 2016).

2.4.3.1.2. Effects in juveniles

One paper that utilised the BTP sheep model investigated 5-month-old lambs. This study reported that following gestational and direct exposure to a chemical mixture through maternal and experimental subjects grazing on BTPs, lambs of

both sexes had increased body weights at weaning and showed increased vocalisation and lower maximal activity levels while restrained compared to controls. Males that had been exposed to the chemical mixture also exhibited increased exploratory behaviours relative to the controls, suggestive of the ability of chemical mixtures in this model to affect cognitive ability (Erhard and Rhind, 2004).

2.4.3.1.3. Effects in adults

Three research articles that utilised the BTP sheep model investigated the effects in adult sheep of gestational exposure to a chemical mixture. Homeostasis of bone tissue was disrupted by exposure, with bone mineral content, thickness, circumference, cross-sectional area, and cavity size all affected (Lind et al., 2009). Interestingly, the adult studies that have used the BTP model also allow for the observation of effects of exposure to a chemical mixture following an extended period of non-exposure. Testicular morphology was altered in adult males (18 months old) following gestational exposure from conception, lactational exposure through weaning, and then direct exposure on BTPs until 7 months old. These rams exhibited a higher occurrence of Sertoli-cell-only seminiferous tubules, and lower numbers and volumes of germ cells (Bellingham et al., 2012). Interestingly these effects were not consistent in all animals, with only a subset of animals (5 of 12) showing a markedly altered phenotype, which may reflect the effects of mixed chemical exposure against the diverse genetic background in this outbred study population. Proteomic analysis of livers from these males and their female counterparts (maintained on treated pastures until 18 months) also identified differentially expressed proteins involved in detoxification and fatty-acid ß-oxidation, as well as albumin and transferrin, in both sexes, with pathway analysis indicating dysregulation of cancer-related and lipid-related pathways (Filis et al., 2019).

2.4.3.2. Drinking water disinfection by-products

A series of whole-mixture studies were conducted by the U.S. Environmental Protection Agency which examined the effects of drinking water disinfection by-products (DBPs) in pregnant rats. Only a small percentage of the >600 identified DBPs have been toxicologically evaluated, but of those that have, many are cytotoxic and genotoxic at concentrations achievable in the disinfection process.

For example, epidemiological studies indicate trihalomethanes (THM4; chloroform, bromodichloromethane, dibromochloromethane, and bromoform) at concentrations >50 µg/L are associated with an increased risk of bladder cancer (Costet et al., 2011). Published research on DBPs have focussed on some of the chemicals currently regulated in the US; specifically total THM4, HAA5 (5 haloacetic acids: chloroacetic acid, bromoacetic acid, dichloroacetic acid, dibromoacetic acid, trichloroacetic acid), and bromate, at 80, 60, and 10 µg/L, respectively. It is of note that in the EU only THM4 and bromate are regulated on this regard, and that THM4 is allowed at levels up to 100 µg/L (Andersson et al., 2019; Li and Mitch, 2018). Three research articles investigated the effects of DBP mixtures from various disinfection methods. The model used in these articles provides a mixture of DBPs at realistic ratios, at concentrations higher than maximum contaminant levels but lower than determined NOAELs for monitored chemicals. While not all the chemicals have determined PODs, these studies were included as regulatory assumed structure-activity relationships and chemical groupings for regulatory conditions can be applied.

Narotsky et al. (2008) supplied water approximately 130x concentrated in DBPs, disinfected by either chlorination or ozonation, to pregnant rats, for 10 days during gestation (GD6 – 16) while controls received boiled, distilled, deionized water. While water consumption was increased and gestation lengths were reduced in dams supplied concentrated water, no adverse developmental effects were reported in pups. In a similar experiment conducted by Narotsky et al. (2012), two strains of rat (F344 and Sprague-Dawley) were exposed to water that had undergone chlorination and then concentration (chlor/conc), or concentration and then chlorination (conc/chlor), over gestation and lactation. Both concentration methods increasing DBPs by around 120x. Dams of both strains given chlor/conc water from early gestation to weaning experienced diarrhoea and polyuria. Sprague-Dawley dams that received concentrated drinking water also had lower body weights at GD20 - PND6 and F344 dams exhibited increased gestation lengths. There were fewer live Sprague-Dawley offspring, with greater perinatal loss/mortality, by PND6, and both strains had reduced bodyweights at PND6. The authors concluded, however, that some of the effects noted may be related to the concentration of inorganic materials such as sodium and sulphate in the concentrated water. However, when dams

were administered conc/chlor water, which was similar in DBP composition but with greatly reduced sodium and sulphate levels to the chlor/conc water, only increased water consumption by dams was noted. Finally, using the chlor/conc method, Narotsky et al. (2013) performed a multi-generational study using water concentrated around 130x, with exposure starting at GD2 for the F1 generation and lasting until termination of the F2 generation. In this study, concentrated water consumption was associated with reduced caput sperm counts in adult F1 males, delayed puberty in juvenile F1 females, thyroid follicular hypertrophy in parental females as well as adult F1 females, and increased birthweights in F2 offspring. While these studies tested concentrated drinking water containing broadly similar concentrations and ratios of DBPs, there are also considerable differences between batches of concentrated drinking water used between studies, which may alter the toxic potential (Li and Mitch, 2018).

2.5. Discussion

A key finding of this review was that in studies where low dose chemical mixtures were tested in parallel with individual component chemicals, most studies reported that the response to mixture exposures were more numerous and/or greater in severity than the responses to individual chemicals. While it is not uncommon to see occasional mild signs of toxicity when individual chemicals are tested at, or close to, NOAEL levels, eight studies summarised in this review reported significant effects from individual chemicals at or below TDI values, theoretically two or three orders of magnitude away from an effective dose. The observation of physiological effects at these very low 'safe' doses calls into question the validity of the POD values used in the calculation of TDI values. Alternatively, these results could indicate significant variation between experiments/laboratories, genomic drift between animal breeders that purport to supply the "same" strain of animals, strain differences in susceptibility, or differences in detection sensitivity. However, all the above still expose potential shortcomings in toxicity assessment. Observations where toxicological effects were more numerous and/or more severe following exposure to a mixture of chemicals rather than the chemical components individually, suggests at least additivity between components of a chemical mixture. However, most studies did not employ experimental designs which allowed for direct comparisons to,

and assessments of, mixture toxicity models, which can definitively distinguish additivity and synergy. Of the seven which provided data appropriate for such comparisons, three reported responses significantly greater than all investigated additivity model predictions, strongly indicating synergy. Although the remaining studies were not appropriate for similar direct critiques of mixture toxicity models, indications to the appropriateness of the mixture toxicity models can be inferred, for example, where there are effects following TDI exposure, which current applications of mixture toxicity models cannot explain. While this work has shown some commonality between various studies, there were also disparities between others. The focus of this review on the most relevant research for translational considerations (mammalian, *in vivo*) also posed the largest limitation, as data and designs between papers were too disparate from each other, and the quantity of literature too small, for true comparative re-evaluations. In a recent systematic review and quantitative reappraisal by Martin et al. (2021), which covered all living organisms, *in vitro* and *in vivo*, most mixtures were found to conform to dose additivity models. This agrees with many reviews which showed dose additivity-based models to be the most accurate across the whole dose-response curve, even when chemical components of a mixture are mechanistically distinct. A most notable example is the additive nature of antiandrogenic chemicals with phthalates, provided by Howdeshell et al. (2017), where additive models accurately predict exposure outcomes, although many of the studies involved did not meet inclusion criteria for this review. However, in twenty percent of literature identified by Martin et al. (2021) effects exceeded dose additivity models substantially, with synergistic interactions more than two-fold. These interactions were attributed to groupings already suspected of synergy (combinations of triazine, azole and pyrethroid pesticides), while also indicating new, potentially synergistic groupings (EDCs within metallic compounds).

Signs of toxicity may be expected from co exposure to mixtures containing chemicals at or close to NOAEL, regardless of additivity type. At this dose level an important consideration is the endpoint used to derive a NOAEL. Where NOAELs have been derived from endpoints dissimilar to those being investigated, study endpoints may have greater or lesser sensitivity to disruption. In these cases, compounds could be dosed either above or below NOAEL for study

endpoints. In the latter case, greater additivity would be needed to cross the effect threshold, prohibitive of detecting effects. Similarly, experiments often used mixtures of too few components. As deviations from additivity are commonly small, simpler mixtures may not have the power to elicit an observable effect. Nearly half of the identified component-based literature used ≤5 mixture components. Additionally, some studies have used pilot data to generate dose-response curves for the specific endpoints being investigated, whereas others have used values derived in some form from regulatory studies or determinations. This could lead to contradictory findings; for example, vinclozolin NOAEL determined at 4 and 5 mg/kg/d by Schneider et al. (2017) and Christiansen et al. (2009) respectively. It is impossible to know if this difference could go towards explaining the differences between the studies (greater than additivity at NOAEL in Christiansen et al. (2009) and no effect at NOAEL in Schneider et al. (2017)), although this was not considered a major issue as PODs were broadly similar for the same chemicals across most studies. Twenty-one of the thirty studies which examined mixtures at NOAEL values reported physiological effects that were attributed to chemical exposure. However, no toxicological or physiological effect should be expected from co-exposure to chemical mixtures where the components are present at or close to TDI. This review identified that in eighteen of the twenty-one studies that tested mixtures at or below TDI values toxicological or physiological effects were reported. The greatest number of chemicals tested at doses equivalent to TDI values was twenty-seven chemicals. In this example, even using dose addition for all components, this level of exposure would still be anticipated to be more than three-fold lower than a dose expected to be able to elicit an effect. This is an indication of interactions between chemical components within the chemical mixtures, unaccounted for by mixture toxicity models.

Of the literature identified which used a whole-mixture methodology, the BTP sheep model is the only which reflects actual human exposure as the drinking water by-products model is orders of magnitude away from realistic exposure. This lack of variation means that inter-species variance remains unaccounted for. While the BTP sheep model could be criticised for a lack of empirically determined PODs, limited quantification of individual chemicals, and no normalisation of dosages, it represents a real-world situation, with a chemical

mixture used according to regulatory guidelines. Such use is deemed appropriate to ensure contaminant levels are below conservative calculations for acceptable human exposure, and thus also for other species for which there is a lack of empirically determined PODs. The BTP sheep model is also representative of human exposure in that many chemicals to which humans are exposed have little or no toxicological data in any species. This is a recognised problem which cannot feasibly be solved by the traditional route of testing of each chemical individually but will most likely rely on new and future methodologies, including read-across, quantitative structure-activity relationship (QSAR) analysis, machine learning, and artificial intelligence (Aschner et al., 2022; NAS, 2017b).

The most examined endpoints in identified research articles were reproductive and/or developmental (nineteen), endocrine disruption (fifteen), hepatic (twelve), and behavioural (eight). There is concern that current guidelines do not sufficiently account for the multitude and ubiquity that characterises human exposure, especially fetal EDC exposure, which could be contributing to current global health problems, including the decline in male reproductive health (Skakkebæk, 2002; Skakkebæk et al., 2001; WHO, 2012). It has been suggested that current inclusion criteria for chemicals in mixture risk assessments, based on shared mechanisms of action, may be too restrictive in terms of mixture risk assessments for male reproductive health (Kortenkamp, 2020). Of the nineteen research articles that reported effects on reproduction and/or development after *in utero* exposure to chemical mixtures, ten used mixtures with individual chemicals at TDI or ≤0.25x NOAEL values. Common responses observed were gonadal dysgenesis, deleterious germ cell alterations, and altered AGD, and specifically in males increased areola number, NR, and genital malformations.

The relevance of systems biology approaches in a toxicological context without additional confirmation of biological effect has previously been questioned (Schneider et al., 2015). The use of omics data was seen in six component-based research articles, and four whole-mixture research articles. However, of the research articles which used omics technologies, four of the six component-based research articles, and three of the four whole-mixture research articles, also had strong supporting morphological data, therefore this was not considered as a factor for exclusion.

2.6. Conclusions

The basis of compounding toxicity from chemical mixtures at low doses, especially at or below TDI values, remains a subject of debate. While there have been great advancements in mixture toxicity assessment, with some acceptance within regulatory bodies, there remains a lack of harmonisation as well as a lack of dose coverage to those far below individually determined NOAEL values. The present work represents a collation and analysis of research articles reporting experiments testing chemical mixtures with individual components at doses believed unable to elicit effects alone. This review, however, did not address other (controversial) aspects of low-dose chemical mixture exposure, such as hormetic, non-monotonic, or biphasic responses.

While the extensive ECETOC literature review concluded no substantial evidence of mixture toxicity not already accounted for (ECETOC, 2012), this review includes many studies which tested mixtures at TDI levels and were published after the ECETOC review. In addition, it should be noted that the ECETOC review focused on the identification of studies that provided evidence of effects greater than additivity model predictions, rather than evidence of effects unaccounted for by additivity model predictions. With this view, most of the literature identified here also falls short, as experimental designs which would have made this possible were typically not employed. However, of those which could, around half found additivity model predictions were inaccurate, and responses significantly greater than additivity model predictions were reported. Additionally, at doses around TDI, this fact is somewhat immaterial, especially when applied towards real-world situations, where co-exposure occurs to thousands of chemicals. It is in this respect that current methodological approaches for cumulative risk assessment fall short, and as such a novel paradigm has been suggested focussing on complex mixtures of chemicals at individual doses around TDI (Tsatsakis et al., 2017, 2016) and to assess risk using a real-life risk simulation (RLRS) approach (Hernández et al., 2020). However, it is logistically impossible to truly simulate real-life exposure by component-based methodologies. For this the BTP sheep model is most realistic, however it was not accepted by ECETOC due to the lack of empirically determined chemical concentrations and dose calculations, despite being common practice on fields

growing crops for human consumption where chemical loads within crops remain below human TDIs. Additionally, due to the expansive chemical nature of biosolids and the limitations of current analytical techniques for such mass chemical quantification, such empirical determination is impractical. Finally, whole-mixture studies cannot give answers to the question of synergy, which can only be addressed through carefully designed component-based studies, but rather give snapshots of an extremely complex and dynamic exposure. Thus, there is little extra to be gained from precise quantification of individual chemicals and exact calculations of dosages resulting from BTP exposure without a greater understanding of biological and chemical interactions between mixture components.

With these considerations in mind, it can be concluded that although no unequivocal evidence to refute conclusions from previous reviews, there is strong evidence to support additional research in this area. However, there were two data gaps identified which would facilitate a greater understanding of low dose chemical mixture toxicity, especially for complex chemical mixtures. Firstly, within the literature there was a lack of data on complex mixtures (with >10 components) at TDI values with data on individual components generated in parallel, likely because of greatly increased study requirements to generate such data. This data gap has been previously identified as impeding improvements to mixture risk assessments (Evans et al., 2015). Secondly, there is a lack of mixture dose-response data at very low-doses, with very few research articles reporting more than two concentrations less than or equal to NOAEL. This data gap deprives analyses of response curves and PODs for mixtures with which to compare to individual components, and thus leaves no basis to assert as evidence for or against current additivity models. Without these gaps being addressed, results cannot be interpreted regarding the nature of any combination effects, nor on the type of interactions or non-interactions occurring. Thus, the appropriateness of current mixture toxicity assessments cannot be critically examined further.

Chapter 3.
Morphological and transcriptomic alterations in neonatal lamb testes following developmental exposure to low-level environmental chemical mixture

3.1. Abstract

Exposure to anthropogenic environmental chemical mixtures could be contributing to the decline in male reproductive health. This study used the biosolid treated pasture (BTP) sheep model to assess the effects of exposure to low-dose chemical mixtures. Maternal BTP exposure was associated with lower plasma testosterone concentrations, a greater proportion of Sertoli cell-only seminiferous tubules, and fewer gonocytes in the testes of neonatal offspring. Transcriptome analysis highlighted changes in testicular mTOR signalling, including lower expression of two mTOR complex components. Transcriptomic hierarchical analysis relative to the phenotypic severity demonstrated distinct differential responses to maternal BTP exposure during pregnancy. Transcriptome analysis between phenotypically normal and abnormal BTP lambs demonstrated separate responses within the cAMP and PI3K signalling pathways towards CREB. Together, the results provide a potential mechanistic explanation for adverse effects. Exposure could lower gonocyte numbers through mTOR mediated autophagy, but CREB mediated survival factors may act to increase germ cell survival.

3.2. Introduction

Over the past 80 years there has been a consistent decline in human semen quality, which has been linked to reductions in male reproductive health and fecundity (Carlsen et al., 1992; Jensen et al., 2008; Merzenich et al., 2010; Paasch et al., 2008). This decline in male reproductive health encompasses increased incidence of disorders such as testicular germ cell cancer, cryptorchidism, hypospadias, hypogonadism and infertility – collectively termed testicular dysgenesis syndrome (TDS; Skakkebæk et al., 2001). Although the exact mechanism(s) underlying TDS remains elusive, it has been linked with dysfunctional Sertoli and Leydig cells within the foetal testes, and abnormal hypothalamic and/or pituitary function (Kumar et al., 2010; Sharpe, 2010). While it has been proposed that the mechanisms underlying TDS may be driven by sedentary lifestyles, stress and improper nutrition, TDS has also been linked with exposure to a wide variety of different anthropogenic chemicals, many of which are ubiquitous in the environment (Checa Vizcaíno et al., 2016; Crean and Senior, 2019; Ilacqua et al., 2018; Sharpe, 2001; Skakkebæk et al., 2001; A.

Wang et al., 2016). Of routinely detected environmental chemicals (ECs), particular attention has fallen on endocrine disrupting chemicals (EDCs) as they have been shown to adversely affect testicular development. For example, *in utero* exposure to phthalates can impair germ cell development, reduce sperm motility, and inhibit testicular testosterone production, in both rats and humans (Boberg et al., 2011; Borch et al., 2006; Hu et al., 2009; Lambrot et al., 2009). In addition to concerns related to high exposure to specific EDCs, there is increasing evidence to suggest that exposure to mixtures of ECs, at doses at or below their respective tolerable daily intake values (TDIs), may result in additive or synergistic physiological effects (Buñay et al., 2018; Docea et al., 2019; Merhi et al., 2010; Tsatsakis et al., 2019b). This possibility is of note, as the human and animal exposome is characterised by chronic, complex, low level EC exposure.

Supplementing acute single EDC exposure studies, a number of studies have used component-based methodologies and exposed animals to mixtures of chemicals with a defined composition and dosage (Kortenkamp, 2014). Despite the increase in mixture complexity, such studies remain insufficient for the investigation of the effects of realistic human exposure, as it is not feasible to produce a chemical mixture that is truly reflective of the human exposome. A more representative model with which to study the health/physiological effects of the human exposome is provide by biosolid treated pasture (BTP) sheep model. Biosolids are a by-product of human domestic/industrial wastewater treatment and are extensively used as a fertiliser on agricultural land, as encouraged by EU and US legislation (EU, 1986; U.S. EPA, 1999), and regulated by the Sludge (Use in Agriculture) Regulations 1989 (DEFRA, 1989) in the UK. As biosolids are derived from human waste their chemical composition reflects the human exposome and it has been shown that biosolids contain extremely low concentrations of many ECs (Rhind et al., 2010, 2002, 2013; Venkatesan and Halden, 2014b). Many of the ECs within biosolids are also measurable in tissue samples collected from sheep which graze within BTP (Bellingham et al., 2012; Filis et al., 2019; Rhind et al., 2010, 2009, 2005) and their offspring (Rhind et al., 2010, 2009, 2005). The array of ECs present and measurable in the BTP sheep model include alkylated phenols, bisphenol A (BPA) and other plasticising agents, dioxin-like compounds, pharmaceuticals and personal care products

(PPCP), phthalates, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and their metabolites (Rhind et al., 2002, 2013; Venkatesan and Halden, 2014b; Zhang et al., 2015).

Previous experiments using the BTP sheep model have demonstrated that gestational exposure to such 'real-world' levels/mixtures of ECs can cause alterations in offspring behaviour, bone composition, cellular and hormonal processes, and liver function, as well as effects on male and female gonadal development (Bellingham et al., 2016, 2013, 2012, 2009; Erhard and Rhind, 2004; Filis et al., 2019; Fowler et al., 2008; Hombach-Klonisch et al., 2013; Lea et al., 2016; Lind et al., 2009; Paul et al., 2005). Specifically in male offspring, maternal grazing on BTPs resulted in lower testis weights and fewer Sertoli cells, Leydig cells and gonocytes in late gestation foetuses (day 110 of 144) when compared to controls (Paul et al., 2005). In addition, maternally EC exposed rams maintained into adulthood showed a subset of animals with reduced germ cell numbers (spermatocytes and round spermatids) and increased incidence of Sertoli cell-only (SCO) seminiferous tubules (Bellingham et al., 2012), phenotypic characteristics which are indicative of TDS (Nistal et al., 2017). In an extension to previous findings, the present study examined the testicular transcriptome of neonatal (1-day old) ram lambs to investigate the mechanistic changes that may underlie adverse morphological and functional outcomes that can occur following gestational mixed EC exposure using the BTP model.

3.3. Methods

3.3.1. Ethics statement

All adult ewes were maintained under normal husbandry conditions at the University of Glasgow Cochno Farm and Research Centre. The research programme was approved by the University of Glasgow School of Veterinary Medicine Research Ethics Committee. All procedures were conducted in accordance with the Home Office Animal (Scientific Procedures) Act (A(SP)A), 1986 regulations.

3.3.2. Experimental animals

Adult Aberdale ewes were maintained, without dietary supplementation, on pastures fertilised with either biosolids, at conventional rates (4 tonnes/ha, twice per annum (April/September); biosolid), or with inorganic fertiliser at a rate which supplied equivalent levels of nitrogen (225 kg N/ha per annum; control) for at least one month prior to being naturally mated with naïve rams, and for the duration of pregnancy. Within 1 day of birth (<24 h) male lambs from ewes exposed to conventionally fertilised pastures (n = 7, Control), and ewes from BTP (n =17, biosolid) were weighed before euthanasia by intravenous barbiturate overdose (140 mg/kg Dolethal, Vetroquinol, UK).

3.3.3. Tissue collection

Plasma was harvested from blood samples collected post-mortem into lithium-heparin tubes (BD Diagnostics - 367885) by centrifugation at 1000 x g for 15 min. Gonads, thyroids, adrenals, and pituitaries were removed and weighed. The left testis was fixed overnight in 10 % neutral buffered formalin (NBF, Thermo Scientific – 16499713), then transferred to 70 % ethanol (VWR – 20821.330) prior to processing and embedding in paraffin wax for histology (Excelsior AS, Thermo Scientific). The right testis was halved and both pieces placed overnight in RNAlater™ Stabilisation Solution (Thermo Scientific – AM7020) before storage at -80 °C until RNA extraction.

3.3.4. Histological staining and analysis

Testicular tissue was sectioned (5 µm) using a Microtome (Leica Biosystems, model RM2125RT). One testis section from each animal was stained (haematoxylin and eosin (H&E)) and four representative images of the lobuli testis captured (Leica DM4000B microscope with a Leica DC480 digital camera using Leica Qwin software) at 40x magnification from separate areas (top, bottom, left, and right). The number of Sertoli cells and gonocytes for each seminiferous tubule captured within each image (mean = 17.7 tubules per image) was determined by manual cell counting in ImageJ (version 1.53a). To be cautious with regards to misidentifying cells as somatic, gonocytes were determined by satisfying any two of three morphological criteria: size (large), shape (circular), or location (within lumen). Mean gonocyte / Sertoli cell per

tubule ratios were calculated relative to the mean control ratio, as well as the proportion of seminiferous tubules without gonocytes (Sertoli cell-only).

3.3.5. RNA extraction and cDNA synthesis

RNA was extracted and purified from approximately 25-30 mg of homogenised tissue with an RNeasy Kit (Qiagen – 74104) supplementing the RLT buffer with 1% (v/v) ß-mercaptoethanol (Sigma-Aldrich – M3148). The purity and concentration of RNA was assessed spectrophotometrically using a NanoDrop (Thermo Scientific, model ND-1000). For each sample, 100 ng of extracted RNA was converted to cDNA using RNaseOUT (Invitrogen – 10777019), Maxima H Minus Reverse Transcriptase (Thermo Scientific – 15259496), primers from a Direct cDNA Sequencing Kit (Oxford Nanopore – SQK-DCS109), RNase Cocktail (Invitrogen – AM2286), LongAmp Taq Master Mix (New England Biolabs – M0287), NEBNext Ultra II End Repair/dA-Tailing Module (New England Biolabs – E7546), and AMPure XP magnetic beads (Beckman Coulter – A63881). cDNA was then assessed spectrophotometrically, as above, for use in Nanopore sequencing.

3.3.6. cDNA library preparation, sequencing, and data analysis

cDNA from each sample was ligated to individual DNA barcodes for multiplexing using a barcode expansion kit (Oxford Nanopore – EXPNBD104) and Blunt/TA Ligase (New England Biolabs – M0367S). As the barcoding kit only contains twelve individual barcodes, samples were split into two groupings of twelve (mixed control and biosolids) for sequencing. 22.4 ng barcoded cDNA from each sample within a grouping was pooled, ligated to direct cDNA sequencing adaptors (Oxford Nanopore – SQK-DCS109), and sequenced using a MinION Nanopore sequencer (Oxford Nanopore) and an R9.4.1 flow cell (Oxford Nanopore – FLO-MIN106D). One half of the sequencing, comprising of 12 animals, was performed by Dr Ana Monteiro. Raw fast5 read data were basecalled, demultiplexed, barcodes trimmed, filtered for quality and minimum length, and concatenated into individual fastq files. Data from both groupings were then combined for alignment to the reference transcriptome (constructed from NCBI's Oar_v4.0 reference genome and annotation files), sorted, and counted. Counts were imported into R, filtered, and normalised for differential expression analysis. Names and version numbers of software used for analysis can be found in Table 3-1. Thresholds for calling differentially expressed genes (DEGs) were p-value <

Table 3-1. sequencing data analysis software details.

Purpose	Software Name	Version Number
Reference Transcriptome Extraction	GFFread	0.12.1
Basecalling	Guppy	4.0.15
Demultiplexing	Deepbinner	0.2.0
Trimming	Deepbinner	0.2.0
Read Filtering	Filtlong	0.2.0
Aligning	Minimap2	2.17
Sorting	Samtools	1.10
Counting	Salmon	0.14.2
Count Filtering	DRIMseq	1.10.1
Normalisation	edgeR	3.24.3
Differential Expression Analysis	edgeR	3.24.3

0.05, log2 fold change <-1 or >1, and false discovery rate (FDR) < 0.1. Hierarchical analysis was performed using iDEP (version 9.2; Ge et al. 2018) on unfiltered count data, using the 1000 most variable genes, regularised log transformation, and default clustering parameters with genes and samples centred and normalised. KEGG pathway analysis was performed using DEG lists in DAVID (version 6.8; Huang et al. 2009). To assess the effect of changes in testicular cellular composition, genes within pathways identified as enriched with DEGs were tested for correlation using generalised linear models on gene counts against the geometric mean of gene counts for tissue specific biomarkers of gonocytes: *CD9* (Kanatsu-Shinohara et al., 2004), *CD14* (Park et al., 2019), *THY1* (Zheng et al., 2014), *NOTCH1* , *GFRA1* (Von Schönfeldt et al., 2004), *CDH1* (Tokuda et al., 2007), and *UCHL1* (Luo et al., 2006).

3.3.7. Determination of phenotypic subtype

Separation of biosolid animals into two phenotypic subgroups was performed in a similar manner to Bellingham et al. (2012), but modified to incorporate two morphological parameters by using the Parzen–Rosenblatt window method for kernel density estimates of testicular morphology. Biosolid animals where morphological values lay within control range or 1 standard deviation of control density estimations, with no visible testicular morphological response, were assigned to the “resistant” biosolid subgroup. Biosolid animals where

morphological values lay outside control value ranges and density estimations were assigned to the "susceptible" biosolid subgroup.

3.3.8. Testosterone determination

Plasma testosterone levels were determined by colorimetric Testosterone ELISA (R&D Systems – KGE010) according to manufacturer's instructions. Briefly, diluted plasma samples (1 in 10) or testosterone standards were equilibrated, in duplicate, with horseradish peroxidase (HRP)-Testosterone conjugate in wells of a 96-well plate primed with anti-testosterone antibodies. Following incubation and washes, a substrate for HRP was added, the plate incubated, and the reaction stopped by the addition of a denaturing stop solution. The plate was then quantified spectrophotometrically on a plate reader (LabTech – LT-4500) at 450 nm corrected to 570 nm. Testosterone concentrations were interpolated from standard curve optical density values using DRC. One data point was excluded as it lay outside the normal physiological range for testosterone (>110 ng/mL).

3.3.9. Statistical analysis

All calculations and statistical analyses were performed in R (version 4.0.2) using base functionality through R Studio (version 1.3.1073). Anatomical, histopathological, and testosterone data were fitted to generalised linear models and groups compared using one-way ANOVA. Boxplots were created using the R package ggplot2 (version 3.3.2). Scatterplots were created using R base functionality plus kernel density estimations from the R package MASS (version 7.3.51.6). Data are presented as mean ± SD.

3.4. Results

3.4.1. Anatomical and histopathological analyses

Mean body weights were not significantly different between control (3.29 ± 0.77 kg) and biosolid males (3.61 ± 0.61 kg). Relative organ to body weight ratios of the gonads, thyroids, and adrenals were not significantly different between control and biosolid groups (Table 3-2). The mean pituitary to body weight ratio, however, was significantly ($p = 0.0025$) lower in biosolid lambs (30.51 µg/kg ± 4.49) compared to controls (37.12 µg/kg ± 3.39) (Table 3-2).

Table 3-2. Mean ± SD for animal body weights and relative organ to body weights in control and biosolid lambs. All p-values derived from generalised linear models of biosolid animals compared with controls.

Morphological indices	Control (n=7)	Biosolid (n=17)	*p*-value
Body Weight (BWT) (kg)	3.29 ± 0.77	3.61 ± 0.61	0.29
Gonads / BWT (mg/kg)	0.20 ± 0.03	0.21 ± 0.03	0.41
Thyroids / BWT (mg/kg)	0.11 ± 0.02	0.11 ± 0.03	0.96
Adrenals / BWT (mg/kg)	0.11 ± 0.03	0.12 ± 0.03	0.54
Pituitary / BWT (µg/kg)	37.12 ± 3.39	30.51 ± 4.49	0.0025

Representative histopathological images from control and biosolid testes are shown in Figure 3-1(A and B). The mean relative gonocyte / Sertoli cell ratio (Figure 3-1C) was significantly ($p = 0.0015$) lower in biosolid lambs (0.61 ± 0.21) compared to controls (1 ± 0.20). The mean percentage of SCO seminiferous tubules (Figure 3-1D) was significantly ($p = 0.0040$) higher in biosolid lambs (32.34 ± 14.19 %) compared to control (11.74 ± 7.83 %).

3.4.2. Testosterone analysis

Mean plasma testosterone concentrations were significantly ($p = 0.049$) lower in biosolid (9.9 ± 9.6 ng/mL) compared to control (20.3 ± 13.9 ng/mL) lambs (Figure 3-1E).

3.4.3. Gene expression and pathway analyses

Differential gene expression analysis of sequenced testes cDNA revealed 296 DEGs (21 with higher expression, and 275 with lower expression, in biosolid compared to control lambs). KEGG pathway analysis revealed 6 pathways with substantial ($p < 0.1$) enrichment between biosolid and control testes (Table 3-3). The most statistically significant enrichment was seen in the mTOR signalling pathway in which a 9-fold enrichment was observed ($p = 0.00048$, FDR = 0.082, Table 3-3). Within the mTOR pathway, the six identified DEGs (*PRKAA1, STRADA, PDPK1, RPS6KB2, MLST8, RICTOR*) showed lower levels of expression in biosolid animals compared to the controls (Table 3-4). The expression of genes within identified pathways did not correlate with the expression of known cell specific gonocyte marker genes (Figure 3-2).

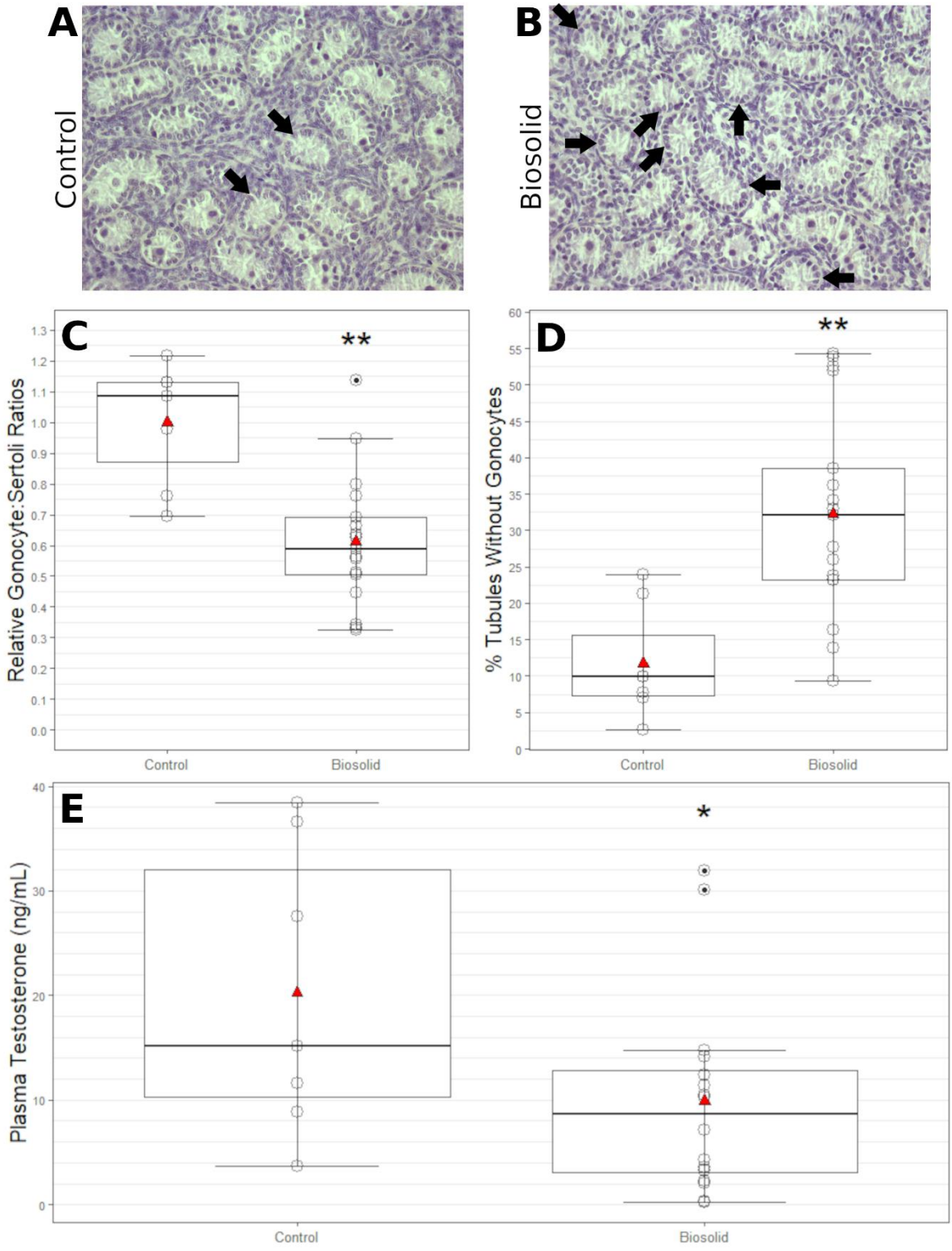


Figure 3-1 Histopathological findings in neonatal control and biosolid lamb testes. Representative images of H&E stained tissue sections from 1-day old control (A) and biosolid (B) lambs taken at 40x magnification. Arrows indicate Sertoli cell-only (SCO) seminiferous tubules. Relative ratios of gonocytes to Sertoli cells (C) and proportions of SCO tubules (D) (p=0.0015 and p=0.0040 respectively). Plasma testosterone concentrations for control and biosolid lambs (E). Boxes represent 25th to 75th percentile, horizontal bar indicates 50th percentile, whiskers indicate range excluding outliers, solid filled circles show outliers, open circles show individual data points, and red triangles show means.

Table 3-3. Results of KEGG pathway analysis of DEGs identified in biosolid lambs compared to controls. All data obtained from DAVID version 6.8 (Huang et al. 2009).

Term	Genes	Enrichment	*p*-Value
mTOR signalling pathway	PRKAA1, STRADA, PDPK1, RPS6KB2, MLST8, RICTOR	8.98	0.00049
Choline metabolism in cancer	CHKB, PDPK1, DGKA, RPS6KB2, PLD2	4.34	0.027
AMPK signalling pathway	PRKAA1, CREB3L3, STRADA, PDPK1, RPS6KB2	3.53	0.051
Insulin secretion	SNAP25, PCLO, CREB3L3, ATP1A3	4.18	0.068
Insulin signalling pathway	PRKAA1, PDPK1, RPS6KB2, CBLB, PHKA2	3.15	0.072
Morphine addiction	GABRA2, PDE1C, GRK6, PDE8A	4.04	0.074

Table 3-4. Differential expression data from for DEGs identified in biosolid animals compared to controls which code for proteins in the mTOR signalling pathway. All p-values were produced using generalised linear models with a quasi-likelihood framework.

Gene	Log_2 Fold Change	P-Value	FDR
RICTOR	-3.07	0.00010	0.026
STRADA	-2.96	0.00011	0.027
MLST8	-2.84	0.00023	0.040
PRKAA1	-2.79	0.00044	0.059
PDPK1	-2.61	0.00081	0.074
RPS6KB2	-2.46	0.00101	0.085

3.4.4. Differential response

Hierarchical analysis of cDNA sequencing data by iDEP indicated significant variation between the testes of biosolid lambs. Exposed lambs were then divided into phenotypes based on observed morphological responses to exposure, which largely corresponded with iDEP hierarchical clustering (Figure 3-3A). Sludge lambs were therefore subset into two subgroups: no visible testicular morphological response ("resistant"), and visible adverse effect on testicular morphology ("susceptible") (Figure 3-3B). Differential gene expression analysis of susceptible biosolid animals vs resistant biosolid animals identified 159 DEGs (123 with higher expression, and 36 with lower expression, in susceptible compared to resistant lambs).

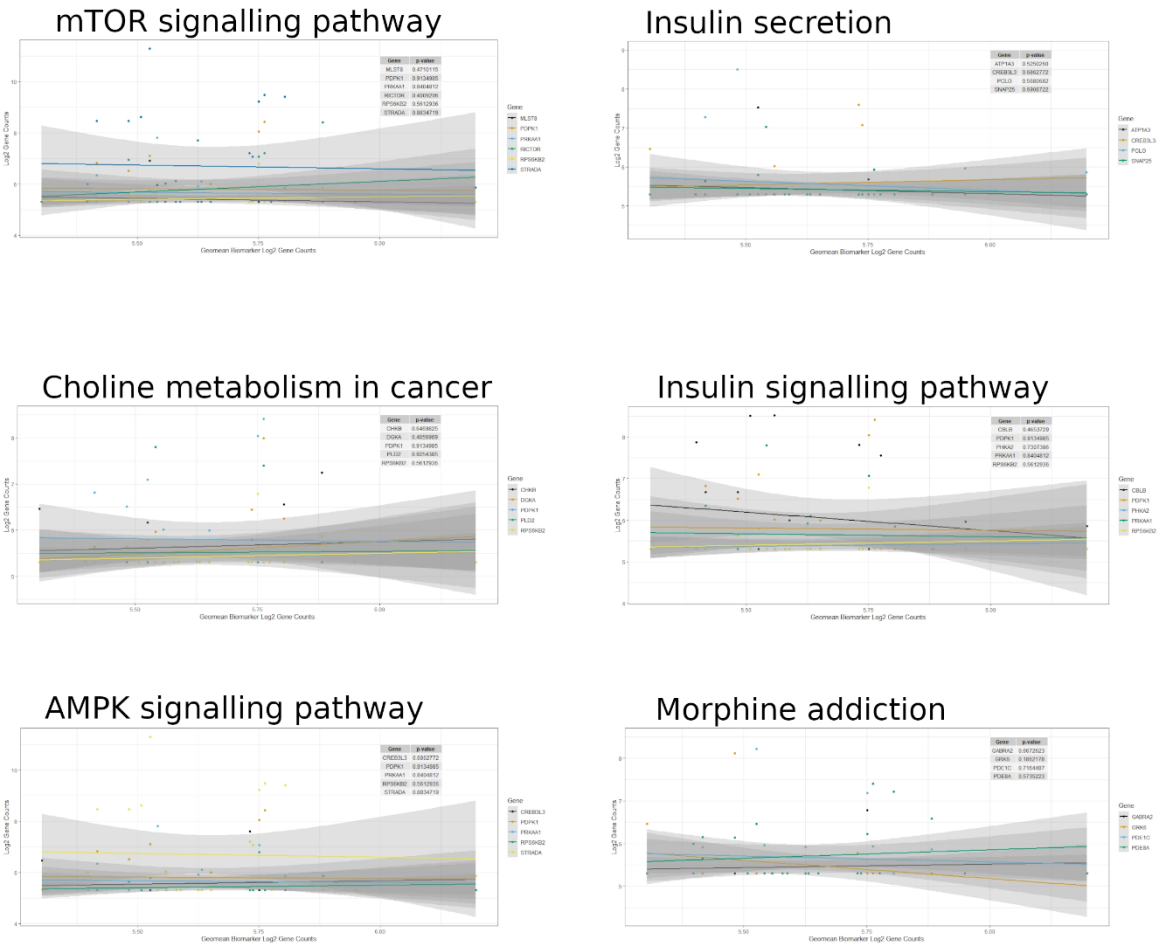


Figure 3-2. Correlations between genes within pathways identified by KEGG analysis between Control and Sludge lambs and the geometric mean of known gonocyte biomarkers.

KEGG pathway analysis revealed 7 pathways with substantial ($p < 0.1$) enrichment. The most statistically significant enrichment was seen in the cAMP signalling pathway in which a 4.7-fold enrichment was observed (p-value = 0.0075, Table 3-5). When analysed against the control data, the expression of 5 of the identified genes within the cAMP signalling pathway were differentially expressed in opposing directions in susceptible and resistant biosolid lambs

Term	Genes	Fold Enrichment	*p*-value
cAMP signalling pathway	RYR2, FXYD1, CREB3L3, ABCC4, NPY1R, PIK3CG	4.73	0.0076
Insulin signalling pathway	MKNK1, SORBS1, PPARGC1A, PIK3CG	4.87	0.046
ABC transporters	ABCA5, ABCC4, ABCA3	8.13	0.050
Arrhythmogenic right ventricular cardiomyopathy	RYR2, ITGA11, CTNNA2	7.64	0.056
Hypertrophic cardiomyopathy	RYR2, ITGA11, TTN	6.30	0.079
Dilated cardiomyopathy	RYR2, ITGA11, TTN	6.15	0.082
Insulin secretion	RIMS2, RYR2, CREB3L3	6.07	0.084

Table 3-5. Results of KEGG pathway analysis of DEGs identified in analysis comparing biosolid resistant lambs compared to biosolid susceptible lambs. All data obtained from DAVID version 6.8 (Huang et al. 2009).

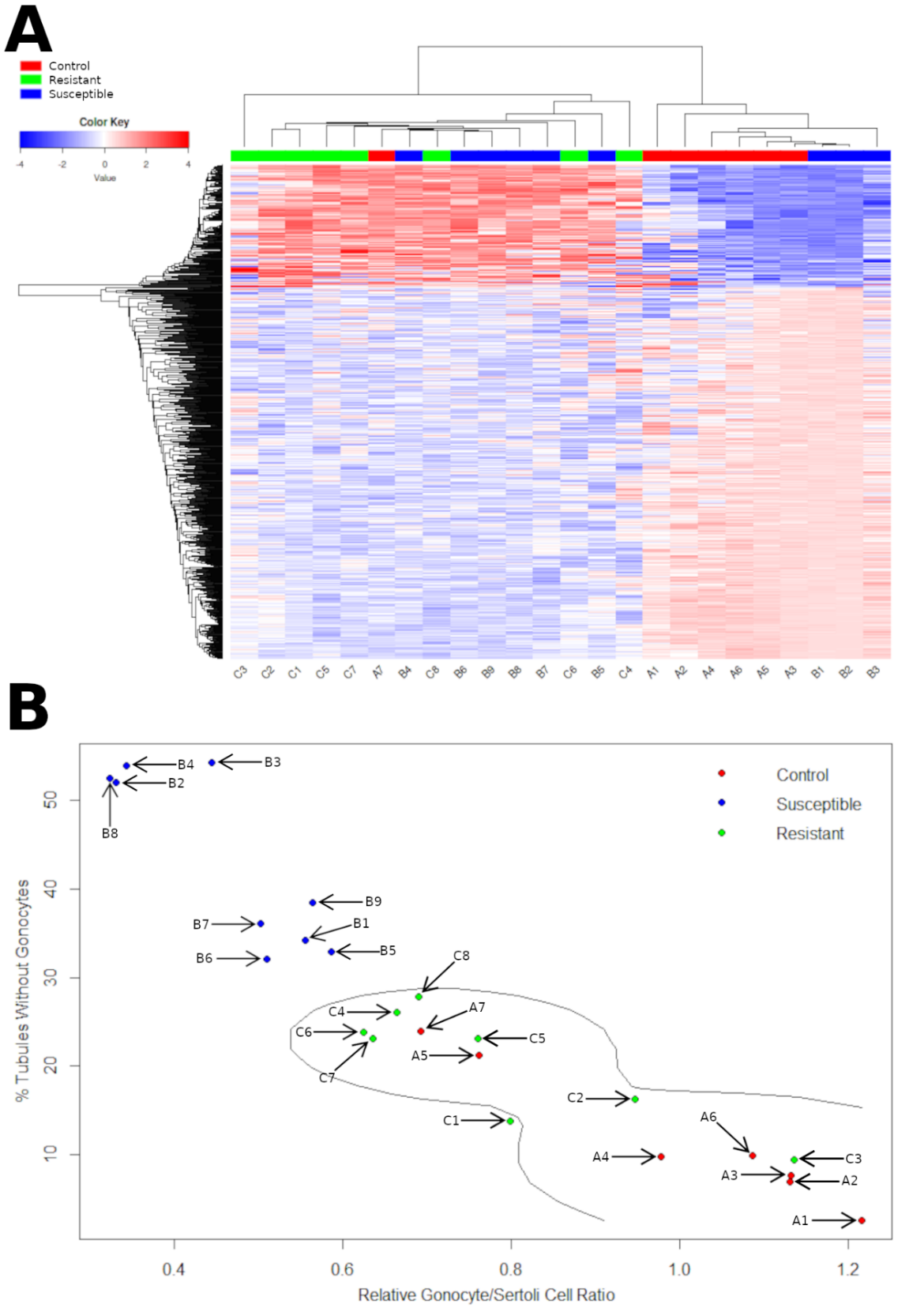


Figure 3-3 Identification and determination of phenotypic subtypes. Hierarchical analysis of cDNA expression counts by iDEP (A) indicates varying response to exposure. Horizontal lines indicate individual genes, colours indicate difference in expression from the control average - red and blue indicate higher or lower gene expression, respectively. Hierarchical clustering trees were produced using genes with maximum expression levels in the top 75%, height of branching is proportional to average linkage correlations. Separation of biosolid lambs based on morphological data into two sub-groups (B): biosolid lambs which saw more of an effect ("susceptible") and those which saw less of an effect ("resistant"), based on a threshold of 1x SD of control kernel density estimates and control value range.

relative to the controls (e.g., higher expression in susceptible, and lower expression in resistant lambs, relative to the controls) or, as in the case of *CREB3L3*, were uniquely expressed in one of the biosolid subgroups alone (Table 3-6). The expression of genes within identified pathways did not correlate with the expression of known cell specific gonocyte marker genes (Figure 3-4).

Table 3-6. Differential expression data for genes within the cAMP signalling pathway, from analysis of biosolid resistant and susceptible lambs compared to controls. Genes shown are those with differential expression in opposing directions with regards to control, and those with unique expression in only one group. All p-values were produced using generalised linear models with a quasi-likelihood framework.

Genes	Log_2 Fold Change Susceptible	Log_2 Fold Change Resistant	FDR	*p*-Value
CREB3L3	0.00	4.27	0.0014	1.04E-06
NPY1R	2.80	-1.01	0.020	0.00016
FXYD1	-2.41	1.62	0.033	0.00037
IKBKG	2.63	-1.08	0.038	0.00054
PIK3CG	1.37	-2.79	0.043	0.00067

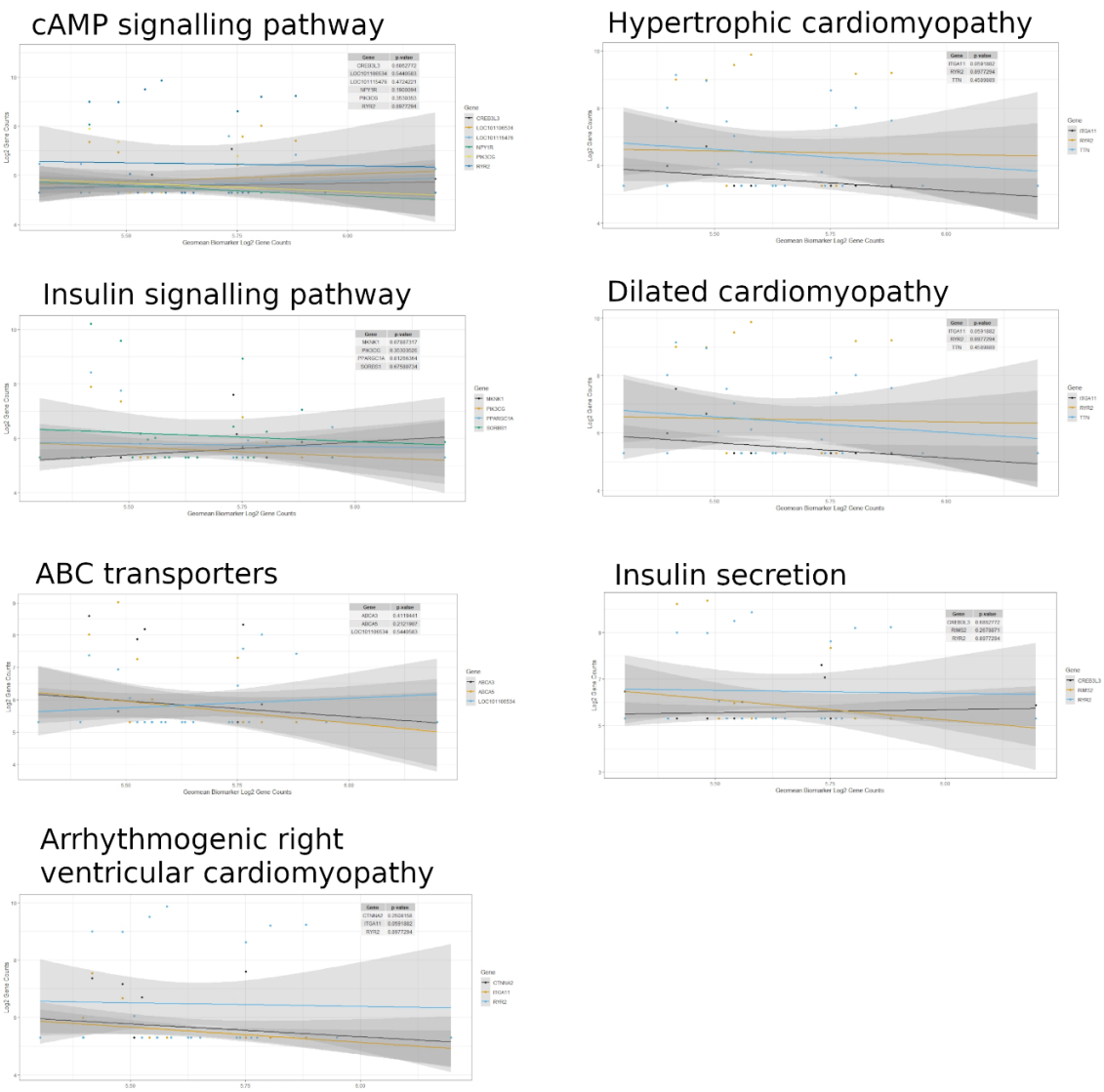


Figure 3-4. Correlations between genes within pathways identified by KEGG analysis between biosolid resistant and biosolid susceptible lambs and the geometric mean of known gonocyte biomarkers.

3.5. Discussion

The results of this study demonstrate that gestational exposure to a complex mixture of ECs results in morphological differences in testicular structure which are similar to those seen in TDS. Gestational exposure to this real-life EC mixture also resulted in differential gene expression within the mTOR signalling pathway, providing a possible mechanism for the observed changes in testicular morphology. Combined DEG, pathway, and morphological analyses demonstrated that variation within the testicular phenotype, in response to EC exposure, is associated with complex changes in differential gene expression. Of specific note were changes in cAMP/CREB (cAMP-response element binding protein) signalling which may underlie compensatory changes that restore/maintain a normal testicular phenotype in some gestationally EC exposed animals.

The lambs in this study were exposed to a real-life mixture of ECs of human relevance, as the exposure model was maternal grazing on pasture treated with biosolids. The results build on previous work with this model which has shown changes in testicular morphology in late gestation foetuses (Paul et al., 2005) and in a subset of adult offspring from ewes exposed to BTP during gestation (Bellingham et al., 2012). The ability of low-level EC mixtures to affect male reproductive health, as seen in this model, is supported by a small number of additional component-based EC mixture studies which have reported deleterious effects of EC mixtures on male sexual development and/or spermatogenesis. Specifically, gestational exposure to a mixture of 5 fungicides affected sexual development in male Han Wistar rat pups (Hass et al., 2012; Jacobsen et al., 2012, 2010), despite all component chemicals being below (as low as 8%) their respective no observable adverse effect levels (NOAEL), and exposure of C57BL/6J mice to an EDC mixture containing 3 phthalate plasticisers, an alkylphenol, and an alkylbenzene at TDI levels, from gestation to adulthood, which resulted in reduced testicular weight and abnormal seminiferous tubule structure and spermatogenesis (Buñay et al., 2018). While the observation of effects in these models could be explained by dose addition, due to similar toxicological modes of action, due to the conservative nature of TDI calculations with an uncertainty factor for inter-species variance (usually ≥100 fold), the EC mixtures in these studies are up to twenty times below the concentrations that

would be predicted to be able to elicit an effect. Together, these results show the ability for even simple chemical mixtures to produce adverse effects on sexual development and/or testicular function.

In this study the changes observed in EC exposed lamb's testes included fewer gonocytes (the precursor germ cells responsible for producing spermatogonial stem cells) and a higher proportion of SCO seminiferous tubules. This phenotypic profile mimics mixed testicular atrophy as seen in TDS (Nistal et al., 2017; Skakkebæk et al., 2001). A further characteristic of TDS is lower plasma testosterone concentrations (Joensen et al., 2008), although this was not observed in the previous studies with this model that investigated testicular changes in foetal or adult sheep, in the current study mean plasma testosterone concentrations were significantly lower in the biosolid lambs. This apparent difference between the current and previous studies using this model could be the result of several factors. First it could be a function of the natural fluctuations in testosterone production that occur throughout development. Alternatively, as the adult sheep had a 12-month period without BTP exposure prior to euthanasia, this may have allowed a degree of recovery from any early effects of EC exposure, in the earlier study. Finally it could be related to differences in biosolid batch variability i.e. the exact chemical composition within the biosolids to which the lamb's mothers were exposed (Clarke and Smith, 2011).

While previous studies using the BTP model have described phenotypic changes induced by EC mixture exposure in foetal and adult offspring, a unique element of the current study was to document changes in the transcriptome of the neonatal testes following EC exposure. The analysis identified almost 300 DEGs in the biosolid compared to control lambs, and KEGG pathway analysis suggested 6 pathways in which EC exposure was associated with substantial differential changes in gene expression. As there were significant changes in the cellular composition of the lamb testes, these transcriptomic differences may reflect the altered cellularity between exposed and control animal testes. However, there were no correlations between the expression of known gonocyte biomarkers and genes identified within enriched pathways, which suggests that changes in cellularity were not a contributing factor. The pathways identified as enriched

were mTOR signalling, choline metabolism in cancer, AMPK signalling, insulin secretion, insulin signalling, and morphine addiction. It is of interest that four of the 6 pathways identified, although not all reaching the statistical threshold of $p < 0.05$, are related to energy metabolism and/or storage. Namely mTOR signalling, a major nutrient-sensitive regulator of cellular growth and metabolism (Sabatini, 2017), AMPK signalling, a key regulator of cellular and organismal metabolism (Mihaylova and Shaw, 2011), insulin secretion, and insulin signalling. It is noteworthy that mTOR signalling is in a complex and reciprocal balance of co-regulation with both AMPK (Inoki et al., 2012) and insulin signalling (Yoon, 2017), and three identified DEGs (PRKAA1, PDPK1, RPS6KB2) are common between all three pathways. AMPK signalling is also important with regard to Sertoli function as it has been reported to be important for the maintenance of Sertoli cell tight junctions and the support of germ cell proliferation and survival (Bertoldo et al., 2015; Nguyen, 2019; Ni et al., 2019), while in Leydig cells AMPK signalling has been associated with testosterone production (Svechnikov et al., 2009). Changes in insulin signalling may also have functional consequences as insulin signalling in Sertoli cells is crucial for lactate production, which is metabolised by developing germ cells (Alves et al., 2013; Pitetti et al., 2013). Significant differential expression was also seen in the choline metabolism in cancer pathway. Abnormal choline metabolism is a potential hallmark of oncogenesis and tumour progression (Glunde et al., 2006) and altered gene expression in this pathway as a result of gestational EC exposure could indicate abnormal cellular processes which may have an impact on testicular germ cell cancer, an additional hallmark of TDS (Skakkebæk et al., 2001).

The pathway which showed the greatest, and most significant enrichment between the biosolid and control testes was the mTOR signalling pathway. Recent work has indicated that mTOR is an important component of testicular development and spermatogenesis (Correia et al., 2020; Jesus et al., 2017; Moreira et al., 2019). Transgenic rodent studies have shown that disruption of mTOR signalling pathways or mTOR complexes (mTORC) in germ cells or Sertoli cells impairs testicular development and spermatogenesis (Boyer et al., 2016; Serra et al., 2017; Xie et al., 2016). The results of the current study add to a growing body of evidence that the mTOR complex or signalling pathway in the

testes can be perturbed by ECs. For instance, the endocrine disruptors 4-nonylphenol (4NP), diethylhexyl phthalate (DEHP), and bisphenol A (BPA) have been shown to inhibit the AMPK-mTOR (4NP) or PI3K-Akt-mTOR (DEHP and BPA) signalling pathways and induce testicular autophagy in pubescent rodents (Duan et al., 2017; Fu et al., 2020; Quan et al., 2017). Similarly, maternal exposure to fine particulate matter during gestation has also been shown to cause testicular autophagy that is associated with decreased gene expression of PI3K-Akt-mTOR pathway components, including mTOR itself (Ren et al., 2020).

Considering the current study contains one time-point, to be definitive with conclusions as to a causative mechanism towards morphological changes can be challenging. Mechanistically, lower gene expression levels within the mTOR signalling pathway of biosolid exposed lambs (especially mTORC components mLST8 and Rictor) could impact gonocyte survival, as in the previous examples. Alternatively, altered mTOR pathway expression may be in addition to altered testis cellularity and not the cause, which may have taken place during gestation and subsided prior to parturition. However, in the present study, testosterone levels were lower in the biosolid lambs, which is associated with altered mTOR signalling through rapamycin use (an mTORC inhibitor) (Kaczmarek et al., 2004). This suggests that reduced mTORC capacity was having a current physiological effect but falls short of conclusive delineation of cause and effect. While there is a solid theoretical connection between morphological, hormonal, and transcriptomic changes, it is also possible these are all latent effects to one or more previous causative agents.

As in the study by Bellingham et al. (2012), the phenotype of the EC exposed males in the current study was not homogeneous; in both studies some male offspring had testes unaffected by exposure and others had a large reduction in germ cells/germ cell precursors. This phenotypic variation could be the result of differences in EC exposure but is more likely to be a facet of the genetic background upon which the EC exposure occurs. The results of the differential gene expression analysis favour the later explanation as there were significant numbers of DEGs in all EC exposed animals relative to the controls. Differential outcome to chemical exposure within outbred populations is not uncommon, and the identification of different phenotypes often precedes the identification of

biological differences. The variable phenotype seen in the present study allowed additional analysis of the data from animals that appear to be susceptible to testicular disruption by EC mixtures and those that appear EC resistant. Given that DEGs were seen in all biosolid lambs, the susceptible and resistant biosolid animals may mount separate mechanisms of physiological compensation to maintain fertility. Examination of genes differentially expressed between animals resistant and susceptible to EC exposure demonstrated potential enrichment in 7 pathways. It is of interest that insulin secretion and insulin signalling pathways were again identified, but apart from CREB3L3 which was identified in both comparisons, with different DEGs to those from the previous comparison (control vs biosolid). Also identified in the comparison of resistant to susceptible biosolid lambs was ABC transporters, which are important in maintaining the blood-testis barrier and are crucial components in gametogenesis, steroidogenesis, and reproductive function (Bloise et al., 2016). The most significantly enriched pathway between the resistant and susceptible biosolid testes was the cAMP signalling pathway. The cAMP signalling pathway is involved in normal testicular development and spermatogenesis through the activation of CREB following FSH stimulation of Sertoli cells (Don and Stelzer, 2002). When patterns of gene expression were compared between both biosolid phenotype subgroups and the controls, 5 cAMP pathway related DEGs were found to be differentially expressed. 4 of these DEGs have commonality in their involvement in CREB activation and/or transcription (CREB3L3, IKBKG, NPY1R, PIK3CG). CREB genes encode proteins within the basic leucine zipper (bZIP) transcription factors family, which drive transcription of target genes by binding to specific cAMP-response elements within promotor regions, and are known to be crucial to testicular development and spermatogenesis (Don and Stelzer, 2002). CREB regulation occurs in Sertoli cells primarily through two mechanisms. Firstly, through the positive regulation of CREB expression *via* the NF-κB pathway, following the phosphorylation, ubiquitination, and degradation of the NF-κB inhibitor I-κB. Phosphorylation of I-κB is performed by the IKK complex, of which the catalytic component IKK-γ is encoded by the gene IKBKG, that can be activated by PI3K. This pathway is multifaceted, as PI3K can also initiate CREB activation through Akt. Secondly, CREB regulation can occur through cAMP mediated PKA activation of CREB, which causes positive autoregulation of CREB

expression (Don and Stelzer, 2002). Interestingly, mRNA for the CREB gene induced in biosolid resistant lambs (CREB3L3) was completely absent in biosolid susceptible and control lambs. The CREB3 family are membrane bound forms of CREB bZIP transcription factors, localised to the endoplasmic reticulum until regulated intramembrane proteolysis release (Zhang et al., 2006). Once within the cytoplasm CREB3 activation and translocation to the nucleus can occur, where CREB3 regulates functions including development, metabolism, secretion, survival, differentiation, tumorigenesis, and cell division, among others (Sampieri et al., 2019). While there is no literature on the role of CREB3L3 specifically within the testis, there is evidence of protein secretion regulation by CREB3L3 in other cell types (Chan et al., 2011). Another identified DEG which was differentially expressed between susceptible and resistant biosolid lambs, relative to the controls, was NPY1R. Neuropeptide Y (NPY) is a secretagogue which influences male reproductive function both through the HPG-axis (Pedrazzini et al., 2003) and direct actions on testicular tissues (Allen et al., 2011). Its actions in the testes are most likely mediated through the Y1 isoform of NPY receptor (encoded by the NPY1R gene) (Allen et al., 2011; Kanzaki et al., 1996; Kopp et al., 2008), which is a $G\alpha_i$ linked GPCR that, following activation by NPY, peptide YY (PYY) or pancreatic polypeptide (PP), results in the inhibition of adenylyl cyclase (AC) and reduced cAMP production (Pedrazzini et al., 2003). However, literature on testicular NYP and NYPR is infrequent, and the exact site of NPY action within the testes remains controversial (Kanzaki et al., 1996; Kopp et al., 2008; Terado et al., 2006).

The main signalling pathways for testis development and spermatogenesis in a Sertoli cell are depicted in Figure 3-5. When the results of the differential gene expression analysis within the phenotypic subgroups of biosolid lambs are considered, both phenotypic subgroups have differential gene expression that may increase CREB activation/expression, but by separate pathways. With regards to components towards CREB *via* NF-κB and Akt, susceptible lambs had higher PI3K and IKBKG expression, whereas resistant lambs had lower PI3K and IKBKG expression. With regards to components towards CREB *via* cAMP, resistant lambs had higher CREB and lower NPYR gene expression, whereas susceptible lambs had no change in CREB and higher NPYR expression. This would be expected to drive CREB activation, increase levels of CREB through

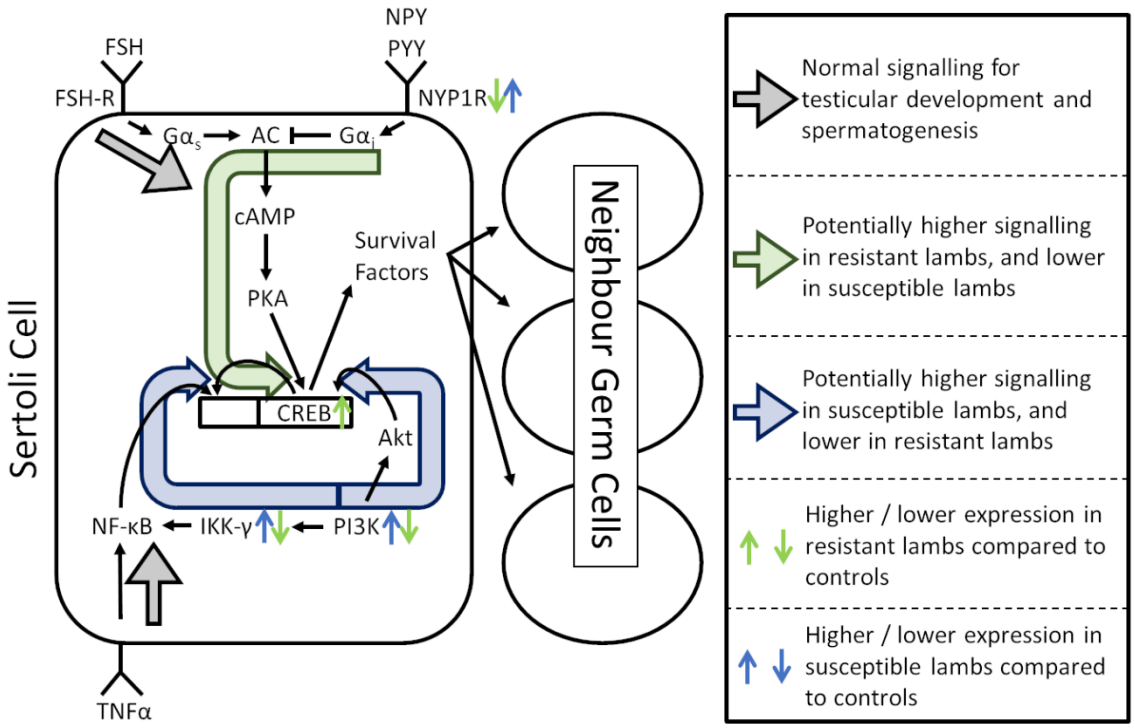


Figure 3-5. Schematic representation of changes to testicular gene expression. Differential expression in biosolid susceptible (blue) and resistant (green) lambs (small arrows) compared to controls, and potential changes to cell signalling (large arrows) from normal developmental and spermatogenic signalling (grey arrows).

autoregulation, and promote the secretion of survival factors in both phenotypic subgroups of biosolid lambs.

In combination our findings indicate that observations of fewer gonocytes in lambs exposed *in utero* to biosolids could potentially be a consequence of reduced mTORC signalling, which has been shown to cause autophagy in response to xenobiotic induced mTORC attenuation. That the expression of mTORC components were lower in all biosolid lambs, yet a proportion did not have a visible morphological response, indicates the presence of differing response types. This is supported by hierarchical transcriptome analysis, is not unexpected given the outbred and heterogeneous nature of sheep, and agrees with previously observed diametric phenotypes in adult rams following similar exposure (Bellingham et al., 2012). The milder adverse response to exposure seen in the resistant biosolid lambs could be attributable to the amplified signal transduction of a secondary messenger and the presence of an additional, pro-secretory form of CREB. Alternatively, the more severe adverse response to exposure seen in the susceptible biosolid lambs could be attributable to reduced CREB activation by cAMP, through increased inhibition of adenylyl cyclase (AC), despite increased activation by NF-κB and Akt *via* PI3K. It is likely that genetic

variance gives lambs a susceptible or resistant phenotype, which if discovered would allow effect predictions and a translatable factor for risk assessment, yet in the absence of deep genomic sequencing this will remain a postulation.

Foetal development is an extremely complex and dynamic period, which increases vulnerability to xenobiotic induced toxicity. This is the first study to show alterations in the expression of mTOR signalling pathway components following gestational exposure to realistic mixtures of chemicals, which may play an important role in the pathogenesis of TDS. These findings add to the body of evidence to suggest that exposure to real-world levels of environmental chemical mixtures during pregnancy may be having an adverse effect on male offspring reproductive health, contributing to the decline in sperm quality and fecundity in humans.

Chapter 4.
Developmental exposure to real-life environmental chemical mixture programs a Testicular Dysgenesis Syndrome-like phenotype in prepubertal lambs

4.1. Abstract

Current declines in male reproductive health may, in part, be driven by anthropogenic environmental chemical (EC) exposure. Using a biosolids treated pasture (BTP) sheep model, this study examined the effects of gestational exposure to a translationally relevant EC mixture. Testes of 8-week-old ram lambs from mothers exposed to BTP during pregnancy contained fewer germ cells and had a greater proportion of Sertoli-cell-only seminiferous tubules. This concurs with previous published data from fetuses and neonatal lambs from mothers exposed to BTP. Comparison between the testicular transcriptome of biosolids lambs and human testicular dysgenesis syndrome (TDS) patients indicated common changes in genes involved in apoptotic and mTOR signalling. Gene expression data and immunohistochemistry indicated increased HIF1α activation and nuclear localisation in Leydig cells of BTP exposed animals. As HIF1α is reported to disrupt testosterone synthesis, these results provide a potential mechanism for the pathogenesis of this testicular phenotype, and TDS in humans.

4.2. Introduction

Male reproductive health has been in decline for the past 80 years (Carlsen et al., 1992). Reports indicate reduced fecundity, semen quality and serum testosterone concentrations, and increased incidence of reproductive disorders (cryptorchidism, hypospadias, hypogonadism, infertility, and testicular germ cell cancer), collectively known as testicular dysgenesis syndrome (TDS) (Sharpe and Skakkebaek, 2008; Skakkebæk et al., 2001). While many contributory factors to the decline in male fertility have been identified, including malnutrition, sedentary lifestyle, and stress, attention has focused on the role of environmental chemicals (ECs) (Crean and Senior, 2019; Ilacqua et al., 2018; Skakkebæk, 2002). Of the many ECs to which humans are routinely exposed, most attention is targeted at endocrine disrupting chemicals (EDCs), to which human epidemiological investigations provide links between fetal anti-androgenic EDC exposure and reduced male reproductive health (Rodprasert et al., 2021a). A variety of ECs are known to adversely affect testicular development; for example, gestational exposure to phthalates is associated with negative effects on germ cell development, sperm motility, and testosterone

production in rodents and humans (Borch et al., 2006; Hu et al., 2009). In addition to studies which have examined the effects of individual ECs, several rodent studies have used component-based methodologies to examine the effects of low dose mixtures of ECs. When presented as mixtures, adverse effects of EC exposure have been reported even when individual chemicals were present at doses at or below their respective tolerable daily intake (TDI) values (Buñay et al., 2018; Kortenkamp, 2014). This type of EC exposure scenario is of note as it more realistically reflects human EC exposure, which is characterised as chronic, extremely complex, and very low-level. However, it is not possible to accurately simulate true human EC exposure using component-based methodologies.

Solids from wastewater treatment, biosolids, are extensively used as an agricultural fertiliser and reflect human EC exposure in terms of complexity and concentration (Rhind et al., 2010, 2002, 2013; Venkatesan and Halden, 2014b, 2014a). When sheep are grazed on biosolids treated pasture (BTP), ECs can be measured in maternal tissues (Bellingham et al., 2012; Filis et al., 2019; Rhind et al., 2010, 2009, 2005), as well as tissues collected from their offspring (Rhind et al., 2010, 2009, 2005). Measured ECs include alkylated phenols, dioxin-like compounds, flame retardants such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), pharmaceuticals and personal care products (PPCPs), plasticising agents such as phthalates and bisphenol A (BPA), polycyclic aromatic hydrocarbons (PAHs), and metabolites thereof (Rhind et al., 2002, 2013; Venkatesan and Halden, 2014b). Therefore, the BTP exposed sheep model is an appropriate model for investigation of the effects of generic human exposure to several different types of EC in parallel. Experiments using the BTP exposed sheep model have previously shown that *in utero* EC exposure can cause a multitude of effects in offspring. These include altered behaviour, differences in bone composition, disruption to cellular and hormonal processes, and changes in liver function, as well as effects on gonadal development in males and females (Bellingham et al., 2016, 2013, 2012, 2009; Elcombe et al., 2021; Erhard and Rhind, 2004; Filis et al., 2019; Fowler et al., 2008; Hombach-Klonisch et al., 2013; Lea et al., 2016, 2022; Lind et al., 2009; Paul et al., 2005). With specific regards to male gonadal development, gestational exposure to BTPs is associated with reduced testicular weight and fewer gonocytes, Leydig cells, and Sertoli

cells in fetuses at gestation day (GD) 110 (Paul et al., 2005). Exposure to BTPs through maternal grazing from GD60 - GD140 is associated with reduced fetal mass, testes weight and adrenal weight, shorter anogenital distances, and reduced testosterone in GD140 fetuses (Lea et al., 2022). Of note is that these largely anti-androgenic effects parallel similar observations in the human (Rodprasert et al., 2021a). In neonatal (1-day-old) lambs, BTP exposure throughout gestation is associated with fewer gonocytes as well as an increased incidence of Sertoli-cell-only (SCO) seminiferous tubules (Elcombe et al., 2021). This study also indicated that there may be two phenotypic responses to EC exposure within male lambs, one in which lambs are more susceptible to disruption and another which appears resistant. This observation mirrors findings in the testes of 19-month-old offspring exposed to BTP *in utero* and for seven months post-natal, where a subset of animals was identified that had reduced germ cell numbers and an increased incidence of SCO seminiferous tubules (Bellingham et al., 2012).

There is currently no information on the effects gestational BTP exposure has on testicular development between parturition and adulthood. Puberty in male sheep begins at approximately 8 weeks of age, therefore the aim of the current study was to examine the morphology and transcriptome of gestationally BTP exposed prepubertal (8-week-old) ram lamb testes to gain insights into the mechanisms underlying observed adverse morphological observations and potential functional outcomes.

4.3. Methods

4.3.1. Ethics statement

All animals were maintained under normal husbandry conditions at the University of Glasgow Cochno Farm and Research Centre. The research programme was approved by the University of Glasgow School of Veterinary Medicine Research Ethics Committee. All procedures were conducted in accordance with the Home Office Animal (Scientific Procedures) Act (A(SP)A), 1986 regulations under licence (PPL PF10145DF).

4.3.2. Experimental animals

EasyCare ewes were maintained on pastures fertilised with either biosolids, at conventional rates (4 tonnes/ha, twice per annum (April/September); biosolids exposed (B)), or with inorganic fertiliser at a rate which supplied equivalent levels of nitrogen (225 kg N/ha per annum; control (C)) for one month prior to mating and for the duration of pregnancy. Mating was by artificial insemination with semen from 4 rams which had only been maintained on control pasture. Ewes were maintained indoors for the final two weeks of pregnancy and fed forage supplemented with concentrates as per normal husbandry practice. Biosolids-exposed ewes received forage harvested from biosolids-treated pastures. After parturition, all ewes and lambs were maintained on control pastures. Therefore, all EC exposure was maternal (i.e., through placental or lactational transfer). At 8-weeks of age, a subset of male offspring from ewes exposed to conventionally fertilised pastures (n=11 ram lambs from separate mothers and balanced across sires (C)) and biosolids treated pastures (BTP) (n=11 rams from separate mothers and balanced across sires (B)), were weighed before euthanasia by intravenous barbiturate overdose (140 mg/kg Dolethal, Vetroquinol, UK) for tissue collection.

4.3.3. Tissue collection

Testes were removed at necropsy. Two transverse slices were taken from the centre of the left testis, fixed overnight in 10% neutral buffered formalin (Thermo Scientific – 16499713), then transferred to 70% ethanol (VWR – 20821.330) prior to processing, and embedding in paraffin wax for histology (Excelsior AS, Thermo Scientific). A 5mm thick transverse slice was taken from the centre of the right testis and frozen on dry ice prior to storage at -80°C until RNA extraction.

4.3.4. Immuno-histochemistry

Formalin-fixed paraffin embedded testicular tissues were sectioned (5µm) using a microtome (Leica Biosystems, model RM2125RT). Immuno-histochemistry for DDX4 was used to identify germ cells. Fluorescent immuno-histochemistry was used to localise HIF1α.

For DDX4 immuno-histochemistry, one section per animal was mounted on a Polysine® coated glass slide, dewaxed, and processed for antigen retrieval (autoclave for 21 minutes while immersed in citrate buffer 10mM, pH 6). Slides were washed in TBS and taken through peroxidase, avidin, and biotin blocking solutions (15 minutes each with TBS washes in between). Non-specific binding was blocked by incubation for 30mins with 20% goat serum in TBS before incubation with the primary antibody (rabbit anti-DDX4 polyclonal antibodies; Abcam – ab13840) diluted 1:1000 in antibody diluent (Agilent DAKO – S2022) overnight at 4°C. Sections were then washed in TBS + 1% Tween20 before being incubated for 30 minutes with a biotinylated secondary antibody (goat anti-rabbit biotinylated polyclonal antibodies; Agilent DAKO – E0432) diluted 1:200 in antibody diluent. Following incubation in secondary antibody, sections were treated with Vectastain ABC-HRP system (Vector Laboratories – PK4000) for 60 minutes before being washed in TBS + 1% Tween20 and stained using DAB for 30 seconds. Slides were then washed in TBS, and counter-stained with haematoxylin and coverslips mounted using DPX. DDX4 staining was performed by Dr Ana Monteiro.

For HIF1α fluorescent immuno-histochemistry, one section per animal was mounted on a Polysine® coated glass slide, dewaxed, and processed for antigen retrieval (microwaved at medium heat in 1 mM EDTA, pH 8.0, for 10 minutes). Slides were washed in TBS and incubated overnight at 4°C with mouse anti-HIF1α antibodies (Invitrogen – MA1-16504) diluted 1:250 in 5% BSA / TBS-T. Slides were washed with TBS and incubated for 1 hour at room temperature with goat anti-mouse antibodies (Abcam – ab150113) diluted 1:1000 in 5% BSA / TBS T. Sections were counter stained with DAPI (Abcam – ab104139) and cover-slipped.

4.3.5. Image capture and analysis

For DDX4 immuno-histochemistry, four images of the lobuli testis were captured (Leica DM4000B microscope with a Leica DC480 digital camera at 100x magnification using Leica Qwin software) from separate areas (top, bottom, left, and right) of each tissue section for each animal, as previously performed (Elcombe et al., 2021). Using ImageJ (version 1.53a), all individual tubules which were entirely captured within images were manually selected (n = 2145 Control, 3673 Biosolid). DDX4 positive and negative cells were counted by automated

macro (pre-validated on a subset of data – Figure 4-1). Mean germ cell to total cell populations, per tubule, were calculated relative to the mean control, as well as the proportion of seminiferous tubules without germ cells (Sertoli-cell-only; SCO). Separately, tubule selections were filtered for circularity using the equation $4\pi(Area/Perimeter)$^2 with a threshold of ≥0.9 (n = 587 Control, 1326 Biosolid) and minimum Feret's diameters measured. Additional images were taken at 400x magnification for increased visual detail but were not part of the analysis.

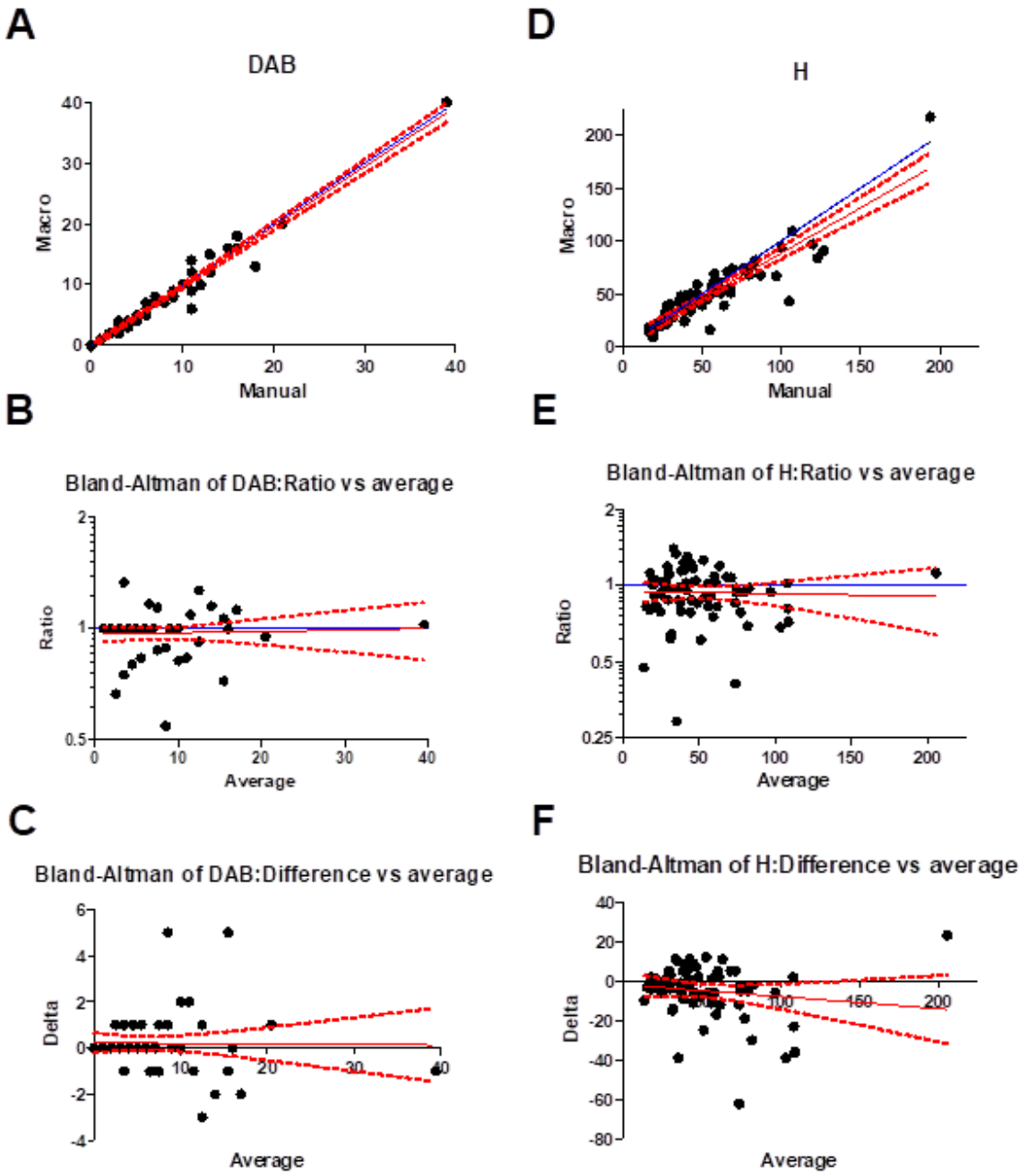


Figure 4-1. Comparative plots, and Ratio and Delta Bland-Altman plots for DAB (A, B, and C) and haematoxylin (D, E, and F) stained cells. Dots represent counts for individual seminiferous tubules. Solid red lines represent regressed line of best fit with 95% confidence intervals represented by dotted red lines. Blue lines represent theoretical complete agreement.

For HIF1α fluorescent immuno-histochemistry, four representative images of the lobuli testis were captured (Leica DM4000B microscope with a Leica DC480 digital camera at 400x magnification using Leica Qwin software) from separate areas (top, bottom, left, and right). Using ImageJ, all areas out-with seminiferous tubules were manually selected and co-localisation analysis performed using the JACoP plugin (Bolte and Cordelières, 2006), which provided Maders' overlap coefficients for the proportion of HIF1α staining that overlapped DAPI staining.

4.3.6. RNA extraction, cDNA library preparation, sequencing, and data analysis

Transcriptome analysis was performed as previously described (Elcombe et al., 2021). Briefly, RNA was extracted, purified, reverse transcribed, and ligated to individual DNA barcodes for multiplexing. As the barcoding kit only contains twelve individual barcodes, samples were split into two groupings of eleven (mixed control and biosolids) for sequencing. Barcoded cDNA samples within a grouping were pooled and sequenced using a MinION Nanopore sequencer (Oxford Nanopore). Data were processed and filtered for quality prior to alignment to the reference transcriptome (constructed from NCBI's Oar_v4.0 reference genome and annotation files) and counted. Aligned data was archived to EMBL's European Bioinformatics Institute, ArrayExpress accession number E-MTAB-11645. Batch effects were assessed by BatchQC (Manimaran et al., 2016). Differential gene expression (DGE) analysis was performed on gene counts by EdgeR. Differentially expressed genes (DEGs) were called using a p-value threshold of 0.05, $\log_2$ fold change threshold of <-1 or >1, and false discovery rate (FDR) threshold of 0.1. Gene ontology (GO) analysis was performed using DEG lists in DAVID (version 6.8).

4.3.7. Quantitative qPCR

RNA was extracted from approximately 30mg of frozen testes using RNeasy Mini Kit (Qiagen – 74104). Genomic DNA was degraded and cDNA synthesised using QuantiTect Reverse Transcription Kit (Qiagen – 205311). qPCR was performed using qPCR Brilliant II SYBR Master Mix (Agilent – 600828) on a Stratagene 3000 qPCR system. Primer details can be found in Table 4-1. Raw fluorescent data were regressed by PCR Miner (Zhao and Fernald, 2005) to produce primer

efficiencies and Ct values which were used in ΔΔCt analysis to produce Log2 Fold Change values.

Table 4-1. Primer details for qPCR.

Gene	Genbank accession number	Forward Primer	Reverse Primer
ACACA	443186	GAAGTCCCTCAGACTCTTAACC	AACCAGACATGCTGGATCTCAT
ACAD11	101118915	ACATCCCTGCCATGAACCAG	GCTACATCGGGCTCTGTCAT
ACLY	654404	GCCCCAAGATTCAGTCCCAA	CGATGGTCCGGATCTGAGTG
ACOX1	101118626	TGGCTGCATTCATCCAAAGGA	ATCAGGCTTCACCTGGGCAT
BIRC5	101123390	ACCAGATGACGACCCTATAGAAGA	CAATGGCACAGCGGACTTTC
CCND1	100144763	GATGCCAACCTCCTCAACGA	TGAACTTCACGTCTGTGGCA
CPT1A	443434	GCGCCGGGTGCATTC	CTCCCACCAGTCACTCACATAA
FABP4	100137067	CATGAAAGAAGTGGGTGTGGG	GGTAGCAGTGACACCGTTCAT
FASN	100170327	GCCTCACTGCCTTCCAGATT	GACTCGGGGCTGATGTCAAT
HK1	100036759	ACCAAGTCAAAAAGATTGACAAGT	TTGGCATCATAGTCCCCACG
LDHA	443089	GGCCCATTAGGGCATCTCT	TGCTGTTACATTATAGTCTTTGCCA
LPL	443408	GACTCCAACGTCATCGTGGT	TGTGGAAACTTCAGGCAGGG
PDPK1	101117211	GACGACGAGGACTGCTATGG	CAGGCAGAGAACCTCAAGGG
VEGFA	443103	CTTGCCTTGCTGCTCTACCT	GCCTCGGCTTGTCACATTTTTC

4.3.8. Western blots

Approximately 15 mg of frozen tissue samples were homogenised using a 1 mL tapered PTFE tissue homogeniser into 19x volume of RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0)) containing protease inhibitors (Merck – 11697498001) and phosphatase inhibitors (Merck – 4906845001). Samples were centrifuged at 12,000 x g for 20 minutes at 4 °C. Following protein determination (Thermo Scientific – 23227), samples were made to a final concentration of 2 µg/µL using LDS Sample Buffer (Invitrogen – NP0007) and Reducing Agent (Invitrogen – NP0004) and heated to 95 °C for 10 minutes. 10 µL of reduced, denatured samples (20 µg protein) were loaded into wells of 4 to 12%, Bis-Tris, 1.0 mm acrylamide gels (Invitrogen – WG1403BOX) using 5 µL of protein reference standard (BioRad – 1610375EDU) in the first and last wells. Gels were run under constant voltage using MOPS SDS Running Buffer (Invitrogen – NP0001) with added antioxidant (Invitrogen – NP0005) and transferred to nitrocellulose membranes (Invitrogen – IB23001 NC) using an iBlot2 (Invitrogen – IB21001). Membranes were blocked using Intercept TBS Blocking Buffer (Licor – 927-60001), washed with TBS-T and TBS, and incubated overnight with primary antibody diluted 1:1000 in 5% BSA/TBS at 4 °C. The next morning membranes were washed with TBS-T and TBS before incubation in secondary

antibody diluted 1:10,000 in 5% BSA/TBS at room temperature for one hour. Membranes were then washed in TBS-T, TBS, and finally MilliQ water before imaging on an Odyssey DLx Imager (Licor – 9142). Primary antibodies used were mouse-anti-HIF1α monoclonal (Invitrogen – MA1-16504) and rabbit-anti-α-tubulin polyclonal (Invitrogen – PA1-38814), with donkey anti-mouse (Invitrogen – SA5-10172) and donkey anti-rabbit (Invitrogen – SA5-10044) fluorophore conjugated secondary antibodies. Fluorescent intensities were quantified using Licor Image Studio Software (version 5.2.5). HIF1α signal intensities were normalised to α Tubulin and expressed as relative to the average of control values.

4.3.9. BaseSpace Analysis

Illumina's BaseSpace correlation Engine enables the comparison and correlation of DEG datasets through a combination of ranked-based enrichment statistics, meta-analyses, and biomedical ontologies (Kupershmidt et al., 2010). BaseSpace correlation Engine employs a rank-based, nonparametric analysis strategy driven by a Running Fisher's test algorithm which performs the rank-based directional enrichment process. This enrichment process utilises a Fisher's exact test to calculate four p-values: two p-values for the genes which are positively correlated between the datasets (genes that are either up or down-regulated in both datasets) and two for the negatively correlated genes (genes that are up-regulated in dataset 1 and down-regulated in dataset 2, or vice versa). The overall correlation p-value was calculated by converting the four p-values to -log10 p-values and subtracting the sum of the negative correlation p-values from the sum of the positive correlation p-values. A p-value threshold for significance of 0.0001 was used. To enable cross-platform and cross-species comparisons BaseSpace correlation engine software uses a database compiled of commonly used gene identifiers and reference identifiers along with ortholog information to standardise mapping across platforms and species.

Expression data for DEGs with $p \leq 0.05$ were analysed for correlation to published data by Illumina's BaseSpace software. Positively correlating DEGs were filtered for those common across each dataset per study. These gene lists were then combined and submitted to DAVID for pathway and GO analyses.

4.3.10. Effect of changes to cellularity

To assess the effect of changes in testicular cellular composition, DEGs with pathways or GO terms identified as enriched were tested for correlation using generalised linear models, on gene counts against the geometric mean of gene counts for germ cell specific biomarkers, and p values corrected for false discovery. Germ cell specific biomarkers used were *CD9, CD14, THY1, NOTCH1, GFRA1, CDH1,* and *UCHL1*.

4.3.11. Statistical analysis

All calculations and statistical analyses were performed in R (version 4.1.1) using base functionality. Unless otherwise stated, data were fitted to generalised linear models with gamma distribution and groups compared by Wald tests using the glm() and summary() base R functions. Models accounted for genetic structure of the data by incorporating sire heritage into calculations. Plots were created using the R package ggplot2 (version 3.3.5). Data are presented as mean ± SD.

4.4. Results

4.4.1. Fewer germ cells and more frequent Sertoli-cell only seminiferous tubules in testes of prepubertal rams prepubertal gestationally exposed to BTP

Histopathology was performed to assess effects of gestational BTP exposure on gross testes morphology and cellularity. Following immunohistochemistry for DDX4, representative images from control (C) and biosolids (B) testes are shown in Figure 4-2 at 100x magnification (A and C) and 400x magnification (B and D). The mean relative germ cell : Sertoli cell ratio (Figure 4-2E) was lower ($p = 0.014$) in B (0.67 ± 0.22) compared to C lambs (1.00 ± 0.29). The mean percentage of SCO seminiferous tubules (Figure 4-2F) was higher ($p = 0.0082$) in B (12.56 ± 11.49 %) compared to C lambs (2.27 ± 1.81 %). The mean minimum Feret's diameter of seminiferous tubules (Figure 4-2G) was smaller ($p = 0.013$) in B (77.51 ± 16.51 µm) than in C (94.89 ± 13.36 µm) lambs.

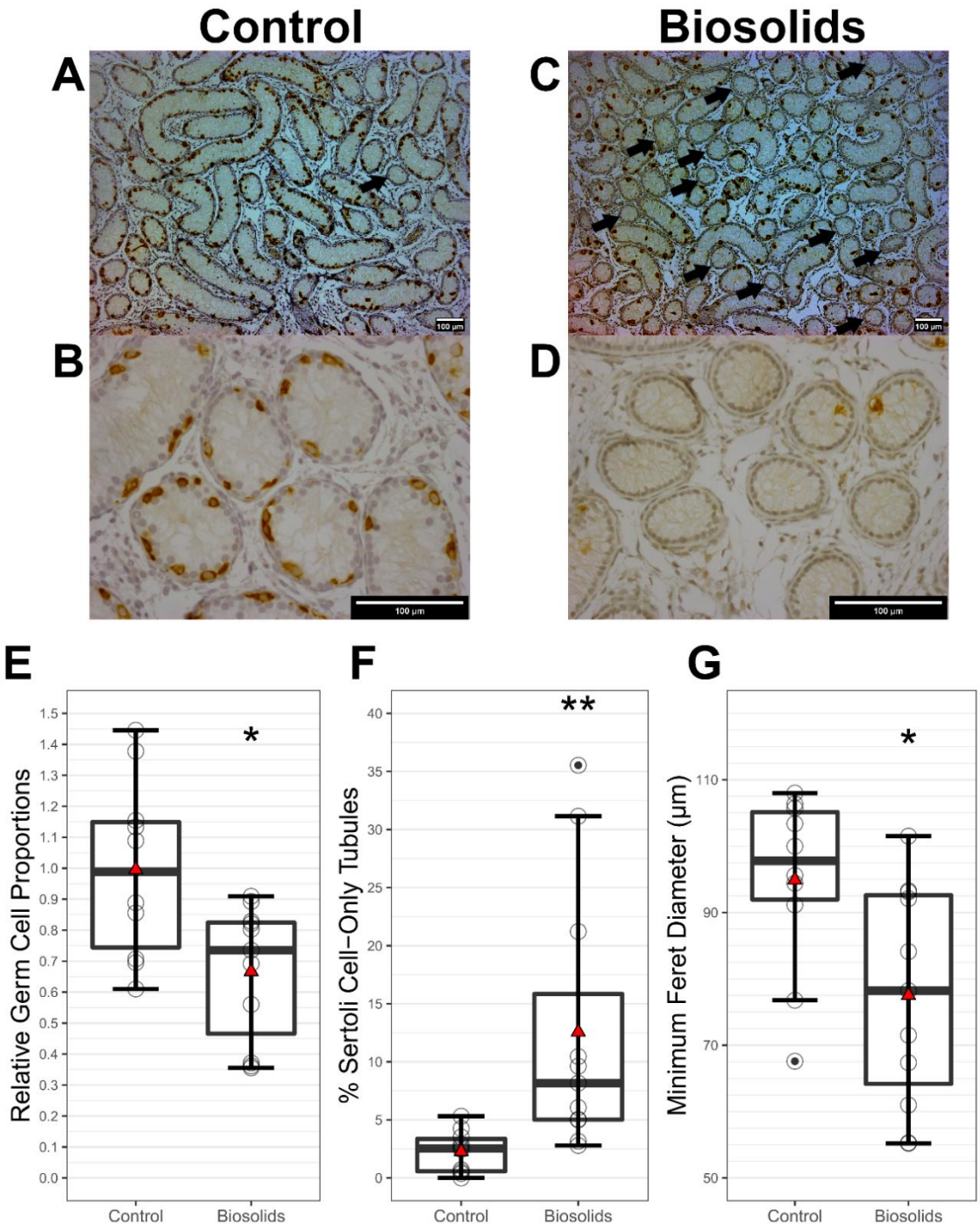


Figure 4-2. Histopathological findings in 8-week-old control and biosolids-exposed lamb testes (n = 11 per group). Representative images of haematoxylin / DDX4-DAB stained tissue sections from 8-week-old control and biosolids lambs, performed by Dr Ana Monteiro, taken with a magnification of 100x (A and C respectively) and 400x (B and D respectively). Scale bars (bottom right of images) show 100 µm. Arrows indicate Sertoli-cell-only (SCO) seminiferous tubules. Relative proportions of germ cells to Sertoli cells (E) and percent of tubules which were SCO (F) (p = 0.014 and p = 0. 0082 respectively). Seminiferous tubule minimum Feret's diameters (p = 0.013) (G). Boxes represent 25th to 75th percentile, horizontal bar indicates 50th percentile, whiskers indicate range excluding outliers, solid filled circles show outliers, open circles show individual data points, and red triangles show means.

4.4.2. Gestational BTP exposure alters testicular transcriptome in prepubertal sheep

Nanopore transcriptome sequencing was performed to identify gene expression affected by gestational BTP exposure. Differential gene expression (DGE) analysis identified 1382 differentially expressed genes (DEGs) between the B and C groups (726 with higher expression and 656 with lower expression in B relative to C). Ninety-nine DEGs had an FDR ≤ 0.1 (60 with higher expression and 39 with lower expression in B relative to C). A z score heatmap of these genes is shown in Figure 4-3 (differential expression data presented in Table 4-2). Gene ontology (GO) analysis of the 99 DEGs indicated 6 GO terms as enriched ($p < 0.05$) (Table 4-3). Two genes, from different GO term groups (*HFM1* and *SIRT4*), had expression levels which correlated ($p < 0.05$) with the geometric mean of germ cell markers (*CD9, CD14, THY1, NOTCH1, GFRA1, CDH1*, and *UCHL1*).

4.4.3. Gestational BTP-induced changes in testicular transcriptome of prepubertal sheep positively correlates with testicular transcriptome of human TDS patients

Illumina's BaseSpace Correlation Engine software was used to identify similar data from public data sets. A positive ($p < 0.0001$) correlation was identified between the biosolids exposed DEG data and human TDS patient testes transcriptome data from 7 published DEG datasets across 3 separate studies (Table 4-4). A gene list was created by identifying correlating DEGs in common across each dataset per study, and then combining between studies. This list comprised of 520 genes and was submitted to DAVID for GO and KEGG pathway analyses, which identified 9 KEGG pathways and 25 GO terms as enriched ($p < 0.05$) (Table 4-5). Five genes, each from different GO term / KEGG pathway groups (*BCLAF1, INPP5A, MAPKAP1, TBC1D15,* and *VPS41*), had expression levels which correlated ($p < 0.05$) with the geometric mean of germ cell markers (*CD9, CD14, THY1, NOTCH1, GFRA1, CDH1,* and *UCHL1*).

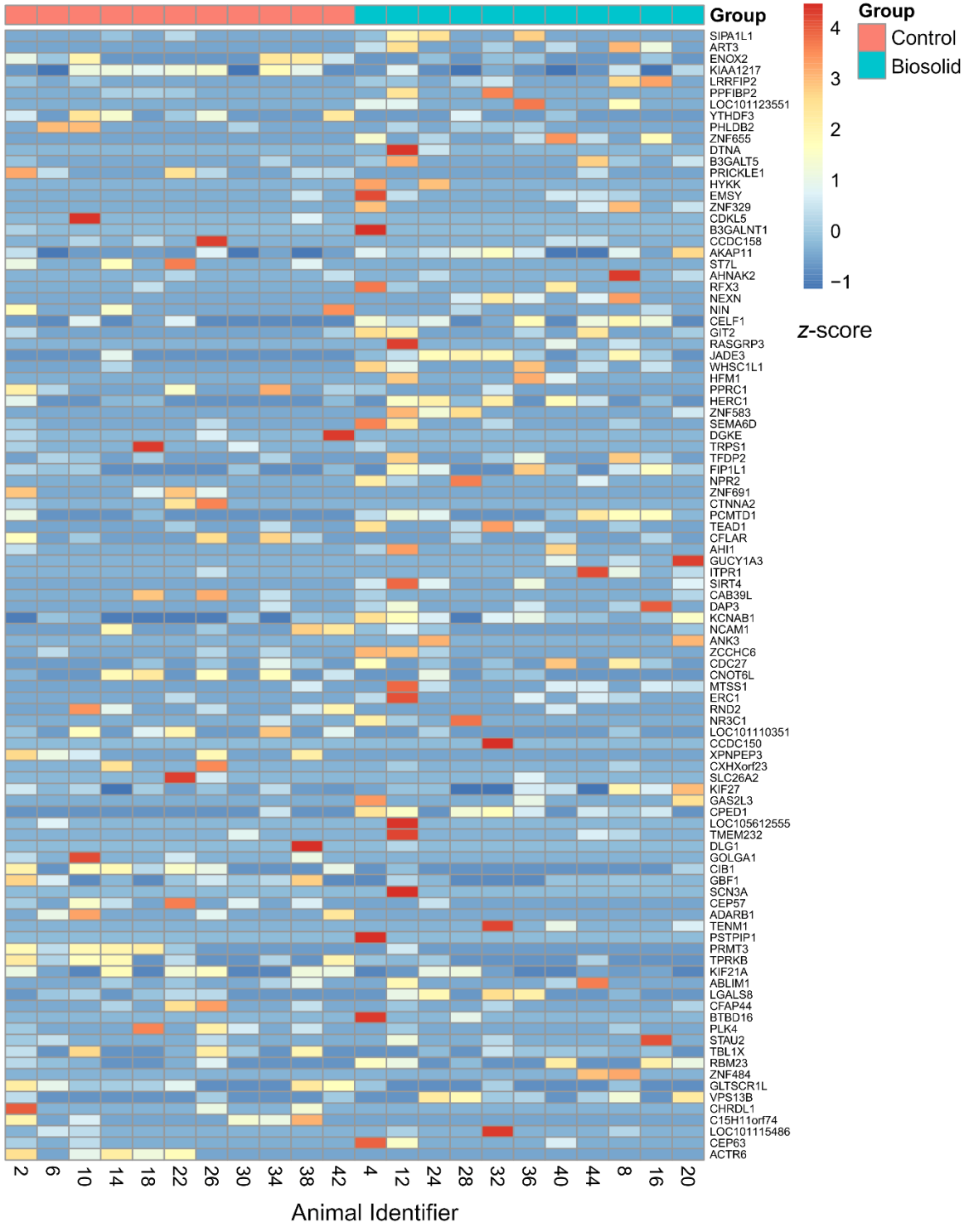


Figure 4-3. Heat map of DEGs plotted against z-score. Genes are ordered from top to bottom by increasing p-value.

Table 4-2. Differential expression data for the 50 genes with lowest p-values. LogFC = Log2 fold change.

Gene	logFC	logCPM	F	PValue	FDR
SIPA1L1	4.58	8.06	32.68	5.82E-08	0.00091
ART3	5.02	7.09	29.85	2.12E-07	0.00166
ENOX2	-3.79	7.59	24.35	2.14E-06	0.00850
KIAA1217	-2.39	9.50	23.69	2.88E-06	0.00850
LRRFIP2	4.04	7.25	23.67	2.91E-06	0.00850
PPFIBP2	4.04	7.52	23.21	3.57E-06	0.00850
ZFP2	4.55	6.86	23.20	3.81E-06	0.00850
YTHDF3	-3.60	7.66	22.49	4.94E-06	0.00964
PHLDB2	-3.71	7.75	22.15	5.76E-06	0.00982
ZNF655	4.41	6.80	22.07	6.29E-06	0.00982
DTNA	4.45	6.82	21.50	8.13E-06	0.01154
B3GALT5	3.79	7.14	21.10	9.29E-06	0.01209
PRICKLE1	-3.71	6.87	19.31	2.11E-05	0.02462
HYKK	4.21	6.72	19.31	2.21E-05	0.02462
EMSY	3.77	6.90	18.67	2.85E-05	0.02965
ZNF329	3.96	6.63	18.27	3.55E-05	0.03467
CDKL5	-4.03	6.65	17.75	4.53E-05	0.03888
B3GALNT1	3.80	6.87	17.67	4.53E-05	0.03888
CCDC158	-3.64	7.10	17.58	4.73E-05	0.03888
AKAP11	2.50	8.16	17.15	5.80E-05	0.04526
ST7L	-3.74	6.55	16.99	6.46E-05	0.04694
AHNAK2	3.49	7.01	16.87	6.61E-05	0.04694
RFX3	3.63	6.83	16.75	6.99E-05	0.04746
NEXN	3.74	6.56	16.65	7.59E-05	0.04771
NIN	-3.41	6.98	16.46	8.00E-05	0.04771
CELF1	2.50	8.32	16.40	8.24E-05	0.04771
GIT2	3.14	7.13	16.40	8.25E-05	0.04771
RASGRP3	3.72	6.55	16.31	8.90E-05	0.04791
JADE3	3.01	7.14	16.20	9.08E-05	0.04791
WHSC1L1	3.48	6.77	16.17	9.20E-05	0.04791
HFM1	3.73	6.56	16.02	1.02E-04	0.05012
PPRC1	-3.17	7.04	15.88	1.06E-04	0.05012
HERC1	2.71	7.79	15.76	1.12E-04	0.05012
ZNF583	3.60	6.52	15.81	1.13E-04	0.05012
SEMA6D	3.28	6.89	15.74	1.13E-04	0.05012
DGKE	-3.74	6.56	15.76	1.16E-04	0.05012
TRPS1	-3.53	6.77	15.56	1.23E-04	0.05193
TFDP2	3.01	7.16	15.31	1.39E-04	0.05695
FIP1L1	2.74	7.50	15.18	1.48E-04	0.05915
NPR2	3.59	6.52	15.12	1.56E-04	0.05958
ZNF691	-3.43	6.46	15.12	1.56E-04	0.05958
CTNNA2	-3.56	6.51	15.05	1.61E-04	0.06002
PCMTD1	2.62	7.69	14.94	1.66E-04	0.06033
TEAD1	3.21	6.89	14.88	1.71E-04	0.06061
CFLAR	-3.07	7.04	14.79	1.79E-04	0.06199
AHI1	3.22	6.84	14.65	1.91E-04	0.06427
GUCY1A3	3.59	6.52	14.59	2.01E-04	0.06427
ITPR1	3.45	6.77	14.54	2.02E-04	0.06427
SIRT4	3.37	6.45	14.59	2.02E-04	0.06427
CAB39L	-3.22	6.87	14.34	2.22E-04	0.06814
DAP3	3.33	6.73	14.33	2.22E-04	0.06814
KCNAB1	2.64	7.14	14.00	2.61E-04	0.07355
NCAM1	-2.76	7.48	14.00	2.62E-04	0.07355
ANK3	3.50	6.50	14.01	2.66E-04	0.07355
ZCCHC6	3.01	7.01	13.96	2.67E-04	0.07355
CDC27	2.79	7.47	13.94	2.68E-04	0.07355
CNOT6L	-2.73	7.48	13.91	2.73E-04	0.07355
MTSS1	3.17	6.67	13.79	2.90E-04	0.07548
ERC1	3.17	6.64	13.74	2.96E-04	0.07548
RND2	-3.21	6.68	13.73	2.98E-04	0.07548
NR3C1	3.30	6.72	13.72	3.00E-04	0.07548
KDM5B	-3.00	6.78	13.68	3.04E-04	0.07548
CCDC150	3.53	6.51	13.62	3.22E-04	0.07852
XPNPEP3	-3.19	6.41	13.55	3.32E-04	0.07903
CXHXorf23	-3.14	6.81	13.48	3.37E-04	0.07903
SLC26A2	-3.27	6.70	13.45	3.41E-04	0.07903
KIF27	2.08	8.53	13.43	3.44E-04	0.07903
GAS2L3	3.32	6.45	13.41	3.57E-04	0.07915
CPED1	3.03	6.62	13.30	3.68E-04	0.07915
LOC105612555	3.24	6.69	13.28	3.72E-04	0.07915
TMEM232	3.19	6.68	13.27	3.72E-04	0.07915
DLG1	-3.36	6.69	13.24	3.79E-04	0.07915
GOLGA1	-3.23	6.42	13.26	3.82E-04	0.07915
CIB1	-2.87	6.71	13.20	3.85E-04	0.07915
GBF1	-2.46	7.50	13.20	3.85E-04	0.07915
SCN3A	3.25	6.41	13.05	4.25E-04	0.08241
CEP57	-3.06	6.61	12.99	4.27E-04	0.08241
ADARB1	-3.20	6.42	13.00	4.34E-04	0.08241
TENM1	3.34	6.46	12.99	4.37E-04	0.08241
PSTPIP1	3.34	6.45	12.98	4.38E-04	0.08241
PRMT3	-2.81	6.81	12.93	4.40E-04	0.08241
TPRKB	-2.66	6.94	12.93	4.41E-04	0.08241
KIF21A	-2.09	8.88	12.91	4.45E-04	0.08241
ABLIM1	2.82	7.34	12.89	4.48E-04	0.08241
LGALS8	2.58	7.46	12.82	4.64E-04	0.08430
CFAP44	-2.89	6.89	12.76	4.79E-04	0.08436
BTBD16	3.27	6.43	12.80	4.80E-04	0.08436
PLK4	-2.98	6.75	12.75	4.81E-04	0.08436
STAU2	2.97	6.96	12.73	4.86E-04	0.08436
TBL1X	-2.52	7.49	12.68	4.99E-04	0.08558
RBM23	2.44	7.64	12.60	5.18E-04	0.08802
ZNF484	3.23	6.43	12.55	5.42E-04	0.09107
GLTSCR1L	-2.69	6.82	12.39	5.73E-04	0.09519
VPS13B	2.61	7.02	12.35	5.87E-04	0.09646
CHRDL1	-3.03	6.37	12.36	5.96E-04	0.09673
C15H11orf74	-3.03	6.38	12.34	6.01E-04	0.09673
AKAP13	3.02	6.82	12.25	6.16E-04	0.09814
CEP63	2.93	6.74	12.21	6.28E-04	0.09899
ACTR6	-2.91	6.35	12.23	6.34E-04	0.09899

Table 4-3. GO terms identified as enriched in the list of 99 DEGs with FDR < 0.1 by DAVID.

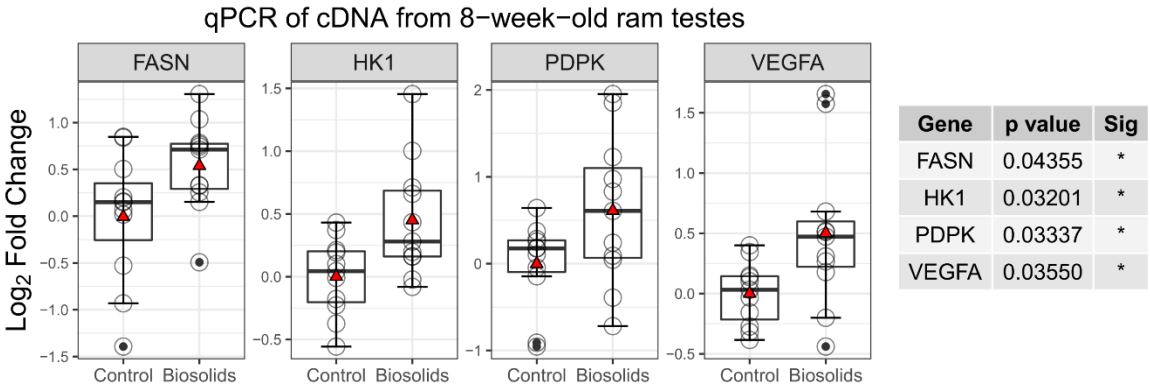
Category	GO Term	Fold Enrichment	*p*-value
Cellular Component	Centrosome	4.03	0.0156
Molecular Function	Nucleic acid binding	2.96	0.0160
Biological Process	Spermatid development	9.60	0.0379
Cellular Component	Node of Ranvier	47.01	0.0412
Molecular Function	Guanylate cyclase activity	45.21	0.0427
Biological Process	Regulation of transcription, DNA-templated	2.95	0.0498

Table 4-4. DEG data sets identified as having significant positive correlation with the biosolids DEG data.

Name of Data Set	p-value	Number of Positively Correlating Genes	Number in Common	Number of Genes Combined
Feig et al (2007) DOI: 10.1093/molehr/gal097				520
Testicular biopsies of azoospermia patients with Sertoli-cell-only sydrome _vs_ full spermatogenesis	3.57E-18	338	105	
Testicular biopsies of azoospermia patients with Sertoli-cell-only _vs_ spermatid stage arrest	2.43E-16	274		
Testicular biopsies of azoospermia patients with Sertoli-cell-only _vs_ spermatocyte stage arrest	2.21E-10	144		
Spiess et al (2012) DOI: 10.1093/humrep/dem292				
Testis from infertile man + Johnsen score 3.2 _vs_ full spermatogenesis	3.44E-07	482	465	
Testis from infertile man + Johnsen score 2 _vs_ full spermatogenesis	8.16E-06	542		
GSE6023				
SCOS patient testicular biopsy - Johnsen Score 10 _vs_ Score 2	2.80E-05	93	47	
SCOS patient testicular biopsy - Johnsen Score 9 _vs_ Score 2	4.48E-07	98		

4.4.4. Gestational BTP exposure increases expression of genes downstream of mTOR activation

As GO and KEGG analysis identified 2 mTOR terms as enriched, qPCR was performed on a range of genes which are downstream products of mTOR activation. Of the fourteen genes quantified by qPCR (*ACACA, ACAD11, ACLY, ACOX1, BIRC5, CCND1, CPT1A, FABP4, FASN, HK1, LDHA, LPL, PDPK1, VEGFA*), four, *FASN* (p = 0.044), *HK1* (p= 0.032), *PDPK* (p = 0.033), and *VEGFA* (p = 0.036), were expressed at a significantly higher level in B than in C animals (Figure 4-4). Three of these genes (*VEGFA, HK1,* and *PDPK1*) are transcribed *via* Hypoxia Inducible Factor 1 Alpha (HIF1α) activation.



Gene	p value	Sig
FASN	0.04355	*
HK1	0.03201	*
PDPK	0.03337	*
VEGFA	0.03550	*

Figure 4-4. Log_2 fold change of testicular gene expression between biosolids-exposed and control animals (n = 11 per group). Boxes represent 25th to 75th percentile, horizontal bar indicates 50th percentile, whiskers indicate range excluding outliers, solid filled circles show outliers, open circles show individual data points, and red triangles show means.

Table 4-5. GO terms and KEGG pathways identified as potentially enriched by DAVID within the list of common DEGs between the biosolids data and at least one correlating study identified by BaseSpace.

Category	Term	Fold Enrichment	*p*-value
GO: Cellular Component	Cytosol	2.00	0.000030
GO: Molecular Function	Zinc ion binding	1.75	0.000140
GO: Cellular Component	Nucleolus	2.04	0.000285
GO: Cellular Component	Intracellular ribonucleoprotein complex	6.03	0.000926
GO: Molecular Function	mRNA 3'-UTR binding	7.51	0.000999
KEGG	Cell cycle	3.52	0.001055
GO: Cellular Component	Early endosome	3.16	0.002503
GO: Molecular Function	Hydrolase activity	3.98	0.003734
KEGG	Purine metabolism	2.76	0.004030
GO: Biological Process	Apoptotic process	3.88	0.004223
KEGG	Metabolic pathways	1.45	0.007357
GO: Biological Process	mTOR signalling	8.96	0.008819
KEGG	Lysine degradation	4.51	0.010057
GO: Molecular Function	GTPase activator activity	2.61	0.014330
KEGG	ErbB signalling pathway	3.33	0.018098
GO: Biological Process	Ras protein signal transduction	4.70	0.020505
GO: Molecular Function	DNA-directed DNA polymerase activity	6.59	0.021242
GO: Biological Process	Positive regulation of gene silencing by miRNA	12.48	0.021909
GO: Cellular Component	mTORC2 complex	11.95	0.024152
GO: Molecular Function	DNA-dependent ATPase activity	5.96	0.027828
GO: Biological Process	Mitochondrial fragmentation involved in apoptotic process	10.92	0.028559
GO: Biological Process	Protein localization to microtubule	10.92	0.028559
KEGG	Pyrimidine metabolism	2.98	0.029146
GO: Biological Process	Platelet-derived growth factor receptor signalling pathway	5.82	0.029464
GO: Molecular Function	RNA polymerase II core promoter proximal region sequence-specific DNA binding	1.92	0.030620
GO: Cellular Component	Nucleus	1.25	0.032457
GO: Biological Process	Retrograde transport, endosome to Golgi	4.04	0.033621
KEGG	Phosphatidylinositol signalling system	2.86	0.034587
GO: Biological Process	Peptidyl-tyrosine autophosphorylation	9.71	0.035899
GO: Molecular Function	ATP binding	1.32	0.036382
KEGG	Biosynthesis of antibiotics	2.07	0.039467
GO: Biological Process	Negative regulation of viral transcription	8.74	0.043875
KEGG	Base excision repair	5.01	0.044115
GO: Biological Process	Mitochondrion organization	4.85	0.047341

4.4.5. Gestational BTP exposure increases nuclear localisation of HIF1α in Leydig cells of prepubertal rams

As qPCR results were indicative of HIF1α activation, immunofluorescent-histochemistry and Western blots were performed. Representative images from C and B testes showing immunofluorescent staining for the transcription factor HIF1α and the nuclear marker DAPI are presented in Figure 4-5A, with the quantified overlap of HIF1α and DAPI signal (Manders' overlap coefficient) presented in Figure 4-5B. In Leydig cells, a greater (p = 0.0032) proportion of HIF1α was seen in the nucleus of B (0.40 ± 0.11) compared to C (0.25 ± 0.10) animals.

Western blots for HIF1α and α-Tubulin are shown in Figure 4-5C with the mean relative normalised abundance of HIF1α in the testes of C and B animals shown in Figure 4-5D. There was less (p = 0.0077) HIF1α detected in the testes of B (0.71 ± 0.22) than C (1.00 ± 0.29) animals.

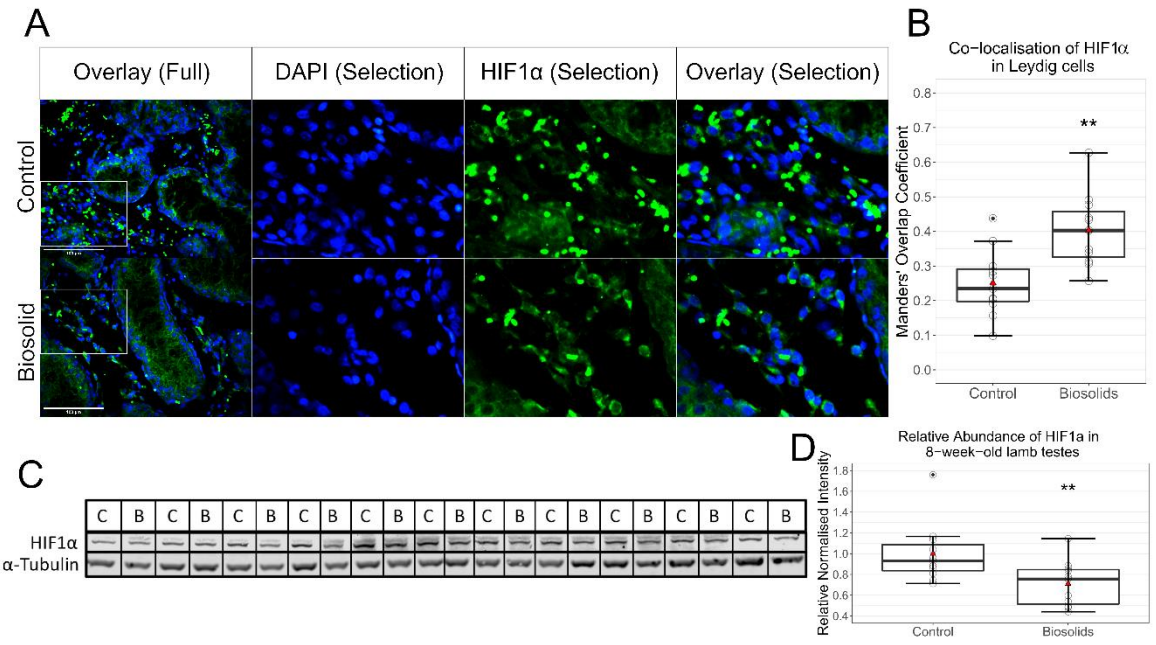


Figure 4-5. HIF1a in testes (n = 11 per group). Immunofluorescent staining of testes sections (A). Images to the right are of selections seen in images to the left, which were taken at 400x magnification. Blue staining is of DAPI and green of HIF1a. Scale bars (bottom left in overlay images) show 100 µm. Co-localisation analysis (B) performed on regions outside of seminiferous tubules shows significantly (p = 0.0032) more nuclear localisation of HIF1a in biosolids-exposed animals than control. Manders' Overlap Coefficients of the proportion of green signal overlapping with blue signal were determined by ImageJ. Western blots for HIF1a and a-Tubulin (C and D) show less (p = 0.0077) HIF1a in biosolids-exposed animals than controls. Boxes represent 25th to 75th percentile, horizontal bar indicates 50th percentile, whiskers indicate range excluding outliers, solid filled circles show outliers, open circles show individual data points, and red triangles show means.

4.5. Discussion

The present study demonstrates the ability for gestational exposure to complex, low-level, real-life chemical mixtures to adversely affect prepubertal testis development. Similarities between morphological and gene expression patterns seen in biosolids exposed lambs and human TDS patients suggest that the changes observed prior to puberty in this model may predispose animals to a TDS-like phenotype and demonstrates the model's utility to investigate the pathogenesis of TDS. Of specific note, biosolids exposure was associated with differential expression of genes related to apoptotic processes and mTOR signalling, and increased expression of a group of mTOR regulated genes which are all transcriptionally controlled by a common nuclear factor (i.e., HIF1α). The expression and activation of HIF1α was also found to be altered. Specifically, HIF1α protein levels were lower in B lamb testes and expression was more localised to the nuclei of Leydig cells. As HIF1α is known to inhibit STAR transcription and testosterone synthesis (Wang et al., 2019), and gestational BTP exposure has been associated with lower serum testosterone levels in fetal and neo-natal offspring (Elcombe et al., 2021; Lea et al., 2022), these changes in HIF1α may provide mechanistic reasoning to the testicular phenotype of biosolids exposed males.

There is increasing evidence of synergistic actions between different chemical components within low dose chemical mixtures leading to adverse effects on male gonadal development. In rodent studies, male offspring exposed gestationally to low doses of some simple chemical mixtures (≤ 10 components) exhibit under-masculinised (aka feminised) phenotypes, genital malformations, pathological testicular morphology, and impaired spermatogenesis (Buñay et al., 2018; Christiansen et al., 2009; Hass et al., 2012; Jacobsen et al., 2012; Rider et al., 2008). The present study found that following gestational exposure to a complex chemical mixture (similar to human exposure) prepubertal male offspring exhibit fewer testicular germ cells and more frequent Sertoli-cell-only seminiferous tubules; a phenotype resemblant of morphological differences in the testes of the late gestation fetus (Paul et al., 2005), neonatal lamb (Elcombe et al., 2021), and a subset of adult offspring (Bellingham et al., 2012) following similar exposure. Importantly, this testicular phenotype also resembles mixed

testicular atrophy, a hallmark of TDS in humans (Nistal et al., 2017; Skakkebæk et al., 2001). Despite the repeatable histological changes seen in the BTP sheep testes, other anatomical correlates of the TDS phenotype of humans (i.e., cryptorchidism, hypospadias, etc) have not been described specifically in BTP exposed sheep. This is likely due to the limited number of animals studied. While these developmental abnormalities are rare in sheep, around 0.1-0.7% cryptorchidism and 0.9% hypospadias (Amann and Veeramachaneni, 2007; Smith et al., 2012b), which may reflect regional incidences in rams selected for culling (Smith et al., 2012a), it is of note that a sibling of one biosolid sheep included in this study was cryptorchidic.

DGE analysis of the testicular transcriptome was undertaken to identify the mechanism by which exposure to the complex mixture of ECs present within biosolids leads to pathogenic changes in testicular morphology. This analysis indicated decreased testicular expression of genes involved in maintaining the blood-testes barrier (BTB) and cell polarity (*PRICKLE, CTNNA2,* and *DLG1*) (Paul and Robaire, 2013; Su et al., 2012; Wang et al., 2022). BTB and cell polarity maintenance are crucial for spermatogonial stem-cell survival, differentiation, and spermatid development (Chen and Cheng, 2016; Mruk and Cheng, 2015). Also poorly expressed in B animals was the gene *ADRB1* (*ADAD2* in humans), which is crucial for male germ cell differentiation, and potentially causative in cohorts of human patients with spermatogenic maturation arrest (Krausz et al., 2020; Snyder et al., 2020). EC exposure also reduced expression of genes essential for spermatogenesis (*CIB1, GBF1, CFAP44,* and *PLK4*) (Au et al., 2015; Miyamoto et al., 2016; Tang et al., 2017; Yuan et al., 2006). EC effects on spermatogenesis were also highlighted by significant enrichment of the GO term “Spermatid Development”. All identified DEGs were tested for correlations to the geometric mean of known germ cell markers to assess the impact of changes to testicular cellularity. However, as only around 2% of genes showed a significant correlation, any effect was deemed minimal. Comparison of the DEG data from this study with DEG data from independent studies in human TDS patients revealed complementarity between the sheep model and TDS patient data and identified EC-responsive genes which may lead to testicular dysfunction. Correlating DEGs between biosolids-exposed animals and TDS patients were also tested against the geometric mean of known germ cell

markers. As <1% of genes showed a significant correlation, any effect from changes to testicular cellularity was also deemed minimal. Of the GO terms and KEGG pathways identified from correlating DEGs between sheep and TDS patient data, two were mTOR and two were apoptotic entries. mTOR is a crucial component of proper testicular development and spermatogenesis (Correia et al., 2020) and mTOR-related pathways have previously been identified in the BTP sheep testicular transcriptome at GD140 (Lea et al., 2022) and in 1-day-old neonates (Elcombe et al., 2021). This would suggest it is a developmentally stable alteration. Given the recognised role of mTOR in testicular development, to investigate the effects of biosolids exposure further the expression of a selection of genes which are upregulated following mTOR activation (Laplante and Sabatini, 2013) was investigated. Of the fourteen genes examined, the testicular expression of four genes was increased in B lambs. Of these, three (*VEGFA, HK1*, and *PDPK1*) are involved in the angiogenic and metabolic adaptive responses to hypoxia *via* Hypoxia Inducible Factor 1 Alpha (HIF1α) activation (Child et al., 2021), indicating a potential common point of EC action.

HIF1α is an important factor in embryonic development which is expressed from early embryonic stages, continuing in germ cells and other tissues into adulthood (Takahashi et al., 2016). Under normoxic conditions, HIF1α is tightly controlled and swiftly degraded *via* polyubiquitination-mediated proteolysis (Child et al., 2021). Under hypoxic conditions, however, the mechanisms for degradation are suppressed and nuclear translocation of HIF1α occurs, which ultimately increases the expression of genes largely involved in angiogenic and metabolic reprogramming (Child et al., 2021). HIF1α activation can also occur independently of oxygen status, triggered by biochemical pathways such as mTOR (Dodd et al., 2015) or small molecules and reactive oxygen species (Bonello et al., 2007; Xia et al., 2009). HIF1α activation in the testes of B animals is likely an adaptive response, as VEGFA and PDPK1 are both critical for spermatogonial stem-cell survival (Fu et al., 2018; Sargent et al., 2016). However, HIF1α activation may have also factored in the observed adverse outcome. HIF1α expression within the testes is highest in Leydig cells (Palladino et al., 2011), a primary function of which is testosterone production. HIF1α has been shown to repress *STAR* transcription in Leydig cells by way of binding site blocking (Wang et al., 2019). As STAR is the main rate-limiting step in steroid

biosynthesis (Manna et al., 2016) a decrease in STAR would be expected to reduce testosterone synthesis, which has also been observed in Leydig cells following HIF1α activation (Wang et al., 2019). While total testicular HIF1α content was reduced in B lambs, there was also a greater proportion of HIF1α localised within the nuclei of the Leydig cells of B animals. However, changes in HIF1α protein levels may be attributable to changes in cellularity and loss of germ cell HIF1α. Additionally, the magnitude of change in HIF1α nuclear localisation (160% of control) outweighs the magnitude of change in HIF1α protein levels (71% of control). Therefore, despite lower overall HIF1α protein levels in the whole testes, it is evident that this was overcome and overall BTP exposure resulted in more activated HIF1α within the nucleus of Leydig cells. An overall EC induced increase in nuclear-localised HIF1α in Leydig cells, as seen in the B animals in the current study, could explain the lower testosterone levels reported in fetal and neonatal B lambs (Elcombe et al., 2021; Lea et al., 2022).

While the present study demonstrates the ability for a complex, low-level, real-life chemical mixture to elicit an adverse effect on the testes of gestationally exposed animals, there are limitations. A major limitation is the lack of knowledge on the precise oral dosage. While the chemical mixture complexity provided by biosolids is a strength of the model in terms of relevance to humans, it also presents a challenge with respect to dose determination. There are published quantifications of various chemicals in organs of directly and gestationally exposed animals (Bellingham et al., 2012; Filis et al., 2019; Rhind et al., 2010, 2009, 2005), but only dioctyl phthalate, octyl phenol, and nonyl phenol have had oral dosage estimations, which were below TDI values (Rhind et al., 2002). There have also been quantifications of 10-12 perfluoroalkyl substances (PFAS) in lettuce, radishes, celery, peas, and tomatoes grown in biosolids fertilised soil, with PFBA and PFPeA having the highest concentrations at >230 ng/g (Blaine et al., 2014, 2013). It is of note that phthalates, alkylphenols, and PFAS can produce reproductive developmental toxicity in offspring when administered individually at higher doses during gestation (Di Nisio et al., 2019; NAS, 2017a; Uguz et al., 2009). However, without precise understanding of dosage, effects from BTP exposure cannot be assessed against mixture toxicity models, and no insight can be gained on possible interactions and/or synergies that may occur. However, this point is somewhat moot as

chemical levels are generally very low and do not differ significantly from control levels; such is the ubiquity of ECs, most are also detectable in control pastures (Evans et al., 2014; Rhind et al., 2010, 2002, 2013). There is also considerable inter-animal variation in organ chemical load (Bellingham et al., 2012; Filis et al., 2019; Rhind et al., 2010, 2009, 2005) resulting from biosolids batch variance, grazing area preferences, and differential uptake across treated pastures. Another significant limitation of the model is the practicalities and resources required to undergo such investigations. Unlike traditional rodent studies, sheep require a great deal of resources, both physical and human. As such, animal numbers and time points are limited. This compounds with the outbred nature of sheep, which is again an advantage in terms of relevance to humans but a disadvantage in terms of complexity. Consequentially, diametric responses to gestational BTP exposure are to be expected, as have indeed been seen previously (Bellingham et al., 2012; Elcombe et al., 2021). This can complicate result interpretation and can be reasonably assumed to mask more subtle effects.

Fetal development is a complex and dynamic period during which there is increased vulnerability to xenobiotic induced toxicity. This study demonstrates that *in utero* exposure to a complex mixture of chemicals that reflects real-life human exposure results in anatomical and molecular changes in the prepubertal testes. It is the first study to show commonality between transcriptomic profiles of a low-level EC exposure animal model and human TDS patients. Investigation of the DEGs common to the model and TDS patients led to evidence of exposure-induced changes to HIF1α activation in Leydig cells, which is linked to steroid biosynthesis. These findings add to the body of evidence to suggest that exposure to real-world levels of environmental chemical mixtures during pregnancy may have an adverse effect on male offspring reproductive health, contributing to the decline in human sperm quality and fecundity.

Chapter 5.

Developmental exposure to a real-life environmental chemical mixture alters testicular transcription factor expression in neonatal and pre-pubertal rams, with morphological changes persisting into adulthood

5.1. Abstract

Environmental chemical (EC) exposure may be impacting male reproductive health. The translationally relevant biosolids treated pasture (BTP) sheep model was used to investigate gestational low-level EC mixture exposure on the testes of F1 male offspring. Adult rams from ewes exposed to BTP 1 month before and throughout pregnancy had more seminiferous tubules with degeneration and depletion of elongating spermatids, indicating "recovery" from previously reported testicular dysgenesis syndrome-like phenotype in neonatal and pre-pubertal BTP lambs. Expression of transcription factors *CREB1* (neonatal) and *BCL11A* and *FOXP2* (pre-pubertal) were significantly higher in the BTP exposed testes, with no changes seen in adults. Increased *CREB1*, which is crucial for testes development and regulation of steroidogenic enzymes, could be an adaptive response to gestational EC exposure to facilitate the phenotypic recovery. Overall, this demonstrates that testicular effects from gestational exposure to low-level mixtures of ECs can last into adulthood, potentially impacting fertility and fecundity.

5.2. Introduction

The past eight decades have seen a consistent decline in the reproductive health of humans and wildlife (Harrison et al., 1997). In men, this presents as reducing sperm counts and semen quality (Levine et al., 2022; Nelson and Bunge, 1974; Swan et al., 2000) concurrent with increasing rates of male reproductive disorders, including testicular cancer (Adami et al., 1994; Chia et al., 2010; Møller, 1998) and anomalies of the male external genitalia, most noticeably cryptorchidism and hypospadias (Campbell et al., 1987; Chilvers et al., 1984; Matlai and Beral, 1985; Paulozzi et al., 1999; Toppari et al., 2010). While endeavours to elucidate the underlying causes of these adverse trends in male reproductive health, now termed testicular dysgenesis syndrome (TDS) (Skakkebæk et al., 2001), have identified many contributory factors, such as malnutrition, sedentary lifestyle, and stress (Crean and Senior, 2019; Ilacqua et al., 2018), much research has focussed on the role of exposure to environmental chemicals (ECs) (Skakkebæk, 2002; Skakkebæk et al., 2022). Epidemiological evidence has shown links between gestational exposure to ECs and negative

reproductive health outcomes for male offspring (Rodprasert et al., 2021), which is mirrored in animal models of gestational exposure to individual ECs, for example, phthalates (Hu et al., 2009; Repouskou et al., 2021).

While many investigative studies have concentrated on potential effects of specific chemicals or families of chemicals, there is increasing attention to the vast numbers of chemicals in the environment to which the population is constantly co-exposed. This is of concern as EC mixture effects can be seen even when the individual mixture components are administered at doses lower than their tolerable daily intake values (TDI) (Buñay et al., 2018; Kortenkamp, 2014). While efforts are being made to model more realistic EC exposure scenarios (Tsatsakis et al., 2017), it is logistically impossible in terms of both the numbers and doses of chemicals to simulate actual EC exposure by traditional component-based methodologies. The biosolids treated pasture (BTP) sheep model, however, utilises a more realistic EC exposure paradigm relative to the human populations; as biosolids (which are a commonly utilised agricultural fertiliser) are derived from domestic and industrial human waste water treatment, they contain a complex mixture of chemicals which reflects human EC exposure (Rhind et al., 2010, 2002, 2013; Venkatesan and Halden, 2014a, 2014b). Grazing of sheep on BTP during pregnancy results in measurable concentrations of many ECs in maternal and offspring organs (Bellingham et al., 2012; Filis et al., 2019; Rhind et al., 2010, 2009, 2005). A TDS-like phenotype (reduced germ cell numbers and greater rates of Sertoli-cell only (SCO) seminiferous tubules) has been reported previously in neonatal (1-day-old) and pre-pubertal (8-weeks-old) lambs whose mothers were grazed on BTP prior to and during pregnancy, with lower plasma testosterone concentrations also reported for the neonatal male offspring (Elcombe et al., 2022b, 2021). In the fetus (GD110 and GD140), across various exposure periods, fewer germ cells, Leydig cells, and Sertoli cells, and lower plasma testosterone concentrations have been reported (Lea et al., 2022; Paul et al., 2005). Results from adult rams would also suggest that BTP exposure (gestationally, and for 7 months during post-natal life, i.e., lactational / direct oral exposure) also results in a TDS-like phenotype in a subset of adult (19-months-old) rams (Bellingham et al., 2012). However, the progression of the maternal BTP exposure induced testicular phenotype seen in male offspring has

not been characterised into adulthood. Testicular transcriptomic profiles have been produced from fetal, neonatal, and pre-pubertal testes, which have indicated perturbations in multiple pathways and led to evidence of alterations in transcription factor (TF) activation, specifically cAMP response element-binding protein (CREB) and hypoxia inducible factor 1 alpha (HIF1α), however, the extent of TF perturbation and the permanence of such alterations is not yet known.

The current study aimed to investigate, in parallel, the morphological and transcriptomic changes in adult (11-months-old) ram testes after pre-conceptional and gestational BTP exposure of their mothers, and to combine this data with that already collected from pre-pubertal rams from the same exposure cohort of animals, and from neonatal rams also born following maternal gestational BTP grazing. By performing analyses across these ages, we aimed to evaluate the progression of the TDS-like phenotype and persistence of TF activity over an extended period without BTP exposure.

5.3. Methods

5.3.1. Ethics statement

All procedures were carried out in line with the UK Home Office Animals (Scientific Procedures) Act (A(SP)A) 1986 regulations, under project licence PF10145DF. The project was also approved by the University of Glasgow School of Biodiversity, One Health, and Veterinary Medicine Research Ethics Committee. Animals were maintained under normal husbandry conditions at the Cochno Farm and Research Centre, University of Glasgow.

5.3.2. Experimental animals

All adult study animals were EasyCare sheep, and siblings or half siblings of the pre-pubertal rams described by Elcombe et al. (2022b), which are also used here. All neonatal animals were Aberdale sheep, and from a separate exposure cohort described in Elcombe et al., (2021). The use of animals from separate exposure cohorts was due to COVID restrictions of movement prohibiting the collection of tissues of EasyCare sheep at 1-day-old. For one month prior to

mating by artificial insemination with semen from 4 rams (4 sire groupings within the same genotype), and for the entirety of pregnancy, ewes were maintained on either biosolids treated pasture (BTP) (biosolids exposed (B)) or pastures fertilised with inorganic fertiliser (control (C)). Pastures were fertilised twice yearly (April and September). BTPs used conventional rates of biosolids (4 tonnes / hectare) as a fertiliser, and C pastures used conventional fertiliser with equivalent amounts of nitrogen (225 kg N/ha per annum). Pregnant ewes were brought indoors two weeks prior to parturition. While maintained indoors, ewes were fed forage harvested from their respective pasture types (i.e., Control vs BTP), supplemented with concentrates as per normal husbandry practice. After birth, pre-pubertal and adult male offspring were maintained on control pastures, whereas neonatal male offspring did not leave birthing pens. At 1 day (neonatal, n = 7 control, n = 17 biosolids), 8 weeks (pre-pubertal, n = 11 control, n = 11 biosolids) or 11 months (adult, n = 11 control, n = 10 biosolids) of age, male offspring were weighed and euthanised by IV barbiturate overdose (140 mg/kg Dolethal, Vetroquinol, UK).

5.3.3. Tissue collection

For all three age groups, at necropsy, testes were dissected. From the left testes, two slices were taken transversely from the centre, quartered, and fixed overnight in 10% neutral buffered formalin (Thermo Scientific – 16499713) before being transferred to 70% ethanol (VWR – 20821.330). Fixed sections of testes were trimmed and processed for embedding in paraffin wax for histology (Excelsior AS, Thermo Scientific). Formalin-fixed, paraffin embedded (FFPE), testicular tissues were stored at room temperature until analysis. From the right testes, transverse slices 5mm thick were taken, quartered, and frozen in liquid nitrogen prior to storage at -70°C for later RNA extraction.

5.3.4. Immuno-histochemistry

Two sections (5µm) of FFPE testicular tissues were taken for each adult animal and mounted on Polysine® coated glass slides. One section per animal underwent immuno-histochemistry (DAB staining), as previously described (Elcombe et al., 2022b), but using a rabbit anti-Sox9 antibody (Sigma-Aldrich AB5535) diluted at 1:1000 to identify Sertoli cells. Sox9 staining was performed

by Dr Ana Monteiro. The other section, and equivalent sections taken from FFPE neonatal testicular tissues, underwent fluorescent immuno-histochemistry for HIF1α as previously described (Elcombe et al., 2022b).

5.3.5. Image capture and analysis

5.3.5.1. SOX9 immuno-histochemistry

Six images from separate areas of the lobuli testis were captured at 100x magnification (Leica DM4000B microscope, Leica DC480 digital camera) for each 11-month-old animal. Individual tubule sections entirely captured within image boundaries (n = 1483 Control, 1423 Biosolids) were manually counted. SOX9 positive Sertoli cells were identified by DAB staining and counted. Spermatogonia, spermatocytes and round spermatids were identified by circular nuclei, and elongating spermatids and spermatozoa were identified by condensed and elongated nuclei with darker haematoxylin staining. Normal seminiferous tubule sections at any stage should have a generation of elongating spermatids or spermatozoa. As per OECD guidelines (OECD, 2009), tubule sections with no or few (<5) elongating spermatids and spermatozoa were classified as showing degeneration and depletion of elongating spermatids, whereas those with many (>5) elongated spermatids and spermatozoa were classified as showing typical spermatogenesis.

5.3.5.2. HIF1α fluorescent immuno-histochemistry

Image capture and analysis of HIF1α fluorescent immuno-histochemistry was performed as previously described (Elcombe et al., 2022b). Briefly, for each section, four images from separate areas of the lobuli testis were captured at 400x magnification (Leica DM4000B microscope, Leica DC480 digital camera). Nuclear HIF1α staining was quantified on areas outside the seminiferous tubules using the JACoP plugin (Bolte and Cordelières, 2006) for ImageJ.

5.3.6. RNA extraction, cDNA library preparation, sequencing, and data analysis

Nanopore transcriptome sequencing and analysis was performed on adult testicular tissues as previously described for the neonatal (Elcombe et al., 2021) and pre-pubertal (Elcombe et al., 2022b) testes. Briefly, cDNA was synthesised

from approximately 30mg of frozen tissue and barcoded for multiplexed sequencing. In two batches, each batch containing half the samples and comprised of an even mix of C and B samples, pooled barcoded sample cDNA was sequenced using a MinION and an R9.4.1 flow cell (Oxford Nanopore – FLO-MIN106D). Reads were basecalled, demultiplexed, barcodes and adapters trimmed, aligned to a reference transcriptome generated using NCBI's Oar_v4.0 reference genome and annotation files, and counted. Differential gene expression analysis was performed on counts by edgeR (Robinson et al., 2010) with differential expression thresholds of $\log_2$ fold change <-1 or >1, p-value < 0.05, and false discovery rate (FDR) < 0.1. Differentially expressed gene (DEG) lists were subjected to gene ontology (GO) and KEGG pathway analyses in DAVID (version 6.8). The DEG list from the adult ram testes, and those previously generated from neonatal (Elcombe et al., 2021) and pre-pubertal (Elcombe et al., 2022b) ram testes, were submitted to ChEA3 for transcription factor (TF) enrichment analysis (Keenan et al., 2019), which produces a list of TFs whose gene products may be over-represented within DEG lists. Sequencing data for gene products of identified transcription factors were extracted from each dataset, geometric means of fold change values calculated, and results filtered for TFs where $\log_2$ of the geometric mean fold change was ≥1 or ≤-1 in any age group.

5.3.7. Quantitative qPCR

RNeasy Mini Kits (Qiagen – 74104) were used to extract RNA from approximately 30mg of frozen neonatal, pre-pubertal, and adult testicular tissues. A QuantiTect Reverse Transcription Kit (Qiagen – 205311) was used to degrade genomic DNA and synthesise cDNA. qPCR was performed on a Stratagene 3000 qPCR system using Brilliant II SYBR Master Mix (Agilent – 600828). Primer sequences are in Table 5-1. Primer efficiencies and Ct values for each sample were calculated by regression of raw fluorescent data by PCR Miner (Zhao and Fernald, 2005). These were used in ΔΔCt analysis to calculate $\log_2$ fold change values.

Table 5-1. Primer details for qPCR.

Gene	Genbank accession number	Forward Primer	Reverse Primer
BCL11A	101119124	GAGAGCCTGATGTTAAAGCCGA	CTCCACGGGATTGGATGCTT
CREB1	443118	GAGCTTGTACCACCGGTAACT	GGTTGCTGGGCACTAGGATT
FOSL1	101111137	GCGGATTGATAAAAGGCGGCG	ACGAGGTGGAACTTCTGCTGG
FOXA1	101106037	TGAAGATGGAAGGGCACGAG	GGAGGAGTAGGCCTCCTGTG
FOXP2	101110051	TGCGGCAACTTGGAAGAATG	CCCAAAGGGCTGGCTTCATA
GATA3	780483	GGCGAGATCCAGCACTCTAGG	GAGAACACAGACACCACGGAA
JUND	443102	GCTCAAGGATGAACCGCAGA	CCTTAATGCGCTCTTGCGTG
HK1	100036759	ACCAAGTCAAAAAGATTGACAAGT	TTGGCATCATAGTCCCCACG
PDPK1	101117211	GACGACGAGGACTGCTATGG	CAGGCAGAGAACCTCAAGGG
VEGFA	443103	CTTGCCTTGCTGCTCTACCT	GCCTCGGCTTGTCACATTTTTC

5.3.8. Statistical analysis

R (version 4.1.1) base functionality was used for all calculations and statistical analyses. Data were fitted to generalised linear models using a gamma distribution and groups compared by Wald tests against their respective controls. Sire (adult and prepubertal cohorts) was incorporated into models to account for the genetic structure of data. The R package ggplot2 (version 3.3.5) was used to produce plots. Data reported as mean ± SD.

5.4. Results

5.4.1. Greater proportions of seminiferous tubules showing degeneration and depletion of elongating spermatids in testes of adult rams gestationally exposed to BTP

In adults, examination of histological images (Figure 5-1A) revealed no differences between B and C ram testes in terms of germ cell numbers or germ cell : Sertoli cell ratios, and no incidences of SCO tubules. The testes of adult B rams contained a higher ($p = 0.0225$) proportion of seminiferous tubule sections showing degeneration and depletion of spermatids (31.62 ± 9.92 %) compared to adult C rams (21.96 ± 6.48 %) (Figure 5-1B).

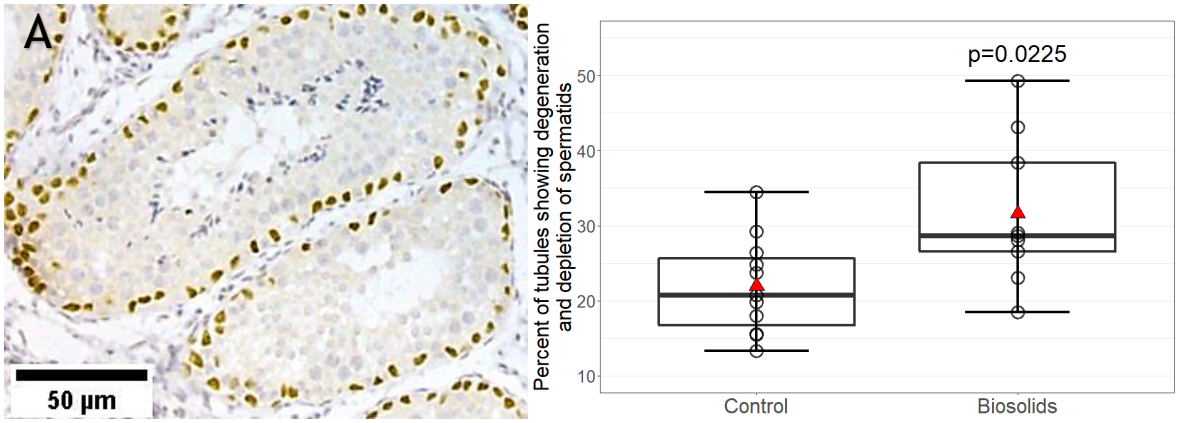


Figure 5-1. SOX9 IHC, performed by Dr Ana Monteiro. (A) Examples of seminiferous tubules showing typical morphology (top) and degeneration and depletion of elongating spermatids (bottom). (B) Biosolids rams have a higher proportion of seminiferous tubules showing degeneration and depletion of elongating spermatids. Boxes represent 25th to 75th percentile, horizontal bar indicates 50th percentile, whiskers indicate range, circles show individual data points, and red triangles show means.

5.4.2. Gestational BTP exposure alters testicular transcriptome in adult rams

Analysis of Nanopore sequenced testicular transcriptomes in adult rams identified 1183 differentially expressed genes (DEGs) between B and C rams (562 with greater levels of expression and 621 with lower levels of expression in B relative to C). Thirty-three DEGs had a false discovery rate ≤ 0.1 (13 with greater levels of expression and 20 with lower levels of expression in B relative to C). Gene ontology (GO) analysis of the 33 DEGs indicated 5 GO terms and 1 KEGG pathway as enriched ($p < 0.05$) (Table 5-2).

Table 5-2. GO terms and KEGG pathways identified as potentially enriched by DAVID.

Category	Term	Fold Enrichment	*p* Value
GO: Cellular Component	Cytosol	3.2	0.0016
KEGG	mTOR signaling pathway	14.7	0.0140
GO: Cellular Component	STAGA complex	104.6	0.0182
GO: Cellular Component	Transcription factor TFTC complex	97.6	0.0195
GO: Biological Process	Histone H3 acetylation	59.4	0.0317
GO: Biological Process	Cellular response to hydrogen peroxide	39.6	0.0472

5.4.3. Extended period without exposure to BTP associated with recovery of TDS-like phenotype, deactivation of HIF1α, and the normalisation of gene expression

Testicular cell counts, proportions of SCO-tubules observed, transcriptomic findings, and HIF1α localisation data are presented in Figure 5-2 for the prepubertal and adult animals, which are from the same BTP exposure (immediately prior to and throughout gestation) cohort, and the neonatal lambs from a separate cohort, but which received a similar exposure (gestational). This represents a synthesis of previously generated and published data (neonatal: cell counts, SCO-tubules, and transcriptomic findings (Elcombe et al., 2021), and pre-pubertal: cell counts, SCO-tubules, transcriptomic findings, and HIF1α localisation (Elcombe et al., 2022b)), new adult data (all), and new neonatal data (HIF1α localisation). As seen in Figure 5-2A and B, reductions in germ cell numbers relative to Sertoli cells and the presence of SCO tubules was most pronounced in neonatal B lambs. Although from a different exposure cohort of animals, these effects of exposure were also present, although to a slightly lesser extent, in the 8-week-old B lambs. There were no differences, however, in the number of SCO tubules or the germ cell: Sertoli cell ratio in the adult

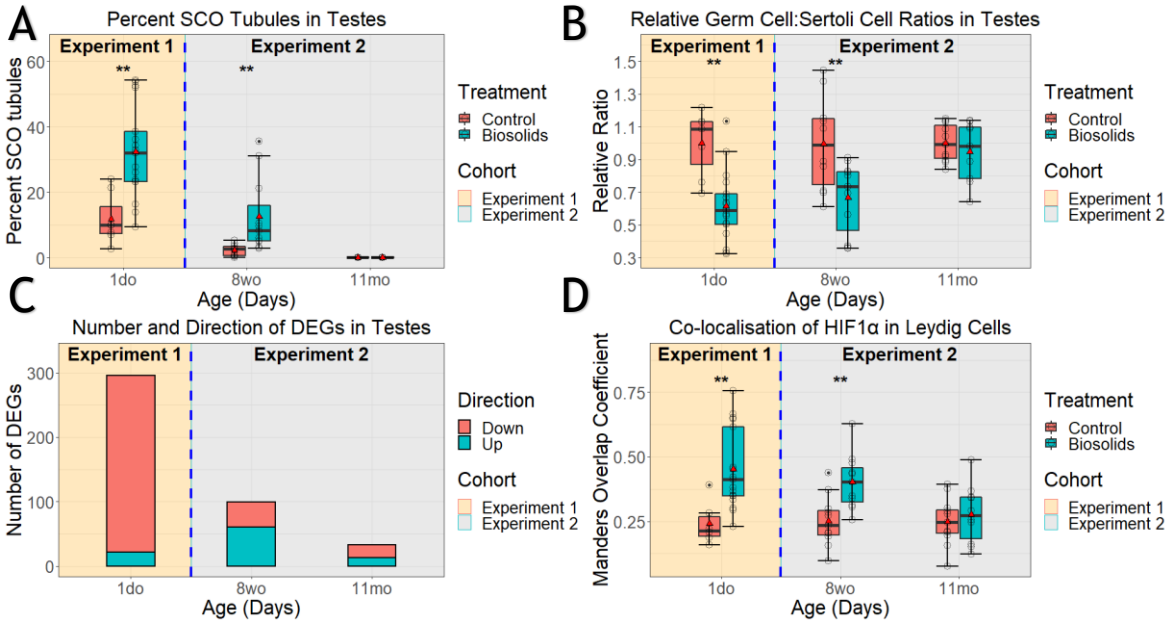


Figure 5-2. Combined data for neonatal, pre-pubertal, and adult ram testes. (A) Frequencies of SCO seminiferous tubules decrease with age. (B) Relative germ cell to Sertoli cell ratios in seminiferous tubules of biosolids rams increases towards control levels with age. (C) Numbers of genes differentially expressed between biosolids and control rams decreases with age. (D) HIF1α nuclear localisation in Leydig cells of biosolids ram testes decreases with age, returning to control levels by adulthood.

rams that were derived from the same exposure cohort of animals as the 8-week-old rams.

Figure 5-2C shows the number and direction of differentially expressed genes between the testes of B and C rams at 1-day-old (neonatal), 8-weeks-old (pre-pubertal), and 11-months-old (adult). The greatest number of DEGs observed was in the neonatal testes (296 DEGs: expression in B rams were higher for 21 genes, and lower for 275 genes, compared to C). The number of DEGs in pre-pubertal ram testes was about one third of the neonatal (99 DEGs: expression in B rams were higher for 60 genes, and lower for 39 genes, compared to C). The number of DEGs identified in the adult ram testes was one third (33 DEGs: expression in B rams were higher for 13 genes, and lower for 20 genes, compared to C) of that seen in the pre-pubertal rams, which were from the same exposure cohort and therefore only differed in age and time since exposure. There was very little overlap between DEGs identified between age groups: 7 shared between the neonatal and pre-pubertal rams, 3 shared between neonatal and adult rams, with only 1 shared between the pre-pubertal and adult rams which were derived from the same exposure cohort of animals (Figure 5-3).

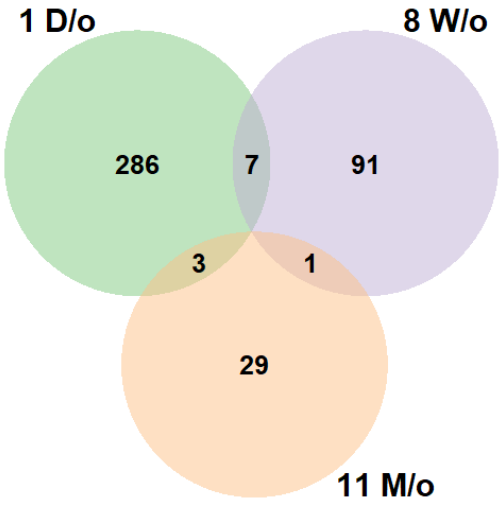


Figure 5-3. VENN diagram showing DEGs identified in neonatal (1D/o), pre-pubertal (8W/o), and adult (11M/o) ram offspring testes.

Nuclear localisation of HIF1α within Leydig cells was investigated in the neonatal maternally B exposed rams and the adult rams born to mothers exposed to BTP immediately before and throughout gestation. The data obtained was compared to the previously reported data from pre-pubertal ram testes (same exposure cohort as the adults) where activation was previously observed (Elcombe et al., 2022b). The proportion of HIF1α nuclear localisation in the C ram testes was

consistent (0.241 ± 0.087, 0.251 ± 0.097, 0.249 ± 0.093) across the neonatal, pre-pubertal, and adult rams, respectively. Within the B exposed rams there was a significantly greater proportion of HIF1α nuclear localisation in the Leydig cells of the testes from the neonatal rams (0.452 ± 0.156, p = 0.0048) relative to C. This pattern was also seen in the pre-pubertal rams (0.403 ± 0.106, p = 0.0032, previously published (Elcombe et al., (2022b)), however, the adults showed no statistically significant difference (0.277 ± 0.113) in the proportion of HIF1α nuclear localisation in the Leydig cells.

To assess the impact greater or equal HIF1α nuclear localisation had on the expression of HIF1α gene products, as before in the pre-pubertal rams, qPCR was performed for *HK1, PDPK, and VEGFA* on cDNA synthesised from neonatal and adult ram testes and combined with previously published expression data for these genes in the pre-pubertal ram offspring (Elcombe et al., 2022b). Significantly greater expression of *HK1* (p = 8.4e-07) and *VEGFA* (p = 0.001) was seen in the neonatal B ram testes compared to C, and no statistically significant differences in gene expression were seen in the adult testes (Figure 5-4). The fold change in expression levels of *HK1* and *VEGFA* for neonatal B rams compared to same age C rams were greater than those for the pre-pubertal B ram testes (previously published in Elcombe et al., (2022b)), but expression levels in the

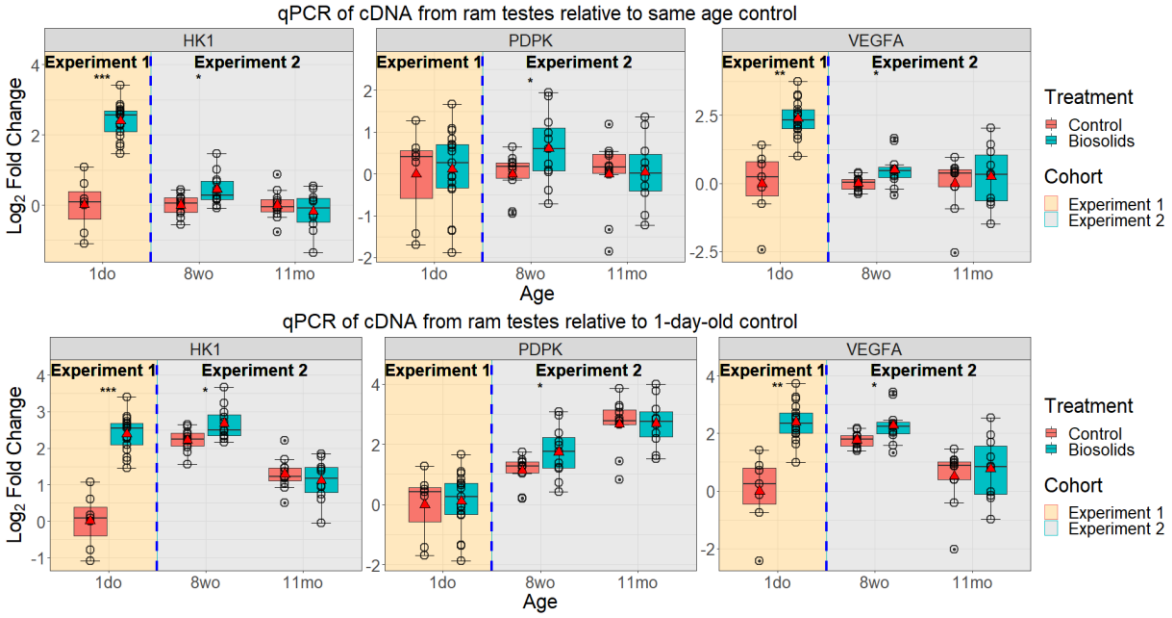


Figure 5-4. qPCR for the genes HK1, PDPK, and VEGFA in neonatal, pre-pubertal, and adult ram testes expressed as Log_2 Fold Change in gene expression relative to neonatal controls. Boxes represent 25th to 75th percentile, horizontal bar indicates 50th percentile, whiskers indicate range excluding outliers, solid filled circles show outliers, open circles show individual data points, and red triangles show means.

neonatal B ram testes were of similar magnitude to the pre-pubertal B ram testes. However, curiously, *PDPK* expression was not different between B and C rams in the neonatal ram testes.

5.4.4. Gestational BTP exposure alters transcription factor expression in testes of neonatal and pre-pubertal lambs, but not adult rams

Transcription factor (TF) analysis of DEG lists from neonatal, pre-pubertal, and adult ram offspring by ChEA3, and subsequent interrogation of sequencing data, identified 50 TFs potentially affected by exposure (Figure 5-5). Of these, 43 showed lower expression of gene products in the neonatal B lamb compared to same age C, with no differences seen in the pre-pubertal or adult offspring that were derived from the same exposure cohort. 1 TF (CREB1) showed higher gene product expression in the neonatal B testes compared to same age C, and 6 TFs (BCL11A, FOSL1, FOXA1, FOXP2, GATA3, and JUND) showed altered gene product expression in either B pre-pubertal or B adult ram testes, which were of the same exposure cohort, compared to same age C (Figure 5-6). Of these, BCL11A, Fox1A and FOXP2 showed lower gene product expression in the neonatal B rams and higher in the pre-pubertal B rams (different exposure cohorts) compared to same age C, and GATA3 showed higher gene product expression in the pre-pubertal and adult rams (same exposure cohort) compared to same age C. To assess if differences in gene product expression were due to changes in TF expression, qPCR was performed on these TFs (Figure 5-7). There were no statistically significant differences in expression levels of *FOSL1, FOXA1, GATA3,* or *JUND* between B and same age C in any age group. In the neonatal testes, *CREB1* expression was significantly ($p = 1.3e-04$) greater in B lambs than in C. In the pre-pubertal testes, significantly greater expression of *BCL11A* ($p = 0.018$) and *FOXP2* ($p = 0.0069$) was seen in the testes of B lambs than same age C.

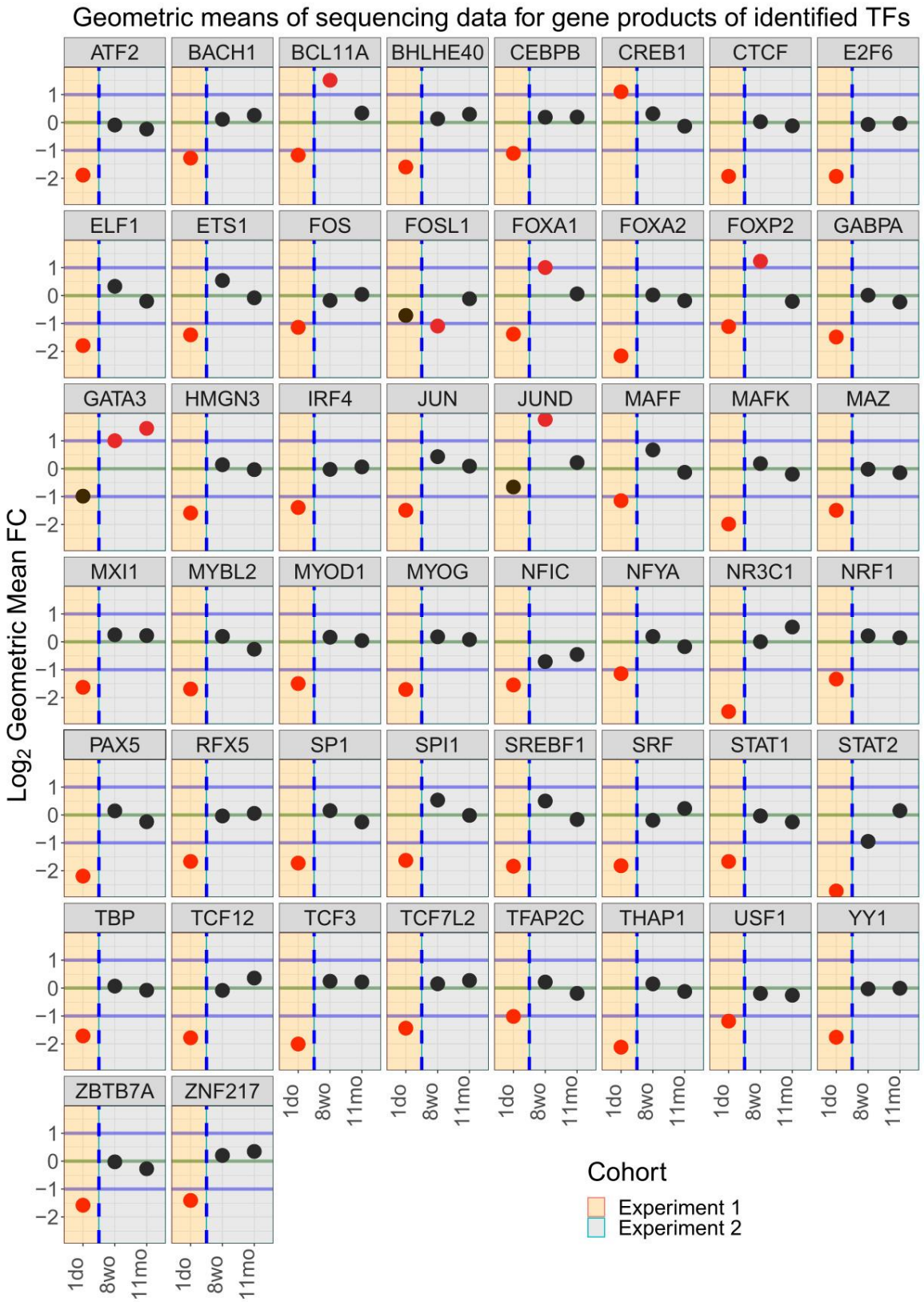


Figure 5-5. TFs which were identified as enriched by ChEA3 analysis, which had log_2 geometric mean fold change of genes products ≥1 or ≤-1 in any age group. Red dots indicate where gene product expression levels passed this threshold.

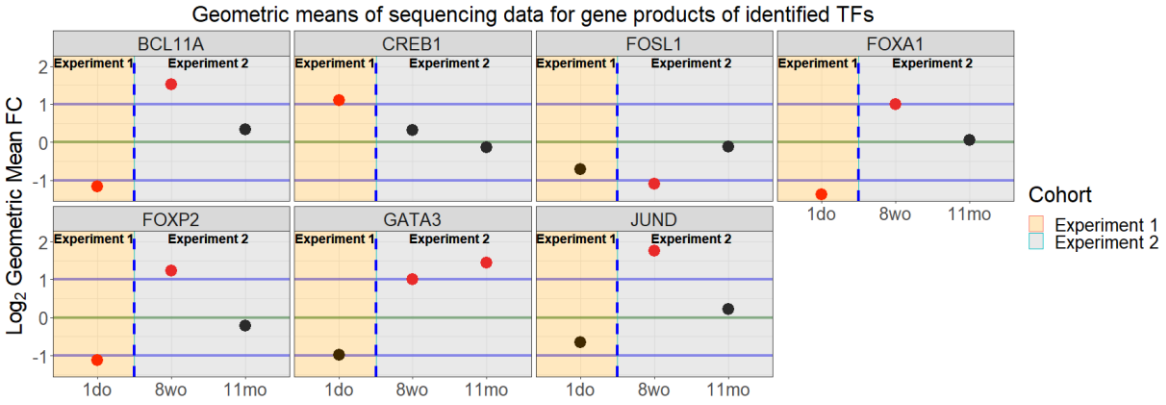


Figure 5-6. Changes in expression of gene products for transcription factors identified by ChEA3. Log_2 of geometric means of fold change based on transcriptomic sequencing data, using a threshold of ±1. Red dots indicate data points which passed the threshold for inclusion.

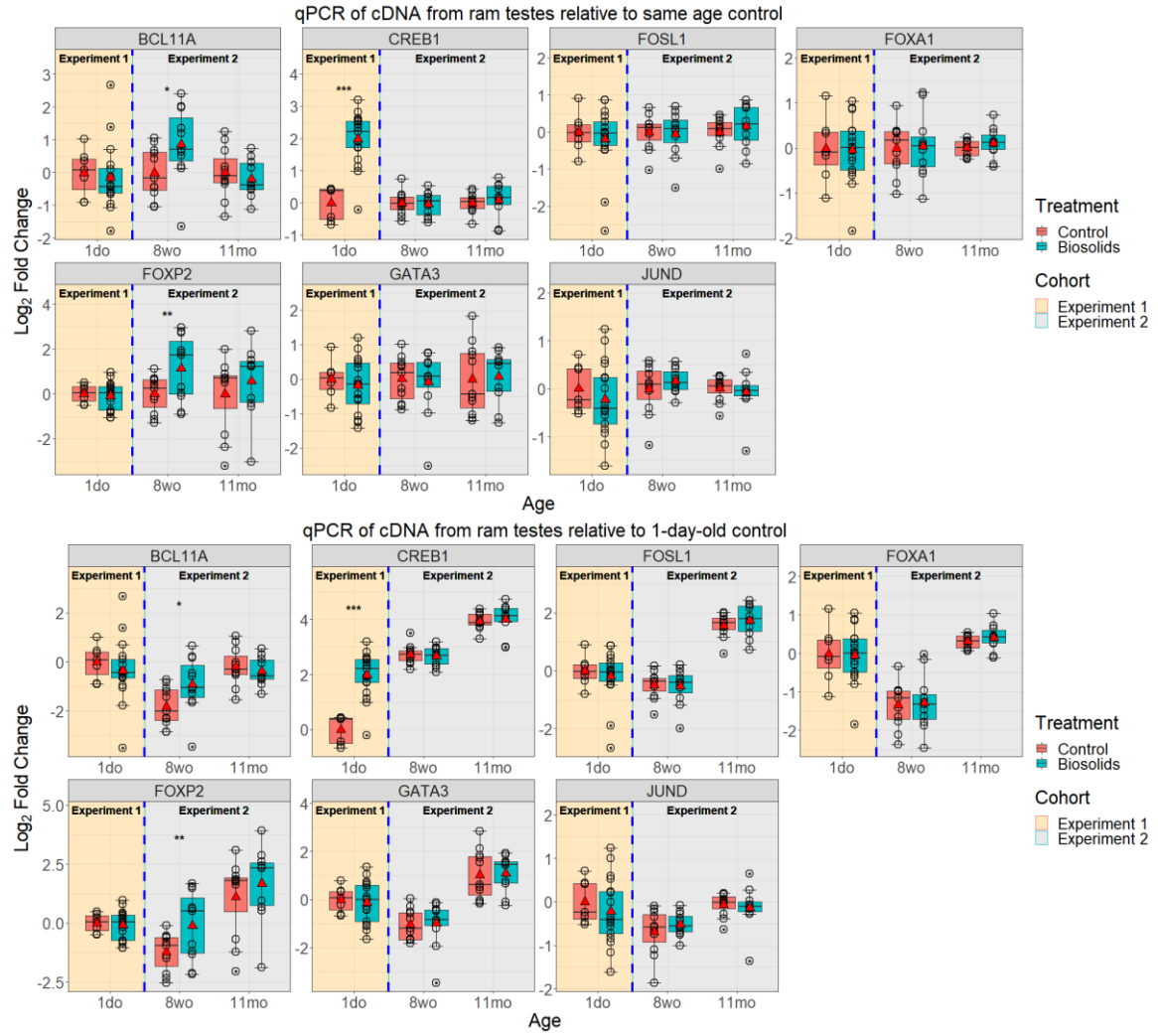


Figure 5-7. qPCR for the genes BCL11A, CREB1, FOSL1, FOXA1, FOXP2, GATA3, and JUND in neonatal, pre-pubertal, and adult ram testes expressed as Log_2 Fold Change in gene expression relative to neonatal controls. Boxes represent 25th to 75th percentile, horizontal bar indicates 50th percentile, whiskers indicate range excluding outliers, solid filled circles show outliers, open circles show individual data points, and red triangles show means.

5.5. Discussion

The long-term consequences of exposure to mixtures of ECs on male reproductive health is of continuing concern. Increasing attention towards the effects of low-dose chemical mixtures has shown that effects can often be seen following exposure to a mixture of chemicals at doses which, individually, would not be expected to elicit an effect (Elcombe et al., 2022a). Low dose gestational exposure to even simple mixtures (containing less than 10 chemicals) have been shown to cause genital malformations, alterations in testicular morphology, inhibited steroidogenesis, and impaired spermatogenesis, in male rodents (Buñay et al., 2018; Christiansen et al., 2009; Jacobsen et al., 2012). However, such component-based studies fall short of simulating the exposure scenario to which humans are constantly exposed, i.e., low concentrations of many hundreds of chemicals. The ability for gestational exposure to a complex, low-level, real-life chemical mixture to affect testicular development, with effects lasting into adulthood, is demonstrated in the present study.

Offspring of sheep grazed on BTP before and during pregnancy have shown a TDS-like phenotype (reduced germ cell numbers and greater rates of Sertoli-cell only seminiferous tubules) in neonatal rams (Elcombe et al., 2021), and in the pre-pubescent half-siblings of the adult rams presented here (Elcombe et al., 2022b). Following similar exposure, reductions in germ cell, Sertoli cell, and Leydig cell numbers has been seen in the mid-gestation (GD110) fetus (Paul et al., 2005), and in the late-gestation fetus (GD140) various exposure timings led to reductions in testes weight, total Sertoli cell numbers, and plasma testosterone concentrations (Lea et al., 2022). In the adult male offspring, there were no differences in terms of total germ cell populations and SCO tubules, which could suggest (partial) recovery from a TDS-like phenotype. Despite this apparent phenotypic recovery, adult B rams exhibited an increased proportion of seminiferous tubules showing degeneration and depletion of elongating spermatids. This phenotype is regarded as the end stage lesion of low intratesticular testosterone (OECD, 2009), which agrees with previously published observations of lower plasma testosterone concentrations in the late gestation fetus and neonatal male offspring (Elcombe et al., 2022b; Lea et al., 2022), and may have an impact on sperm production, semen quality, fertility,

and fecundity. When the adult phenotype is compared to younger male offspring (neonatal and pre-pubertal) with very similar gestational BTP exposure, a lessening of morphological changes with increasing time without exposure was mirrored in both the decrease in the numbers of differentially expressed genes, and the normalisation of transcription factor expression and activity. The persistence of a TDS-like phenotype in a subset of adult males that had been grazed on BTP for 7 post-natal months (Bellingham et al., 2012) contrasts with the results seen in the adults in this study. While this phenotypic difference in adult BTP sheep testes could be a factor of susceptibility differences between breeds, as those used in Bellingham et al. (2012) were Texel sheep, it also suggests that while the TDS-like phenotype may originate during fetal development, continued EC exposure post-parturition may be required to maintain the TDS-like phenotype into adulthood. Indeed, this latter scenario is more reflective of consistent real-life exposure in humans.

The results of transcriptomic analyses are another indication of the enduring effects of fetal exposure, and while fewer DEGs were identified in the present adult ram testes than in the neonatal and pre-pubertal analyses, GO and pathway analyses still identified several changes compared to control animals. Of specific note was the identification of the mTOR signalling pathway as a site of disruption. mTOR has previously been identified as affected by EC exposure in transcriptomic analyses of fetal, neonatal, and pre-pubertal biosolids ram testes (Elcombe et al., 2022b, 2021; Lea et al., 2022). Alterations with mTOR signalling pathways due to low-level exposure should be of concern as mTOR is a crucial component of proper testicular development and spermatogenesis (Correia et al., 2020) and its disruption by chemical exposure (e.g., 4NP, DEHP, or BPA) has been shown to induce testicular autophagy in pubescent rodents (Duan et al., 2017; Fu et al., 2020; Quan et al., 2017). Additionally, mTOR is also crucial in spermatogonial stem cell maintenance, and induction or inhibition of mTOR activity can deplete the pool of spermatogonial stem cells (Hobbs et al., 2015; Xiong et al., 2015). Based on the results of our previous work in which investigations into disrupted mTOR signalling led to evidence of Hypoxia Inducible Factor 1 Alpha (HIF1α) activation and nuclear localisation in Leydig cells of biosolids pre-pubertal lambs (Elcombe et al., 2022b), this was examined

in the current study in both neonatal lambs, and adult animals from the same exposure cohort as the pre-pubertal offspring. A greater proportion of HIF1α activation and nuclear localisation in Leydig cells in the neonatal lambs indicates disruption of this signalling pathway by ECs is likely to be of fetal origin. As the Leydig cells contain the highest amounts of HIF1α in the testes (Palladino et al., 2011) and, through binding site blocking, HIF1α activation can reduce the transcription of STAR, the rate limiting step in steroidogenesis, and lower testosterone production (Manna et al., 2016; Wang et al., 2019). In this respect, changes in HIF1α activation may have a role in the pathogenesis of the TDS-like phenotype seen in younger biosolids animals, where lower testosterone levels were observed in the GD140 fetus and neonatal lamb (Elcombe et al., 2021; Lea et al., 2022). However, the primary function of HIF1α is that of angiogenic and metabolic reprogramming in response to hypoxia (Child et al., 2021), and its activation could occur *via* changes in biochemical pathways (e.g. mTOR) (Dodd et al., 2015) or in direct response to xenobiotics (Bonello et al., 2007; Xia et al., 2009).

The persistent changes in activation of the TF HIF1α in biosolids animals at different ages led to the investigation of alterations in other TFs. By scrutinising transcriptomic data of TFs highlighted by ChEA3, fifty TFs were identified as potentially affected by EC exposure. Most (forty-four) had gene product expression levels altered only in the neonatal lambs, and of these all but one (*CREB1*) was associated with lower expression of gene products in the neonatal lamb. Therefore, expression levels of the six TFs identified in the other age groups, and *CREB1*, were quantified to investigate if the increased gene product transcription was a result of increased TF transcription. Higher transcription levels of *CREB1* in the neonatal, and *BCL11A* and *FOXP2* in the pre-pubertal testes matched the increased transcription of gene products of those TFs identified within the sequencing data for those age groups. While there is no literature on the roles of BCL11A or FOXP2 within the testes, Cyclic AMP (cAMP) signalling and CREB (cAMP-response element binding protein) have previously been identified as affected by EC exposure in the neonatal biosolids lamb (Elcombe et al., 2021), and are known to play important roles within the testes. Within Sertoli cells, cAMP signalling and CREB are crucial in testicular

development and spermatogenesis (Don and Stelzer, 2002), and within Leydig cells CREB regulates the expression of important steroidogenic genes (Kumar et al., 2018). It is therefore possible that the activation and increased transcription of *CREB1* in the biosolid ram testes is an adaptive response to EC exposure.

A challenge and limitation in the interpretation of the present results are that two age groups (pre-pubertal and adult) are from the same exposure cohort, are of the same breed of sheep, and are full or half-siblings, whereas the other age group (neonatal) were different in all these respects. As the chemical contents of biosolids has batch variability, and chemical uptake varies based on factors of soil content (Clarke and Smith, 2011; Rhind et al., 2013; Zhang et al., 2015), there were undoubtedly differences in chemical composition between the exposure received by the neonatal rams and that to which the pre-pubertal and adult rams received. The genetic differences between Aberdale (neonatal rams) and EasyCare (pre-pubertal and adult rams) sheep is another variable of unknown impact on the results, which could affect susceptibility or resistance to exposure-induced effects and must be considered while interpreting. However, that the pre-pubertal rams morphologically resembled the neonatal rams more than their older exposure cohort counterparts, the adults, and that the pre-pubertal and neonatal rams showed very similar HIF1α activation patterns, these concerns were not considered to be major factors with regards to the present study. Therefore, this allowed us to compare the effects of exposure over multiple ages, which allows considerations of directionality and longevity, which is a strength of the present study. An additional strength comes from using a more translationally relevant animal system, rather than traditional laboratory rodents. Sheep are precocial with organ development more similar at birth to humans than altricial rodents and are more physiologically like humans in terms of reproductive cycle, gestational periods, lifespan, steroidal biosynthesis, and start and duration of puberty. Crucially, similarities in testis development between sheep and humans, in terms of hypothalamic–pituitary–gonadal axis function onset, plasma androgen and anti-Mullerian hormone levels, genital tubercle formation, and external genitalia differentiation, are much greater than for other species, including rats and mice (O'Shaughnessy and Fowler, 2011).

It is well recognised that fetal development is a period of increased risk to xenobiotic induced toxicity, especially that mediated by endocrine disruption. The present study exemplifies this, evidencing that exposure to an environmental chemical mixture, at realistically low doses, during gestation alone is sufficient to produce observable morphological and molecular effects in the testes, which persist into adulthood. Differences between the adult rams in this study and adult rams from a similar study, with a period of post-natal exposure, indicates a crucial period of life whereby, with continued exposure, TDS-like traits persist into adulthood. This also suggests adverse effects evident in early life are not permanent and may be at least partially recoverable, dependant on removal from the source of exposure. Increased HIF1α activation in Leydig cells is shown to be present from birth in exposed offspring, which may be linked to lowered steroidogenesis during fetal development and early life. Increased transcription of *CREB1* could be a compensatory mechanism against this action, and therefore may be important in the partial recovery observed. The current findings add to the increasing body of evidence suggesting that exposure to real-world levels of environmental chemical mixtures during pregnancy may be having a negative impact on the reproductive health of male offspring, contributing to declining male reproductive health, including sperm counts, semen quality, fertility and fecundity.

Chapter 6.

Discussion

6.1. Overview

Exposure to a multitude of ECs may be contributing to the currently observed negative trends in male reproductive health. The work included in this thesis used the biosolids treated pasture (BTP) sheep model to explore the developmental testicular effects of gestational exposure to a translationally relevant, low level EC mixture. Using next generation Nanopore sequencing alongside traditional biochemical, histological, and molecular techniques, the effects of exposure on the testes were assessed in neonatal (1-day-old), pre-pubertal (8-week-old), and adult (11-month-old) male offspring. These investigations revealed changes in testicular morphology, gene expression and transcription factor activation status, which indicates that EC exposure during development may play a role in the observed deterioration of male reproductive health in humans and wildlife. The results also provide crucial leads for future research; possible mechanisms through which EC mixtures may perturb normal testicular development and function and could inform the formation of strategies to combat the decline in human male reproductive health.

Chapter 1 detailed the extent to which humans are exposed to high numbers of ECs at low doses through the interrogation of biomonitoring data of the USA population. Current declines in human male reproductive health were then discussed, as well as the conceptualisation of testicular dysgenesis syndrome (TDS) and the hypothesised links between these phenomena and developmental EC exposure. In Chapter 2, a published review paper, literature surrounding low dose mixture toxicity was reviewed and critically analysed. This analysis demonstrated the uniqueness of the BTP sheep model for the investigation of real-life human EC exposure. The subsequent 3 chapters, 2 of which have been published, document primary research conducted using the BTP sheep model. These chapters described EC associated changes in testicular morphology and transcriptomics at specific ages of post-parturitional development, as well as analyses from birth to early adulthood, in animals born to mothers grazed on BTP pasture prior to mating and during gestation.

EC exposure induced changes to testicular morphology were observed in the neonatal, prepubertal and adult animals. The most severe testicular phenotype observed, fewer germ cells and more frequent Sertoli-cell-only (SCO)

seminiferous tubules, characteristics which are considered histopathological hallmarks of TDS (Nistal et al., 2017), was seen in the 1-day-old and 8-week-old animals. While this phenotype was not apparent in the adult testes, a greater proportion of seminiferous tubules showed degeneration and depletion of elongated spermatids in the adult testes; changes that could affect fertility and fecundity. This type of histopathological phenotype seen in the adult biosolid rams has been associated with chronic or severe testosterone depletion (OECD, 2009) and is therefore consistent with the observation that neonatal biosolids rams had lower plasma testosterone concentrations, and published work showing lower plasma testosterone concentrations in the late gestation male fetus (Lea et al., 2022). Interestingly, this possibility and early patterns of testosterone secretion contrasts with unpublished results which show an early pubertal elevation of testosterone concentrations in biosolids compared to control rams. This may be a compensatory mechanism, or a "rebound" effect from lessening transcription factor activation (discussed later). As the results demonstrate that an abnormal testicular phenotype was present at birth, they indicate a fetal origin to the pathogenesis of this testicular phenotype. While the less severe phenotype seen in the adult BTP rams contrasts with previously published BTP data, in the previous study the rams were both older and had received post-natal EC exposure (Bellingham et al., 2012). This implicates a direct involvement of EC exposure in the testicular phenotypic pathogenesis seen in the BTP model and suggests that crucial periods of post-natal development may also be important for the continuation of the phenotypic changes into adulthood. This is highly relevant to human health, as EC exposure is not limited to gestation, but is continuous throughout life.

EC exposure-induced changes to testicular gene expression, as identified by transcriptome sequencing, were observed in the neonatal, prepubertal and adult animals. The total number of differentially expressed genes appears to reduce with age, which also corresponds with increased time since EC exposure. Mirroring this pattern in gene expression, transcription factor (TF) enrichment analysis also showed a continual reduction in the total number of enriched TFs with age. These observations may be due to the release of ECs sequestered by tissues during gestational development being later released back into systemic circulation during periods of enhanced growth where fat stores are utilised, thus

maintaining EC exposure postnatally. Alternatively, it could be a result of biological processes requiring time to re-equilibrise, gene transcription compensating for exposure induced alterations, or any combination of these. An important transcriptomic finding came from the comparison of pre-pubertal BTP transcriptomic data with publicly available transcriptomic data, which showed a high degree of similarity to those of human TDS patients. As the differentially expressed genes in common between the BTP data and TDS patient data only showed a small number of genes with expression levels which correlated to that of germ cell markers, this similarity in gene expression was not due to common morphological changes in cellularity. Therefore, the differentially expressed genes in common between the BTP data and TDS patient data are EC-responsive genes which are also altered in TDS patients. While these could be due to altered function secondary to TDS, or causative factors of TDS, this evidence coupled with histopathological similarities provides the evidence base to use these findings in a translational manner.

An extremely interesting finding, which was seen across all ages studied, was the identification of mTOR signalling as a testicular target that appears to be permanently affected by developmental EC exposure. mTOR is the major regulator of growth, mass accumulation, and cell survival in animals. mTOR is the main functional component of two complexes, mTORC1 and mTORC2, which respond to nutrient availability, growth factors and environmental stresses, and control most anabolic and catabolic processes (Sabatini, 2017). mTOR is an important component of testicular development and spermatogenesis (Correia et al., 2020; Jesus et al., 2017), especially as part of mTORC1 where mTOR signalling is crucial to maintain spermatogonial stem cells (SSC) (Moreira et al., 2019). Indeed, mTORC1 induction or inhibition disturbs SSC pool maintenance, and can lead to SSC depletion (Hobbs et al., 2015; Xiong et al., 2015). mTOR has also been identified as susceptible to EC disruption in testicular transcriptome analysis of the late gestation fetus (Lea et al., 2022). Interrogation of the neonatal transcriptomic data suggested that the observed testicular phenotype could be associated with lower expression levels of components of mTORC1 and mTORC2. This is in agreement with published accounts that EC exposure induced reductions in mTOR signalling can result in reproductive toxicity of the testes; specifically, in pubescent rodents, testicular autophagy can be induced by 4-

nonylphenol through inhibition of the AMPK-mTOR pathway (Duan et al., 2017), or by diethylhexyl phthalate or bisphenol A through actions on the PI3K-Akt-mTOR pathway (Fu et al., 2020; Quan et al., 2017). As efforts to investigate EC induced alterations to expression of mTORC components and mTOR phosphorylation at the protein level for this thesis were impeded by anti-body problems, an alternative approach of examining gene transcription downstream of mTOR activation was employed. This resulted in evidence of increased HIF1α activation and nuclear localisation in Leydig cells, but lower HIF1α protein levels in the whole testes in the biosolids group relative to the controls. As mTORC1 is a positive regulator of HIF1α expression (Düvel et al., 2010; Hudson et al., 2002), reduced testicular HIF1α protein levels, consequent to EC exposure, may be explained by reduced expression of mTORC components resulting in reduced mTORC1 activity. However, as HIF1α activation is also positively regulated by mTORC1 (Laplante and Sabatini, 2013), increased transcription of HIF1α gene products would suggest higher mTOR activation in biosolids animals. Thus, increased HIF1α activation and HIF1α gene products transcription is likely unconnected to changes in mTOR pathway related gene expression, and a more probable explanation is through interactions between HIF1α and small molecules or reactive oxygen species (Bonello et al., 2007; Xia et al., 2009). Therefore, it is still unknown if changes in the expression of mTOR signalling components are involved in any observed effects from BTP exposure. Nevertheless, the identification of the mTOR signalling pathway in transcriptome analyses of the late fetus, neonatal, pre-pubertal, and adult testes following gestational EC exposure is a novel finding of significant interest, with the potential to be, at least in part, causative of the adverse outcome, and therefore highly worthy of future research.

Several findings related to the EC induced activation or expression of TFs were identified in the testes of the neonatal and pre-pubertal ram offspring. The most prominent finding was increased activation and nuclear localisation of HIF1α in the Leydig cells of both the neonatal and pre-pubertal testes of EC exposed rams. As HIF1α activation within Leydig cells has been shown to reduce testosterone production through the inhibition of *STAR* transcription (Wang et al., 2019), this provides a putative mechanism for lower plasma testosterone concentrations seen in the neonatal lambs and the late gestation fetus (Lea et

al., 2022). As HIF1α activation was highest in the neonatal lambs, lower than the neonates but still higher than control in the pre-pubertal rams, and not different to control in the adult rams, this may also explain the "rebound" effect in relation to unpublished plasma testosterone results mentioned earlier. If lower plasma testosterone concentrations were driven by HIF1α inhibition of *STAR* transcription, as STAR is the rate limiting factor in steroidal biosynthesis (Manna et al., 2016), any compensatory mechanisms to increase steroidogenesis would likely be ineffective; HIF1α activation would maintain the steroidogenic bottleneck. Thus, it is reasonable to believe that in this scenario a lessening of HIF1α activation, and thus more *STAR* transcription, would result in an initial over production of testosterone followed by a later normalisation. Indeed, HIF1α activation normalised between the ages of 8-weeks-old and 11-months-old, and unpublished results show higher plasma testosterone concentrations in ram offspring from 9.5-weeks-old to 6-months-old, and no statistically significant difference from 6.5-months-old to 10.5-months-old. This supports the hypothesis that HIF1α activation and nuclear localisation within Leydig cells is involved in lowering plasma testosterone concentrations.

An additional TF of note that may have been affected by EC exposure was CREB1. In the neonatal testes, the expression of *CREB1* was found to be increased and differential expression patterns of genes involved in CREB activation were identified. Specifically, transcriptome analysis of neonatal testes indicated two distinct gene expression profiles in biosolids rams, which appeared to correspond to observed phenotypic severity. When transcriptomic analysis was performed with biosolids rams separated into those affected by EC exposure and those phenotypically resembling controls, two separate mechanisms towards CREB activation where identified, which can mediate survival factor secretion. Rams more susceptible to exposure induced changes in testicular morphology may have higher NF-κB / PI3K signalling towards CREB, whereas rams more resistant to exposure induced changes in testicular morphology may have higher cAMP / PKA signalling towards CREB, as well as expressing a pro-secretory form of CREB (*CREB3L3*). As activated CREB can bind to its own promotor and amplify CREB transcription (Don and Stelzer, 2002), higher *CREB1* transcription in all neonatal BTP rams suggests both resistant and susceptible neonatal pathways towards CREB activation were successful.

Differences in susceptibility may, therefore, result from expression of *CREB3L3* in resistant lambs, or alternatively may be a consequence of other, yet to be identified factor(s), potentially resulting from genetic variation within the flock (discussed later). Indeed, differential responses to exposure in an outbred population is not to be unexpected and was seen previously in the testes of adult BTP rams (Bellingham et al., 2012).

Together this research showed that low level exposure to many chemicals during gestation can cause adverse effects on testicular development, with effects still evident in adulthood. It provides value to the field by using a model with much greater translatability than other currently employed models of chemical mixture exposure, and links exposure outcomes to human TDS patient data.

6.2. Strengths

The BTP sheep model is unique in its ability to capture an exposure dynamic similar to that which humans and wildlife are continually exposed in terms of concentrations and numbers of chemicals, i.e., it investigates a realistic exposure paradigm which cannot be achieved experimentally by component-based methodologies. While also producing limitations (discussed later), the use of a translationally relevant whole-mixture methodology is important as it incorporates chemicals which are not routinely assessed toxicologically. Such unknown or data poor chemicals include metabolites and industrial intermediary chemicals, secondary reaction products, contaminants, and impurities. Equally as important as capturing the possible effects of unknown or data poor chemicals, a further strength of the model is the multitude of chemicals present within biosolids. Mathematical models of mixture toxicity perform best for simple mixtures, with few chemical components. As shown in Chapter 2, more complex chemical mixtures are more likely to produce more numerous effects, or effects with a greater magnitude, than these models predict. Therefore, capturing as many chemicals as possible to which the population is exposed is crucial to assessing real-life chemical mixture exposure, and currently the BTP sheep model is by far the animal model closest to a true reflection of human exposure in this respect.

Another strength to the BTP sheep model is the use of a more translationally relevant precocial species. While using sheep instead of more traditional altricial laboratory species (i.e., rodents) does pose limitations (discussed later), physiological similarities between sheep and humans are closer than rodents and humans. Crucially, for this research, sheep and human testicular development share many similarities which are markedly different in other species, including rats and mice. These include the age of onset for hypothalamic–pituitary–gonadal axis function, changes in plasma levels of androgen and anti-Mullerian hormone, and timings for genital tubercle formation and external genitalia differentiation (O'Shaughnessy and Fowler, 2011). Other especially relevant aspects of physiological similarity between sheep and humans are longer life spans and gestation lengths, reproductive cycle similarities, stage of developmental progress by birth, steroidal biosynthesis pathways, and later start and longer duration of puberty. Many of these factors give a greater period of exposure during critical periods of development which are associated with xenobiotic vulnerability or allow for greater bioaccumulation of chemicals. Additionally, the sheep used are outbred animals, which provides a genetic background that is more comparable to humans than inbred animals such as common laboratory rodents, a known limitation to their use in toxicological research. This genetic variation is an important factor in hazard identification and risk assessment of chemical exposure, as even small polymorphisms can cause an increased risk to exposure, e.g., NAT2 slow acetylation polymorphisms increase the risk of bladder cancer following exposure to N-substituted aryl compounds (Cartwright et al., 1982; Hanke and Krajewska, 1990; Marcus et al., 2000). This genetic variety may have been a factor in the different responses to developmental EC exposure seen in the neonatal sheep, and adult sheep in Bellingham et al. (2012).

From a technical perspective, a major strength of this work comes from the use of next generation sequencing for testicular transcriptomes. Advancements in sequencing technology have provided a more accessible route for transcriptomic investigations, with greatly reduced hardware and running costs. Having these facilities within the investigating laboratory allowed transcriptomic analyses to be conducted within the budgetary constraints of this research. Omics investigations allow a system wide view of an experiment and can identify

changes which would likely otherwise be missed by focussed investigations. Indeed, within the research presented here, all findings out-with anatomical and morphological observations originated from analyses of sequencing data.

6.3. Limitations

As alluded to in the previous section, there are certain limitations to this research. The main limitation of the BTP sheep model is that there is no precise knowledge of chemical exposure in terms of concentration or chemical types. The literature contains values for many chemicals within biosolids, biosolids treated soil, plants grown on biosolids treated soil, and organ chemical loads of animals put to pasture on BTPs. However, chemicals investigated account for only a small proportion of chemicals present within biosolids, and only three chemicals have had oral dosage estimations for BTP sheep. This is further complicated as often a flock of sheep will congregate into smaller groups, which graze on differing areas, thereby producing a varied chemical mixture exposure across a flock. This is likely to have also contributed to the seemingly diametric responses between groups of exposed animals within the same flock, as seen in the neonatal sheep, and adults from previously published work (Bellingham et al., 2012). These issues are then compounded by batch and temporal variances in biosolids chemical content, and differential chemical uptake as a function of soil type (Clarke and Smith, 2011; Rhind et al., 2013; Zhang et al., 2015). Parallel projects are currently ongoing to characterise the full extent of chemical exposure received by BTP sheep, and the temporal changes in BTP soil chemical load.

While these limitations do not impact the utility of the BTP sheep model, as exposure is so low that exposure assessments based on organ chemical loads show no significant differences, it does preclude analysis into the appropriateness of mixture toxicity models, as precise dosage knowledge is required for prediction calculations. This limitation is, however, largely moot, as many of the chemicals present are data poor, and are missing the required knowledge for integrating into mixture toxicity models, such as mode of actions and PODs. It is also noteworthy that, as with human EC exposure, biosolids chemical load has geographical variability (Clarke and Smith, 2011), although biosolids content of major ECs are mostly similar across the globe. Uptake of ECs

by pastures will equally have geographical variability due to climatic factors. However, these limitations are also largely moot, as so far, all BTP sheep experiments have been performed in Scotland, which precludes any geographical variability as a factor of variance between experiments. Logistical limitations related to the use of large animals are also present. The physical requirements for flock maintenance inhibits the generation of larger numbers, which reduces the power to detect more subtle changes due to exposure. This is compounded by the extra genetic diversity within outbred animals discussed previously.

6.4. Future Directions

Of the research findings discussed in the present work, future research using the BTP sheep model could investigate the fetal origins of HIF1α activation within Leydig cells and the impact this has on testicular development. As HIF1α activation has the potential to be a causative agent in the pathogenesis of the TDS-like phenotype, discerning whether this results from direct chemical interactions or upstream biochemical pathways could identify the molecular initiating event to the adverse outcome. If this were achieved, high throughput screening could be employed against common ECs to identify potentially contributory chemicals, which could then be a focus in efforts to reverse the negative trends in male reproductive health. Another worthy avenue for future research is mTOR signalling. While the present research did not produce further evidence for or against the involvement of aberrant mTOR signalling, the repeated transcriptomic identification of mTOR pathways is very interesting, and mTOR could feasibly be involved in the testicular phenotypic pathogenesis, recovery, or both. CREB would also present an interesting research stream. Investigations into downstream effects of CREB activation may reveal if this is involved in the apparent phenotypic recovery by adulthood, and if so, which facets of which downstream pathways are important. Finally, genomic sequencing of the rams investigated here and previously could be of value, as this would allow genotyping based on outcome severity. This may identify genetic polymorphisms associated with resistance and/or susceptibility to exposure. If such findings were made this could be applied to the human population to produce variables for effect predictions and a translatable factor for risk assessment.

Future research could investigate other issues of declining health trends and increasingly prevalent diseases and disorders in which EC exposure has been implicated. These include female reproductive health, various cancers, disorders of metabolism, such as diabetes, fatty liver, and obesity, cardiovascular complications, such as hypertension and dysrhythmia, and neurodegenerative disorders, such as Alzheimer's and Parkinson's disease. Limitations identified in the previous section could also be addressed. Exhaustive chemical analysis of samples would be of great value to BTP sheep model research. As sheep consume a mixture of soil and herbage during grazing, oral estimations are difficult and subject to wide variability, therefore quantification of chemical levels in plasma samples would be most ideal. While traditional approaches for empirical chemical level determination are not appropriate due to the number of chemicals to which the sheep are exposed, non-targeted methods such as 2D-LC/MS/MS would be appropriate. As this would give a complete picture of circulating chemicals (including metabolites), there would be scope to compare observations (past and future) to predictions by mixture toxicity models. However, a great many of the chemicals identified would not have sufficient data to include within these models, and as such a lot of effort would need to be given in determining or deriving the required information. This could be done by expert judgement using methods such as read-across, or newer approaches such as computational toxicology models (artificial intelligence, machine learning, etc). Most likely such an endeavour would require a mixture of multiple approaches. Finally, the limitations produced specifically by using sheep could be countered by translating the model into another species. A possible route for this would be to feed a smaller herbivore (e.g., guinea pigs) herbage from control and BTPs. Such an approach would allow greater numbers of animals to be used, which would increase the power to detect exposure induced effects, and by using an aggregate of herbage collected from across BTPs, doses would be normalised across all animals.

6.5. Concluding Remarks

The persistent decline in human male reproductive health is of continuing concern. The research conducted and presented here adds further evidence to the involvement of environmental chemicals in driving this negative trend.

Furthermore, exposure induced changes in testicular morphology were accompanied by transcriptomic investigations which facilitated the identification of potential mechanisms for the pathogenesis of, and potential recovery from, an adverse outcome.

The phenotypic similarities between the testicular morphology of the young biosolids ram offspring investigated in the present work, and that of human TDS patients, are stark. Furthering this are the transcriptomic correlations between the testicular transcriptomes of pre-pubertal biosolids lambs and TDS patients. Together, these provide a confident position to call this a TDS-like phenotype arising from gestational exposure to a low-level environmental chemical mixture, allowing these findings to be used translationally. Evidence is presented for an amount of recovery in the testicular phenotype of adult ram offspring. As this contrasts with previous biosolids work where exposure lasted for 7 months post-parturition, the involvement of environmental chemical mixture exposure in the pathogenesis of TDS is further implicated, and it is demonstrated that fetal development is not the sole period of xenobiotic vulnerability in terms of a TDS-like adverse outcome.

Mechanistic investigations into the pathogenesis, progression, and potential recovery of this phenotype led to numerous interesting findings, most notably changes in the activation or transcription of multiple transcription factors. As each transcription factor is a master regulator of many genes, the dysregulation of transcription factors can have widespread and long-lasting repercussions. The persistent activation and nuclear localisation of HIF1α is a likely candidate for contributing to the adverse developmental outcome of the biosolids ram testes, due to the fetal origin and the known involvement in inhibiting steroidogenesis. Conversely, CREB is a likely candidate for contributing to the recovery of the adverse developmental outcome of the biosolids ram testes, due to its involvement in testicular development and steroidogenesis. As differential phenotypic responses were seen the neonatal biosolids ram lambs investigated in the present work, and in the adult biosolids ram offspring from previous work, the balance between these two factors may be crucial in determining individual risk to environmental chemical mixture exposure.

In conclusion, this body of work convincingly implicates gestational EC exposure in the development of TDS, and thus as a contributing factor to declines in male reproductive health. Further research in this area may be able to determine conclusively the origins and mechanisms of this adverse effect, which could inform efforts to reverse these negative trends. Due to the scope of these findings and the widespread occurrence and impact of declines in male reproductive health, this research should be of interest to environmental scientists and toxicologists, health care professionals, governmental and regulatory agencies, and the public.

References

Abdelouahab, N., AinMelk, Y., Takser, L., 2011. Polybrominated diphenyl ethers and sperm quality. Reprod. Toxicol. 31, 546–550. https://doi.org/10.1016/j.reprotox.2011.02.005

Adami, H.-O., Bergström, R., Möhner, M., Zatoôski, W., Storm, H., Ekbom, A., Tretli, S., Teppo, L., Ziegler, H., Rahu, M., Gurevicius, R., Stengrevics, A., 1994. Testicular cancer in nine northern european countries. Int. J. Cancer 59, 33–38. https://doi.org/10.1002/ijc.2910590108

Adnan, M.R., Lee, C.N., Mishra, B., 2022. Adverse effects of phytoestrogens on mammalian reproductive health. Vet. Med. Anim. Sci. 10, 1. https://doi.org/10.7243/2054-3425-10-1

Akingbemi, B.T., Braden, T.D., Kemppainen, B.W., Hancock, K.D., Sherrill, J.D., Cook, S.J., He, X., Supko, J.G., 2007. Exposure to Phytoestrogens in the Perinatal Period Affects Androgen Secretion by Testicular Leydig Cells in the Adult Rat. Endocrinology 148, 4475–4488. https://doi.org/10.1210/en.2007-0327

Akre, O., Richiardi, L., 2009. Does a testicular dysgenesis syndrome exist? Hum. Reprod. 24, 2053–2060. https://doi.org/10.1093/humrep/dep174

Allen, C.D., Waser, B., Körner, M., Reubi, J.C., Lee, S., Rivier, C., 2011. Neuropeptide Y acts within the rat testis to inhibit testosterone secretion. Neuropeptides 45, 55–61. https://doi.org/10.1016/j.npep.2010.10.006

Alves, M.G., Martins, A.D., Rato, L., Moreira, P.I., Socorro, S., Oliveira, P.F., 2013. Molecular mechanisms beyond glucose transport in diabetes-related male infertility. Biochim. Biophys. Acta - Mol. Basis Dis. 1832, 626–635. https://doi.org/10.1016/j.bbadis.2013.01.011

Amann, R.P., Veeramachaneni, D.N.R., 2007. Cryptorchidism in common eutherian mammals. Reproduction 133, 541–561. https://doi.org/10.1530/REP-06-0272

Andersson, A., Ashiq, M.J., Shoeb, M., Karlsson, S., Bastviken, D., Kylin, H., 2019. Evaluating gas chromatography with a halogen-specific detector for the determination of disinfection by-products in drinking water. Environ. Sci. Pollut. Res. 26, 7305–7314. https://doi.org/10.1007/s11356-018-1419-2

Annamalai, J., Namasivayam, V., 2015. Endocrine disrupting chemicals in the atmosphere: Their effects on humans and wildlife. Environ. Int. 76, 78–97. https://doi.org/10.1016/j.envint.2014.12.006

Aschner, M., Mesnage, R., Docea, A.O., Paoliello, M.M.B., Tsatsakis, A., Giannakakis, G., Papadakis, G.Z., Vinceti, S.R., Santamaria, A., Skalny, A. V., Tinkov, A.A., 2022. Leveraging artificial intelligence to advance the understanding of chemical neurotoxicity. Neurotoxicology 89, 9–11. https://doi.org/10.1016/j.neuro.2021.12.007

Assinder, S., Davis, R., Fenwick, M., Glover, A., 2007. Adult-only exposure of male rats to a diet of high phytoestrogen content increases apoptosis of meiotic and post-meiotic germ cells. Reproduction 133, 11–19. https://doi.org/10.1530/rep.1.01211

Au, C.E., Hermo, L., Byrne, E., Smirle, J., Fazel, A., Simon, P.H.G., Kearney, R.E., Cameron, P.H., Smith, C.E., Vali, H., Fernandez-Rodriguez, J., Ma, K., Nilsson, T., Bergeron, J.J.M., 2015. Expression, sorting, and segregation of Golgi proteins during germ cell differentiation in the testis. Mol. Biol. Cell 26, 4015–4032. https://doi.org/10.1091/mbc.E14-12-1632

Auger, J., Le Denmat, D., Berges, R., Doridot, L., Salmon, B., Canivenc-Lavier, M.C., Eustache, F., 2013. Environmental levels of oestrogenic and antiandrogenic compounds feminize digit ratios in male rats and their unexposed male progeny. Proc. R. Soc. B Biol. Sci. 280, 20131532. https://doi.org/10.1098/rspb.2013.1532

Auxietre, T.-A., Dumontier, M.-F., Balguy, I., Frapart, Y., Canivenc-Lavier, M.-C., Berges, R., Boudalia, S., Auger, J., Corvol, M.-T., Savouret, J.-F., 2014. Sub-NOAEL amounts of vinclozolin and xenoestrogens target rat chondrogenesis in vivo. Biochimie 99, 169–177. https://doi.org/10.1016/j.biochi.2013.12.001

Bal, H.S., 1984. Effect of Methoxychlor on Reproductive Systems of the Rat. Exp. Biol. Med. 176, 187–196. https://doi.org/10.3181/00379727-176-41861

Barbonetti, A., Castellini, C., Di Giammarco, N., Santilli, G., Francavilla, S., Francavilla, F., 2016. In vitro exposure of human spermatozoa to bisphenol A induces pro-oxidative/apoptotic mitochondrial dysfunction. Reprod. Toxicol. 66, 61–67. https://doi.org/10.1016/j.reprotox.2016.09.014

Bellingham, M., Amezaga, M.R., Mandon-Pepin, B., Speers, C.J.B., Kyle, C.E., Evans, N.P., Sharpe, R.M., Cotinot, C., Rhind, S.M., Fowler, P.A., 2013. Exposure to chemical cocktails before or after conception - The effect of timing on ovarian development. Mol. Cell. Endocrinol. 376, 156–172. https://doi.org/10.1016/j.mce.2013.06.016

Bellingham, M., Fowler, P.A., Amezaga, M.R., Rhind, S.M., Cotinot, C., Mandon-Pepin, B., Sharpe, R.M., Evans, N.P., 2009. Exposure to a Complex Cocktail of Environmental Endocrine-Disrupting Compounds Disturbs the Kisspeptin/GPR54 System in Ovine Hypothalamus and Pituitary Gland. Environ. Health Perspect. 117, 1556–1562. https://doi.org/10.1289/ehp.0900699

Bellingham, M., Fowler, P.A., MacDonald, E.S., Mandon-Pepin, B., Cotinot, C., Rhind, S., Sharpe, R.M., Evans, N.P., Mandon-Pepin, B., Cotinot, C., Rhind, S., Sharpe, R.M., Evans, N.P., 2016. Timing of maternal exposure and foetal sex determine the effects of low-level chemical mixture exposure on the foetal neuroendocrine system in sheep. J. Neuroendocrinol. 28, jne.12444. https://doi.org/10.1111/jne.12444

Bellingham, M., Mckinnell, C., Fowler, P.A., Amezaga, M.R., Zhang, Z., Rhind,

S.M., Cotinot, C., Mandon-Pepin, B., Evans, N.P., Sharpe, R.M., 2012. Foetal and post-natal exposure of sheep to sewage sludge chemicals disrupts sperm production in adulthood in a subset of animals. Int. J. Androl. 35, 317–329. https://doi.org/10.1111/j.1365-2605.2011.01234.x

Berger, A., Ziv-Gal, A., Cudiamat, J., Wang, W., Zhou, C., Flaws, J.A., 2016. The effects of in utero bisphenol A exposure on the ovaries in multiple generations of mice. Reprod. Toxicol. 60, 39–52. https://doi.org/10.1016/j.reprotox.2015.12.004

Bertoldo, M.J., Faure, M., Dupont, J., Froment, P., 2015. AMPK: A master energy regulator for gonadal function. Front. Neurosci. 9, 1–11. https://doi.org/10.3389/fnins.2015.00235

Blaine, A.C., Rich, C.D., Hundal, L.S., Lau, C., Mills, M.A., Harris, K.M., Higgins, C.P., 2013. Uptake of perfluoroalkyl acids into edible crops via land applied biosolids: Field and greenhouse studies. Environ. Sci. Technol. 47, 14062–14069. https://doi.org/10.1021/es403094q

Blaine, A.C., Rich, C.D., Sedlacko, E.M., Hundal, L.S., Kumar, K., Lau, C., Mills, M.A., Harris, K.M., Higgins, C.P., 2014. Perfluoroalkyl acid distribution in various plant compartments of edible crops grown in biosolids-amended soils. Environ. Sci. Technol. 48, 7858–7865. https://doi.org/10.1021/es500016s

Bloise, E., Ortiga-Carvalho, T.M., Reis, F.M., Lye, S.J., Gibb, W., Matthews, S.G., 2016. ATP-binding cassette transporters in reproduction: A new frontier. Hum. Reprod. Update 22, 164–181. https://doi.org/10.1093/humupd/dmv049

Boas, M., Feldt-Rasmussen, U., Skakkebæk, N.E., Main, K.M., 2006. Environmental chemicals and thyroid function. Eur. J. Endocrinol. 154, 599–611. https://doi.org/10.1530/eje.1.02128

Boberg, J., Christiansen, S., Axelstad, M., Kledal, T.S., Vinggaard, A.M., Dalgaard, M., Nellemann, C., Hass, U., 2011. Reproductive and behavioral effects of diisononyl phthalate (DINP) in perinatally exposed rats. Reprod. Toxicol. 31, 200–209. https://doi.org/10.1016/j.reprotox.2010.11.001

Bolte, S., Cordelières, F.P., 2006. A guided tour into subcellular colocalization analysis in light microscopy. J. Microsc. 224, 213–232. https://doi.org/10.1111/j.1365-2818.2006.01706.x

Bonello, S., Zähringer, C., BelAiba, R.S., Djordjevic, T., Hess, J., Michiels, C., Kietzmann, T., Görlach, A., 2007. Reactive oxygen species activate the HIF-1α promoter via a functional NFκB site. Arterioscler. Thromb. Vasc. Biol. 27, 755–761. https://doi.org/10.1161/01.ATV.0000258979.92828.bc

Boobis, A., Budinsky, R., Collie, S., Crofton, K., Embry, M., Felter, S., Hertzberg, R., Kopp, D., Mihlan, G., Mumtaz, M., Price, P., Solomon, K., Teuschler, L., Yang, R., Zaleski, R., 2011. Critical analysis of literature on low-dose synergy for use in screening chemical mixtures for risk assessment. Crit. Rev. Toxicol. 41, 369–383.

https://doi.org/10.3109/10408444.2010.543655

Bopp, S.K., Barouki, R., Brack, W., Dalla Costa, S., Dorne, J.L.C.M., Drakvik, P.E., Faust, M., Karjalainen, T.K., Kephalopoulos, S., van Klaveren, J., Kolossa-Gehring, M., Kortenkamp, A., Lebret, E., Lettieri, T., Nørager, S., Rüegg, J., Tarazona, J. V., Trier, X., van de Water, B., van Gils, J., Bergman, Å., 2018. Current EU research activities on combined exposure to multiple chemicals. Environ. Int. 120, 544–562. https://doi.org/10.1016/j.envint.2018.07.037

Bopp, S.K., Kienzler, A., Richarz, A.N., van der Linden, S.C., Paini, A., Parissis, N., Worth, A.P., 2019. Regulatory assessment and risk management of chemical mixtures: challenges and ways forward. Crit. Rev. Toxicol. 49, 174–189. https://doi.org/10.1080/10408444.2019.1579169

Borch, J., Axelstad, M., Vinggaard, A.M., Dalgaard, M., 2006. Diisobutyl phthalate has comparable anti-androgenic effects to di-n-butyl phthalate in fetal rat testis. Toxicol. Lett. 163, 183–190. https://doi.org/10.1016/j.toxlet.2005.10.020

Boyer, A., Girard, M., Thimmanahalli, D.S., Levasseur, A., Céleste, C., Paquet, M., Duggavathi, R., Boerboom, D., 2016. mTOR regulates gap junction alpha-1 protein trafficking in sertoli cells and is required for the maintenance of spermatogenesis in mice. Biol. Reprod. 95, 13–13. https://doi.org/10.1095/biolreprod.115.138016

Braun, J.M., Sathyanarayana, S., Hauser, R., 2013. Phthalate exposure and children's health. Curr. Opin. Pediatr. 25, 247–254. https://doi.org/10.1097/MOP.0b013e32835e1eb6

Buha Djordjevic, A., Antonijevic, E., Curcic, M., Milovanovic, V., Antonijevic, B., 2020. Endocrine-disrupting mechanisms of polychlorinated biphenyls. Curr. Opin. Toxicol. 19, 42–49. https://doi.org/10.1016/j.cotox.2019.10.006

Buñay, J., Larriba, E., Patiño-Garcia, D., Cruz-Fernandes, L., Castañeda-Zegarra, S., Rodriguez-Fernandez, M., del Mazo, J., Moreno, R.D., 2018. Editor's Highlight: Differential Effects of Exposure to Single Versus a Mixture of Endocrine-Disrupting Chemicals on Steroidogenesis Pathway in Mouse Testes. Toxicol. Sci. 161, 76–86. https://doi.org/10.1093/toxsci/kfx200

Burroughs, C.D., Mills, K.T., Bern, H.A., 1990. Long-term genital tract changes in female mice treated neonatally with coumestrol. Reprod. Toxicol. 4, 127–135. https://doi.org/10.1016/0890-6238(90)90007-I

Campbell, D.M., Webb, J.A., Hargreave, T.B., 1987. Cryptorchidism in Scotland. Br. Med. J. (Clin. Res. Ed). 295, 1235–1236. https://doi.org/10.1136/bmj.295.6608.1235

Carlsen, E., Giwercman, A., Keiding, N., Skakkebaek, N.E., 1992. Evidence for decreasing quality of semen during past 50 years. Obstet. Gynecol. Surv. 48, 200–202. https://doi.org/10.1097/00006254-199303000-00023

Carpy, S.A., Kobel, W., Doe, J., 2000. Health Risk of Low-Dose Pesticides

Mixtures: A Review of the 1985-1998 Literature On Combination Toxicology and Health Risk Assessment. J. Toxicol. Environ. Heal. Part B 3, 1–25. https://doi.org/10.1080/109374000281122

Cartwright, R., Rogers, H., Barham-Hall, D., Glashan, R., Ahmad, R., Higgins, E., Kahn, M., 1982. Role of N-acetyltransferase Phenotypes in Bladder Carcinogenesis: A Pharmacogenetic Epidemiological Approach to Bladder Cancer. Lancet 320, 842–846. https://doi.org/10.1016/S0140-6736(82)90810-8

Cederroth, C.R., Zimmermann, C., Beny, J.-L., Schaad, O., Combepine, C., Descombes, P., Doerge, D.R., Pralong, F.P., Vassalli, J.-D., Nef, S., 2010. Potential detrimental effects of a phytoestrogen-rich diet on male fertility in mice. Mol. Cell. Endocrinol. 321, 152–160. https://doi.org/10.1016/j.mce.2010.02.011

Chan, C.P., Kok, K.H., Jin, D.Y., 2011. CREB3 subfamily transcription factors are not created equal: Recent insights from global analyses and animal models. Cell Biosci. 1, 14–16. https://doi.org/10.1186/2045-3701-1-6

Checa Vizcaíno, M.A., González-Comadran, M., Jacquemin, B., 2016. Outdoor air pollution and human infertility: a systematic review. Fertil. Steril. 106, 897-904.e1. https://doi.org/10.1016/j.fertnstert.2016.07.1110

Chen, H., Cheng, C.Y., 2016. Planar cell polarity (PCP) proteins and spermatogenesis. Semin. Cell Dev. Biol. 59, 99–109. https://doi.org/10.1016/j.semcdb.2016.04.010

Chen, Y., Liu, S., Xu, H., Zheng, H., Bai, C., Pan, W., Zhou, H., Liao, M., Huang, C., Dong, Q., 2019. Maternal exposure to low dose BDE209 and Pb mixture induced neurobehavioral anomalies in C57BL/6 male offspring. Toxicology 418, 70–80. https://doi.org/10.1016/j.tox.2019.02.016

Chia, V.M., Quraishi, S.M., Devesa, S.S., Purdue, M.P., Cook, M.B., McGlynn, K.A., 2010. International Trends in the Incidence of Testicular Cancer, 1973-2002. Cancer Epidemiol. Biomarkers Prev. 19, 1151–1159. https://doi.org/10.1158/1055-9965.EPI-10-0031

Child, F., Frost, J., Shakir, D., Wilson, J.W., Rocha, S., 2021. Transcription | Regulation of Gene Transcription by Hypoxia-Inducible Factor 1α, in: Encyclopedia of Biological Chemistry III. Elsevier, pp. 480–489. https://doi.org/10.1016/B978-0-12-819460-7.00033-5

Chilvers, C., Forman, D., Pike, M.C., Fogelman, K., Wadsworth, M.E.J., 1984. Apparent Doubling of Frequency of Undescended Testis in England and Wales in 1962-81. Lancet 324, 330–332. https://doi.org/10.1016/S0140-6736(84)92697-7

Christiansen, S., Kortenkamp, A., Axelstad, M., Boberg, J., Scholze, M., Jacobsen, P.R., Faust, M., Lichtensteiger, W., Schlumpf, M., Burdorf, A., Hass, U., 2012. Mixtures of endocrine disrupting contaminants modelled on human high end exposures: an exploratory study in rats. Int. J. Androl. 35, 303–316. https://doi.org/10.1111/j.1365-2605.2011.01242.x

Christiansen, S., Scholze, M., Dalgaard, M., Vinggaard, A.M., Axelstad, M., Kortenkamp, A., Hass, U., 2009. Synergistic disruption of external male sex organ development by a mixture of four antiandrogens. Environ. Health Perspect. 117, 1839–1846. https://doi.org/10.1289/ehp.0900689

Clarke, B.O., Smith, S.R., 2011. Review of "emerging" organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. Environ. Int. 37, 226–247. https://doi.org/10.1016/j.envint.2010.06.004

Coats, J.R., 1990. Mechanisms of toxic action and structure-activity relationships for organochlorine and synthetic pyrethroid insecticides. Environ. Health Perspect. 87, 255–262. https://doi.org/10.1289/ehp.9087255

Conley, J.M., Lambright, C.S., Evans, N., Cardon, M., Furr, J., Wilson, V.S., Gray, L.E., 2018. Mixed "antiandrogenic" chemicals at low individual doses produce reproductive tract malformations in the male rat. Toxicol. Sci. 164, 166–178. https://doi.org/10.1093/toxsci/kfy069

Correia, B., Sousa, M.I., Ramalho-Santos, J., 2020. The mTOR pathway in reproduction: From gonadal function to developmental coordination. Reproduction 159, R173–R188. https://doi.org/10.1530/REP-19-0057

Costet, N., Villanueva, C.M., Jaakkola, J.J.K., Kogevinas, M., Cantor, K.P., King, W.D., Lynch, C.F., Nieuwenhuijsen, M.J., Cordier, S., 2011. Water disinfection by-products and bladder cancer: Is there a European specificity? A pooled and meta-analysis of European caseecontrol studies. Occup. Environ. Med. 68, 379–385. https://doi.org/10.1136/oem.2010.062703

Cousins, I.T., Ng, C.A., Wang, Z., Scheringer, M., 2019. Why is high persistence alone a major cause of concern? Environ. Sci. Process. Impacts 21, 781–792. https://doi.org/10.1039/c8em00515j

Crean, A.J., Senior, A.M., 2019. High-fat diets reduce male reproductive success in animal models: A systematic review and meta-analysis. Obes. Rev. 20, 921–933. https://doi.org/10.1111/obr.12827

D'Cruz, S.C., Jubendradass, R., Jayakanthan, M., Rani, S.J.A., Mathur, P.P., 2012. Bisphenol A impairs insulin signaling and glucose homeostasis and decreases steroidogenesis in rat testis: An in vivo and in silico study. Food Chem. Toxicol. 50, 1124–1133. https://doi.org/10.1016/j.fct.2011.11.041

Davis, B.J., Maronpot, R.R., Heindel, J.J., 1994. Di-(2-ethylhexyl) Phthalate Suppresses Estradiol and Ovulation in Cycling Rats. Toxicol. Appl. Pharmacol. 128, 216–223. https://doi.org/10.1006/taap.1994.1200

DEFRA, 1989. The Sludge (Use in Agriculture) Regulations. UK.

Delclos, K.B., Camacho, L., Lewis, S.M., Vanlandingham, M.M., Latendresse, J.R., Olson, G.R., Davis, K.J., Patton, R.E., da Costa, G.G., Woodling, K.A., Bryant, M.S., Chidambaram, M., Trbojevich, R., Juliar, B.E., Felton, R.P., Thorn, B.T., 2014. Toxicity Evaluation of Bisphenol A Administered by Gavage to Sprague Dawley Rats From Gestation Day 6 Through Postnatal Day

90. Toxicol. Sci. 139, 174–197. https://doi.org/10.1093/toxsci/kfu022

Demur, C., Métais, B., Canlet, C., Tremblay-Franco, M., Gautier, R., Blas-Y-Estrada, F., Sommer, C., Gamet-Payrastre, L., 2013. Dietary exposure to a low dose of pesticides alone or as a mixture: The biological metabolic fingerprint and impact on hematopoiesis. Toxicology 308, 74–87. https://doi.org/10.1016/j.tox.2013.03.004

den Braver-Sewradj, S.P., van Spronsen, R., Hessel, E.V.S., 2020. Substitution of bisphenol A: a review of the carcinogenicity, reproductive toxicity, and endocrine disruption potential of alternative substances. Crit. Rev. Toxicol. 50, 128–147. https://doi.org/10.1080/10408444.2019.1701986

Di Nisio, A., Sabovic, I., Valente, U., Tescari, S., Rocca, M.S., Guidolin, D., Dall'Acqua, S., Acquasaliente, L., Pozzi, N., Plebani, M., Garolla, A., Foresta, C., 2019. Endocrine disruption of androgenic activity by perfluoroalkyl substances: Clinical and experimental evidence. J. Clin. Endocrinol. Metab. 104, 1259–1271. https://doi.org/10.1210/jc.2018-01855

Diamanti-Kandarakis, E., Bourguignon, J.P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T., Gore, A.C., 2009. Endocrine-disrupting chemicals: An Endocrine Society scientific statement. Endocr. Rev. 30, 293–342. https://doi.org/10.1210/er.2009-0002

Dixon, R.A., 2004. Phytoestrogens. Annu. Rev. Plant Biol. 55, 225–261. https://doi.org/10.1146/annurev.arplant.55.031903.141729

Docea, A.O., Gofita, E., Goumenou, M., Calina, D., Rogoveanu, O., Varut, M., Olaru, C., Kerasioti, E., Fountoucidou, P., Taitzoglou, I., Zlatian, O., Rakitskii, V.N., Hernandez, A.F., Kouretas, D., Tsatsakis, A., 2018. Six months exposure to a real life mixture of 13 chemicals' below individual NOAELs induced non monotonic sex-dependent biochemical and redox status changes in rats. Food Chem. Toxicol. 115, 470–481. https://doi.org/10.1016/j.fct.2018.03.052

Docea, A.O., Goumenou, M., Calina, D., Arsene, A.L., Dragoi, C.M., Gofita, E., Pisoschi, C.G., Zlatian, O., Stivaktakis, P.D., Nikolouzakis, T.K., Kalogeraki, A., Izotov, B.N., Galateanu, B., Hudita, A., Calabrese, E.J., Tsatsakis, A., 2019. Adverse and hormetic effects in rats exposed for 12 months to low dose mixture of 13 chemicals: RLRS part III. Toxicol. Lett. 310, 70–91. https://doi.org/10.1016/j.toxlet.2019.04.005

Dodd, K.M., Yang, J., Shen, M.H., Sampson, J.R., Tee, A.R., 2015. mTORC1 drives HIF-1α and VEGF-A signalling via multiple mechanisms involving 4E-BP1, S6K1 and STAT3. Oncogene 34, 2239–2250. https://doi.org/10.1038/onc.2014.164

Dodds, E.C., Lawson, W., 1936. Synthetic strogenic Agents without the Phenanthrene Nucleus. Nature 137, 996–996. https://doi.org/10.1038/137996a0

Don, J., Stelzer, G., 2002. The expanding family of CREB/CREM transcription factors that are involved with spermatogenesis. Mol. Cell. Endocrinol. 187,

115–124. https://doi.org/10.1016/S0303-7207(01)00696-7

Dorato, M.A., Engelhardt, J.A., 2005. The no-observed-adverse-effect-level in drug safety evaluations: Use, issues, and definition(s). Regul. Toxicol. Pharmacol. 42, 265–274. https://doi.org/10.1016/j.yrtph.2005.05.004

Drakvik, E., Altenburger, R., Aoki, Y., Backhaus, T., Bahadori, T., Barouki, R., Brack, W., Cronin, M.T.D., Demeneix, B., Hougaard Bennekou, S., van Klaveren, J., Kneuer, C., Kolossa-Gehring, M., Lebret, E., Posthuma, L., Reiber, L., Rider, C., Rüegg, J., Testa, G., van der Burg, B., van der Voet, H., Warhurst, A.M., van de Water, B., Yamazaki, K., Öberg, M., Bergman, Å., 2020. Statement on advancing the assessment of chemical mixtures and their risks for human health and the environment. Environ. Int. 134. https://doi.org/10.1016/j.envint.2019.105267

Du, L., Li, S., Qi, L., Hou, Y., Zeng, Y., Xu, W., Wang, H., Zhao, X., Sun, C., 2014. Metabonomic analysis of the joint toxic action of long-term low-level exposure to a mixture of four organophosphate pesticides in rat plasma. Mol. BioSyst. 10, 1153–1161. https://doi.org/10.1039/C4MB00044G

Duan, P., Hu, C., Quan, C., Yu, T., Huang, W., Chen, W., Tang, S., Shi, Y., Martin, F.L., Yang, K., 2017. 4-Nonylphenol induces autophagy and attenuates mTOR-p70S6K/4EBP1 signaling by modulating AMPK activation in Sertoli cells. Toxicol. Lett. 267, 21–31. https://doi.org/10.1016/j.toxlet.2016.12.015

Dunnick, J.K., Kissling, G., Gerken, D.K., Vallant, M.A., Nyska, A., 2007. Cardiotoxicity of Ma Huang/caffeine or ephedrine/caffeine in a Rodent model system. Toxicol. Pathol. 35, 657–664. https://doi.org/10.1080/01926230701459978

Düvel, K., Yecies, J.L., Menon, S., Raman, P., Lipovsky, A.I., Souza, A.L., Triantafellow, E., Ma, Q., Gorski, R., Cleaver, S., Vander Heiden, M.G., MacKeigan, J.P., Finan, P.M., Clish, C.B., Murphy, L.O., Manning, B.D., 2010. Activation of a metabolic gene regulatory network downstream of mTOR complex 1. Mol. Cell 39, 171–183. https://doi.org/10.1016/j.molcel.2010.06.022

ECETOC, 2012. Effects of Chemical Co-exposures at Doses Relevant for Human Safety Assessments. Eur. Cent. Ecotoxicol. Toxicol. Chem.

EFSA Scientific Committee, More, S.J., Hardy, A., Bampidis, V., Benford, D., Hougaard Bennekou, S., Bragard, C., Boesten, J., Halldorsson, T.I., Hernández-Jerez, A.F., Jeger, M.J., Knutsen, H.K., Koutsoumanis, K.P., Naegeli, H., Noteborn, H., Ockleford, C., Ricci, A., Rychen, G., Schlatter, J.R., Silano, V., Nielsen, S.S., Schrenk, D., Solecki, R., Turck, D., Younes, M., Benfenati, E., Castle, L., Cedergreen, N., Laskowski, R., Leblanc, J.C., Kortenkamp, A., Ragas, A., Posthuma, L., Svendsen, C., Testai, E., Dujardin, B., Kass, G.E.N., Manini, P., Zare Jeddi, M., Dorne, J.L.C., Hogstrand, C., 2019. Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. EFSA J. 17.

https://doi.org/10.2903/j.efsa.2019.5634

Elcombe, C.R., Wolf, C.R., Westwood, L.A., 2013. Compositions Comprising Perfluorooctanoic Acid. US 2013/0029928.

Elcombe, C.S., Evans, N.P., Bellingham, M., 2022a. Critical review and analysis of literature on low dose exposure to chemical mixtures in mammalian in vivo systems. Crit. Rev. Toxicol. 52, 221–238. https://doi.org/10.1080/10408444.2022.2091423

Elcombe, C.S., Monteiro, A., Elcombe, M.R., Ghasemzadeh-Hasankolaei, M., Sinclair, K.D., Lea, R., Padmanabhan, V., Evans, N.P., Bellingham, M., 2022b. Developmental exposure to real-life environmental chemical mixture programs a testicular dysgenesis syndrome-like phenotype in prepubertal lambs. Environ. Toxicol. Pharmacol. 94, 103913. https://doi.org/10.1016/j.etap.2022.103913

Elcombe, C.S., Monteiro, A., Ghasemzadeh-Hasankolaei, M., Evans, N.P., Bellingham, M., 2021. Morphological and transcriptomic alterations in neonatal lamb testes following developmental exposure to low-level environmental chemical mixture. Environ. Toxicol. Pharmacol. 86, 103670. https://doi.org/10.1016/j.etap.2021.103670

Endocrine Society, 2022. Common EDCs and Where They Are Found [WWW Document]. URL https://www.endocrine.org/topics/edc/what-edcs-are/common-edcs (accessed 6.21.22).

Erhard, H.W., Rhind, S.M., 2004. Prenatal and postnatal exposure to environmental pollutants in sewage sludge alters emotional reactivity and exploratory behaviour in sheep. Sci. Total Environ. 332, 101–108. https://doi.org/10.1016/j.scitotenv.2004.04.012

Eskenazi, B., Rauch, S.A., Tenerelli, R., Huen, K., Holland, N.T., Lustig, R.H., Kogut, K., Bradman, A., Sjödin, A., Harley, K.G., 2017. In utero and childhood DDT, DDE, PBDE and PCBs exposure and sex hormones in adolescent boys: The CHAMACOS study. Int. J. Hyg. Environ. Health 220, 364–372. https://doi.org/10.1016/j.ijheh.2016.11.001

EU, 1986. On the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. Off. J. Eur. Communities 29, 1–27.

European Commission, 2021. COMMISSION REGULATION (EU) 2021/1297 of 4 August 2021. Off. J. Eur. Union L282/29-L282/33.

European Commission, 2018. Commission Regulation 2018/2005 of 17 December 2018. Off. J. Eur. Union 6, L 322/14-L 322/19. https://doi.org/10.3000/17252555.L_2011.012.eng

Eustache, F., Mondon, F., Canivenc-Lavier, M.C., Lesaffre, C., Fulla, Y., Berges, R., Cravedi, J.P., Vaiman, D., Auger, J., 2009. Chronic Dietary Exposure to a Low-Dose Mixture of Genistein and Vinclozolin Modifies the Reproductive Axis, Testis Transcriptome, and Fertility. Environ. Health Perspect. 117,

1272–1279. https://doi.org/10.1289/ehp.0800158

Evans, N.P., Bellingham, M., Sharpe, R.M., Cotinot, C., Rhind, S.M., Kyle, C., Erhard, H., Hombach-Klonisch, S., Lind, P.M., Fowler, P.A., 2014. Reproduction Symposium: Does grazing on biosolids-treated pasture pose a Pathophysiological risk associated with increased exposure to endocrine disrupting compounds? J. Anim. Sci. 92, 3185–3198. https://doi.org/10.2527/jas.2014-7763

Evans, R.M., Scholze, M., Kortenkamp, A., 2015. Examining the feasibility of mixture risk assessment: A case study using a tiered approach with data of 67 pesticides from the Joint FAO/WHO Meeting on Pesticide Residues (JMPR). Food Chem. Toxicol. 84, 260–269. https://doi.org/10.1016/j.fct.2015.08.015

Farrow, S., 1994. Falling sperm quality: fact or fiction? BMJ 309, 1–2. https://doi.org/10.1136/bmj.309.6946.1

Fenton, S.E., Ducatman, A., Boobis, A., DeWitt, J.C., Lau, C., Ng, C., Smith, J.S., Roberts, S.M., 2021. Per- and Polyfluoroalkyl Substance Toxicity and Human Health Review: Current State of Knowledge and Strategies for Informing Future Research. Environ. Toxicol. Chem. 40, 606–630. https://doi.org/10.1002/etc.4890

Ferris, J., Mahboubi, K., MacLusky, N., King, W.A., Favetta, L.A., 2016. BPA exposure during in vitro oocyte maturation results in dose-dependent alterations to embryo development rates, apoptosis rate, sex ratio and gene expression. Reprod. Toxicol. 59, 128–138. https://doi.org/10.1016/j.reprotox.2015.12.002

Filis, P., Walker, N., Robertson, L., Eaton-Turner, E., Ramona, L., Bellingham, M., Amezaga, M.R., Zhang, Z., Mandon-Pepin, B., Evans, N.P., Sharpe, R.M., Cotinot, C., Rees, W.D., O'Shaughnessy, P., Fowler, P.A., 2019. Long-term exposure to chemicals in sewage sludge fertilizer alters liver lipid content in females and cancer marker expression in males. Environ. Int. 124, 98–108. https://doi.org/10.1016/j.envint.2019.01.003

Fountoucidou, P., Veskoukis, A.S., Kerasioti, E., Docea, A.O., Taitzoglou, I.A., Liesivuori, J., Tsatsakis, A., Kouretas, D., 2019. A mixture of routinely encountered xenobiotics induces both redox adaptations and perturbations in blood and tissues of rats after a long-term low-dose exposure regimen: The time and dose issue. Toxicol. Lett. 317, 24–44. https://doi.org/10.1016/j.toxlet.2019.09.015

Fowler, P.A., Dora, N.J., McFerran, H., Amezaga, M.R., Miller, D.W., Lea, R.G., Cash, P., McNeilly, A.S., Evans, N.P., Cotinot, C., Sharpe, R.M., Rhind, S.M., 2008. In utero exposure to low doses of environmental pollutants disrupts fetal ovarian development in sheep. Mol. Hum. Reprod. 14, 269–280. https://doi.org/10.1093/molehr/gan020

Fu, G., Dai, J., Li, Z., Chen, F., Liu, L., Yi, L., Teng, Z., Quan, C., Zhang, L., Zhou, T., Donkersley, P., Song, S., Shi, Y., 2020. The role of STAT3/p53 and

PI3K-Akt-mTOR signaling pathway on DEHP-induced reproductive toxicity in pubertal male rat. Toxicol. Appl. Pharmacol. 404, 115151. https://doi.org/10.1016/j.taap.2020.115151

Fu, H., Zhang, W., Yuan, Q., Niu, M., Zhou, F., Qiu, Q., Mao, G., Wang, H., Wen, L., Sun, M., Li, Z., He, Z., 2018. PAK1 Promotes the Proliferation and Inhibits Apoptosis of Human Spermatogonial Stem Cells via PDK1/KDR/ZNF367 and ERK1/2 and AKT Pathways. Mol. Ther. - Nucleic Acids 12, 769–786. https://doi.org/10.1016/j.omtn.2018.06.006

Gangolli, S.D., 1982. Testicular effects of phthalate esters. Environ. Health Perspect. 45, 77–84. https://doi.org/10.1289/ehp.824577

Gao, H.T., Xu, R., Cao, W.X., Qian, L.L., Wang, M., Lu, L., Xu, Q., Yu, S.Q., 2017. Effects of six priority controlled phthalate esters with long-term low-dose integrated exposure on male reproductive toxicity in rats. Food Chem. Toxicol. 101, 94–104. https://doi.org/10.1016/j.fct.2017.01.011

Ge, S.X., Son, E.W., Yao, R., 2018. iDEP: An integrated web application for differential expression and pathway analysis of RNA-Seq data. BMC Bioinformatics 19, 1–24. https://doi.org/10.1186/s12859-018-2486-6

Glüge, J., Scheringer, M., Cousins, I.T., DeWitt, J.C., Goldenman, G., Herzke, D., Lohmann, R., Ng, C.A., Trier, X., Wang, Z., 2020. An overview of the uses of per- and polyfluoroalkyl substances (PFAS). Environ. Sci. Process. Impacts 22, 2345–2373. https://doi.org/10.1039/D0EM00291G

Glunde, K., Jacobs, M.A., Bhujwalla, Z.M., 2006. Choline metabolism in cancer: Implications for diagnosis and therapy. Expert Rev. Mol. Diagn. 6, 821–829. https://doi.org/10.1586/14737159.6.6.821

Golden, R., Gandy, J., Vollmer, G., 2005. A Review of the Endocrine Activity of Parabens and Implications for Potential Risks to Human Health. Crit. Rev. Toxicol. 35, 435–458. https://doi.org/10.1080/10408440490920104

Grande, S.W., Andrade, A.J.M., Talsness, C.E., Grote, K., Chahoud, I., 2006. A Dose-Response Study Following In Utero and Lactational Exposure to Di(2-ethylhexyl)phthalate: Effects on Female Rat Reproductive Development. Toxicol. Sci. 91, 247–254. https://doi.org/10.1093/toxsci/kfj128

Gray, L.E., Laskey, J., Ostby, J., 2006. Chronic Di-n-butyl Phthalate Exposure in Rats Reduces Fertility and Alters Ovarian Function During Pregnancy in Female Long Evans Hooded Rats. Toxicol. Sci. 93, 189–195. https://doi.org/10.1093/toxsci/kfl035

Gray, L.E., Ostby, J., Ferrell, J., Rehnberg, G., Linder, R., Cooper, R., GOLDMAN, J., SLOTT, V., LASKEY, J., 1989. A Dose-Response Analysis of Methoxychlor-Induced Alterations of Reproductive Development and Function in the Rat. Toxicol. Sci. 12, 92–108. https://doi.org/10.1093/toxsci/12.1.92

Gray, T.J.B., Gangolli, S.D., 1986. Aspects of the testicular toxicity of phthalate esters. Environ. Health Perspect. 65, 229–235.

https://doi.org/10.1289/ehp.8665229

Grier, J.W., 1982. Ban of DDT and Subsequent Recovery of Reproduction in Bald Eagles. Science (80-.). 218, 1232–1235. https://doi.org/10.1126/science.7146905

Guillette, L.J., Crain, D.A., 1996. Endocrine Disrupting Contaminants Abnormalitie in Reptiles. Comments Toxicol. 5, 381–399.

Guillette, L.J., Crain, D.A., Rooney, A.A., Pickford, D.B., 1995. Organization versus activation: the role of endocrine-disrupting contaminants (EDCs) during embryonic development in wildlife. Environ. Health Perspect. 103, 157–164. https://doi.org/10.1289/ehp.95103s7157

Guillette, L.J., Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.F., Woodward, A.R., 1994. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ. Health Perspect. 102, 680–688. https://doi.org/10.1289/ehp.94102680

Hadrup, N., Svingen, T., Mandrup, K., Skov, K., Pedersen, M., Frederiksen, H., Frandsen, H.L., Vinggaard, A.M., 2016. Juvenile male rats exposed to a low-dose mixture of twenty-seven environmental chemicals display adverse health effects. PLoS One 11, 1–22. https://doi.org/10.1371/journal.pone.0162027

Hanke, J., Krajewska, B., 1990. Acetylation Phenotypes and Bladder Cancer. J. Occup. Med. 32, 917–918.

Harrison, P.T.C., Holmes, P., Humfrey, C.D.N., 1997. Reproductive health in humans and wildlife: Are adverse trends associated with environmental chemical exposure? Sci. Total Environ. 205, 97–106. https://doi.org/10.1016/S0048-9697(97)00212-X

Hass, U., Boberg, J., Christiansen, S., Jacobsen, P.R., Vinggaard, A.M., Taxvig, C., Poulsen, M.E., Herrmann, S.S., Jensen, B.H., Petersen, A., Clemmensen, L.H., Axelstad, M., 2012. Adverse effects on sexual development in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. Reprod. Toxicol. 34, 261–274. https://doi.org/10.1016/j.reprotox.2012.05.090

Hass, U., Christiansen, S., Axelstad, M., Scholze, M., Boberg, J., 2017. Combined exposure to low doses of pesticides causes decreased birth weights in rats. Reprod. Toxicol. 72, 97–105. https://doi.org/10.1016/j.reprotox.2017.05.004

Herbstman, J.B., Sjödin, A., Kurzon, M., Lederman, S.A., Jones, R.S., Rauh, V., Needham, L.L., Tang, D., Niedzwiecki, M., Wang, R.Y., Perera, F., 2010. Prenatal Exposure to PBDEs and Neurodevelopment. Environ. Health Perspect. 118, 712–719. https://doi.org/10.1289/ehp.0901340

Hernández, A.F., Docea, A.O., Goumenou, M., Sarigiannis, D., Aschner, M., Tsatsakis, A., 2020. Application of novel technologies and mechanistic data

for risk assessment under the real-life risk simulation (RLRS) approach. Food Chem. Toxicol. 137, 111123. https://doi.org/10.1016/j.fct.2020.111123

Hernández, A.F., Gil, F., Lacasaña, M., 2017. Toxicological interactions of pesticide mixtures: an update. Arch. Toxicol. 91, 3211–3223. https://doi.org/10.1007/s00204-017-2043-5

Heys, K.A., Shore, R.F., Pereira, M.G., Jones, K.C., Martin, F.L., 2016. Risk assessment of environmental mixture effects. RSC Adv. 6, 47844–47857. https://doi.org/10.1039/c6ra05406d

Hlisníková, H., Petrovičová, I., Kolena, B., Šidlovská, M., Sirotkin, A., 2021. Effects and mechanisms of phthalates' action on neurological processes and neural health: a literature review. Pharmacol. Reports 73, 386–404. https://doi.org/10.1007/s43440-021-00215-5

Hobbs, R.M., La, H.M., Mäkelä, J., Kobayashi, T., Noda, T., Pandolfi, P.P., 2015. Distinct germline progenitor subsets defined through Tsc2–<scp>mTORC</scp> 1 signaling. EMBO Rep. 16, 467–480. https://doi.org/10.15252/embr.201439379

Hofe, C.R., Feng, L., Zephyr, D., Stromberg, A.J., Hennig, B., Gaetke, L.M., 2014. Fruit and vegetable intake, as reflected by serum carotenoid concentrations, predicts reduced probability of polychlorinated biphenyl–associated risk for type 2 diabetes: National Health and Nutrition Examination Survey 2003-2004. Nutr. Res. 34, 285–293. https://doi.org/10.1016/j.nutres.2014.02.001

Hombach-Klonisch, S., Danescu, A., Begum, F., Amezaga, M.R., Rhind, S.M., Sharpe, R.M., Evans, N.P., Bellingham, M., Cotinot, C., Mandon-Pepin, B., Fowler, P.A., Klonisch, T., 2013. Peri-conceptional changes in maternal exposure to sewage sludge chemicals disturbs fetal thyroid gland development in sheep. Mol. Cell. Endocrinol. 367, 98–108. https://doi.org/10.1016/j.mce.2012.12.022

Howdeshell, K.L., Hotchkiss, A.K., Gray, L.E., 2017. Cumulative effects of antiandrogenic chemical mixtures and their relevance to human health risk assessment. Int. J. Hyg. Environ. Health 220, 179–188. https://doi.org/10.1016/j.ijheh.2016.11.007

Hu, G.X., Lian, Q.Q., Ge, R.S., Hardy, D.O., Li, X.K., 2009. Phthalate-induced testicular dysgenesis syndrome: Leydig cell influence. Trends Endocrinol. Metab. 20, 139–145. https://doi.org/10.1016/j.tem.2008.12.001

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57. https://doi.org/10.1038/nprot.2008.211

Hudson, C.C., Liu, M., Chiang, G.G., Otterness, D.M., Loomis, D.C., Kaper, F., Giaccia, A.J., Abraham, R.T., 2002. Regulation of Hypoxia-Inducible Factor 1α Expression and Function by the Mammalian Target of Rapamycin. Mol. Cell. Biol. 22, 7004–7014. https://doi.org/10.1128/MCB.22.20.7004-7014.2002

Ilacqua, A., Izzo, G., Emerenziani, G. Pietro, Baldari, C., Aversa, A., 2018. Lifestyle and fertility: The influence of stress and quality of life on male fertility. Reprod. Biol. Endocrinol. 16, 115. https://doi.org/10.1186/s12958-018-0436-9

Inoki, K., Kim, J., Guan, K.L., 2012. AMPK and mTOR in cellular energy homeostasis and drug targets. Annu. Rev. Pharmacol. Toxicol. 52, 381–400. https://doi.org/10.1146/annurev-pharmtox-010611-134537

Jacobsen, H., Østergaard, G., Lam, H.., Poulsen, M.., Frandsen, H., Ladefoged, O., Meyer, O., 2004. Repeated dose 28-day oral toxicity study in Wistar rats with a mixture of five pesticides often found as residues in food: alphacypermethrin, bromopropylate, carbendazim, chlorpyrifos and mancozeb. Food Chem. Toxicol. 42, 1269–1277. https://doi.org/10.1016/j.fct.2004.03.003

Jacobsen, P.R., Axelstad, M., Boberg, J., Isling, L.K., Christiansen, S., Mandrup, K.R., Berthelsen, L.O., Vinggaard, A.M., Hass, U., 2012. Persistent developmental toxicity in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. Reprod. Toxicol. 34, 237–250. https://doi.org/10.1016/j.reprotox.2012.05.099

Jacobsen, P.R., Christiansen, S., Boberg, J., Nellemann, C., Hass, U., 2010. Combined exposure to endocrine disrupting pesticides impairs parturition, causes pup mortality and affects sexual differentiation in rats. Int. J. Androl. 33, 434–442. https://doi.org/10.1111/j.1365-2605.2009.01046.x

Jayaraj, R., Megha, P., Sreedev, P., 2016. Organochlorine pesticides, their toxic effects on living organisms and their fate in the environment. Interdiscip. Toxicol. 9, 90–100. https://doi.org/10.1515/intox-2016-0012

Jefferson, W., Newbold, R., Padilla-Banks, E., Pepling, M., 2006. Neonatal Genistein Treatment Alters Ovarian Differentiation in the Mouse: Inhibition of Oocyte Nest Breakdown and Increased Oocyte Survival. Biol. Reprod. 74, 161–168. https://doi.org/10.1095/biolreprod.105.045724

Jefferson, W.N., Padilla-Banks, E., Newbold, R.R., 2005. Adverse Effects on Female Development and Reproduction in CD-1 Mice Following Neonatal Exposure to the Phytoestrogen Genistein at Environmentally Relevant Doses. Biol. Reprod. 73, 798–806. https://doi.org/10.1095/biolreprod.105.041277

Jensen, T.K., Sobotka, T., Hansen, M.A., Pedersen, A.T., Lutz, W., Skakkebæk, N.E., 2008. Declining trends in conception rates in recent birth cohorts of native Danish women: A possible role of deteriorating male reproductive health. Int. J. Androl. 31, 81–92. https://doi.org/10.1111/j.1365-2605.2007.00827.x

Jesus, T.T., Oliveira, P.F., Sousa, M., Cheng, C.Y., Alves, M.G., 2017. Mammalian target of rapamycin (mTOR): a central regulator of male fertility? Crit. Rev. Biochem. Mol. Biol. 52, 235–253. https://doi.org/10.1080/10409238.2017.1279120

Jirsová, S., Mašata, J., Jech, L., Zvárová, J., 2010. Effect of polychlorinated

biphenyls (PCBs) and 1,1,1-trichloro-2,2,-bis (4-chlorophenyl)-ethane (DDT) in follicular fluid on the results of in vitro fertilization–embryo transfer (IVF-ET) programs. Fertil. Steril. 93, 1831–1836. https://doi.org/10.1016/j.fertnstert.2008.12.063

Joensen, U.N., Jørgensen, N., Meyts, E.R. De, Skakkebæk, N.E., 2008. Testicular dysgenesis syndrome and Leydig cell function. Basic Clin. Pharmacol. Toxicol. 102, 155–161. https://doi.org/10.1111/j.1742-7843.2007.00197.x

Jones, H.B., Garside, D.A., Liu, R., Roberts, J.C., 1993. The Influence of Phthalate Esters on Leydig Cell Structure and Function in Vitro and in Vivo. Exp. Mol. Pathol. 58, 179–193. https://doi.org/10.1006/exmp.1993.1016

Jørgensen, N., Meyts, E.R.-D., Main, K.M., Skakkebaek, N.E., 2010. Testicular dysgenesis syndrome comprises some but not all cases of hypospadias and impaired spermatogenesis. Int. J. Androl. 33, 298–303. https://doi.org/10.1111/j.1365-2605.2009.01050.x

Jouannet, P., Wang, C., Eustache, F., Kold-Jensen, T., Auger, J., 2001. Semen quality and male reproductive health: The controversy about human sperm concentration decline. Apmis 109, 333–344. https://doi.org/10.1034/j.1600-0463.2001.090502.x

Julien, B., Pinteur, C., Vega, N., Labaronne, E., Vidal, H., Naville, D., Le Magueresse-Battistoni, B., 2018. Evidence for estrogeno-mimetic effects of a mixture of low-dose pollutants in a model of ovariectomized mice. Environ. Toxicol. Pharmacol. 57, 34–40. https://doi.org/10.1016/j.etap.2017.11.008

Julien, B., Pinteur, C., Vega, N., Vidal, H., Naville, D., Le Magueresse-Battistoni, B., 2019. Estrogen withdrawal and replacement differentially target liver and adipose tissues in female mice fed a high-fat high-sucrose diet: impact of a chronic exposure to a low-dose pollutant mixture☆. J. Nutr. Biochem. 72, 108211. https://doi.org/10.1016/j.jnutbio.2019.07.002

Jurewicz, J., Hanke, W., 2011. Exposure to phthalates: Reproductive outcome and children health. A review of epidemiological studies. Int. J. Occup. Med. Environ. Health 24, 115–141. https://doi.org/10.2478/s13382-011-0022-2

Kaczmarek, I., Groetzner, J., Adamidis, I., Landwehr, P., Mueller, M., Vogeser, M., Gerstorfer, M., Uberfuhr, P., Meiser, B., Reichart, B., 2004. Sirolimus impairs gonadal function in heart transplant recipients. Am. J. Transplant. 4, 1084–1088. https://doi.org/10.1111/j.1600-6143.2004.00456.x

Kamrin, M.A., 2009. Phthalate Risks, Phthalate Regulation, and Public Health: A Review. J. Toxicol. Environ. Heal. Part B 12, 157–174. https://doi.org/10.1080/10937400902729226

Kanatsu-Shinohara, M., Toyokuni, S., Shinohara, T., 2004. CD9 Is a Surface Marker on Mouse and Rat Male Germline Stem Cell. Biol. Reprod. 70, 70–75. https://doi.org/10.1095/biolreprod.103.020867

Kanzaki, M., Fujisawa, M., Okuda, Y., Okada, H., Arakawa, S., Kamidono, S., 1996. Expression and regulation of neuropeptide Y messenger ribonucleic acid in cultured immature rat Leydig and Sertoli cells. Endocrinology 137, 1249–1257. https://doi.org/10.1210/endo.137.4.8625896

Kassotis, C.D., Vandenberg, L.N., Demeneix, B.A., Porta, M., Slama, R., Trasande, L., 2020. Endocrine-disrupting chemicals: economic, regulatory, and policy implications. Lancet Diabetes Endocrinol. 8, 719–730. https://doi.org/10.1016/S2213-8587(20)30128-5

Keenan, A.B., Torre, D., Lachmann, A., Leong, A.K., Wojciechowicz, M.L., Utti, V., Jagodnik, K.M., Kropiwnicki, E., Wang, Z., Ma'ayan, A., 2019. ChEA3: transcription factor enrichment analysis by orthogonal omics integration. Nucleic Acids Res. 47, W212–W224. https://doi.org/10.1093/nar/gkz446

Kopp, J., Collin, O., Villar, M., Mullins, D., Bergh, A., Hökfelt, T., 2008. Regulation of neuropeptide Y Y1 receptors by testosterone in vascular smooth muscle cells in rat testis. Neuroendocrinology 88, 216–226. https://doi.org/10.1159/000138250

Kortenkamp, A., 2020. Which chemicals should be grouped together for mixture risk assessments of male reproductive disorders? Mol. Cell. Endocrinol. 499, 110581. https://doi.org/10.1016/j.mce.2019.110581

Kortenkamp, A., 2014. Low dose mixture effects of endocrine disrupters and their implications for regulatory thresholds in chemical risk assessment. Curr. Opin. Pharmacol. 19, 105–111. https://doi.org/10.1016/j.coph.2014.08.006

Kortenkamp, A., 2008. Low dose mixture effects of endocrine disrupters: Implications for risk assessment and epidemiology. Int. J. Androl. 31, 233–240. https://doi.org/10.1111/j.1365-2605.2007.00862.x

Kortenkamp, A., Backhaus, T., Faust, M., 2009. State of the Art Report on Mixture Toxicity - Final Report. ECHA 070307/2007/485103/ETU/D.1.

Kouidhi, W., Desmetz, C., Nahdi, A., Bergès, R., Cravedi, J.-P., Auger, J., May, M. El, Canivenc-Lavier, M.C., 2012. In Utero and Lactational Exposure to Low-Dose Genistein-Vinclozolin Mixture Affects the Development and Growth Factor mRNA Expression of the Submandibular Salivary Gland in Immature Female Rats. Toxicol. Pathol. 40, 593–604. https://doi.org/10.1177/0192623311436183

Kouki, T., Kishitake, M., Okamoto, M., Oosuka, I., Takebe, M., Yamanouchi, K., 2003. Effects of neonatal treatment with phytoestrogens, genistein and daidzein, on sex difference in female rat brain function: estrous cycle and lordosis. Horm. Behav. 44, 140–145. https://doi.org/10.1016/S0018-506X(03)00122-3

Krausz, C., Riera-Escamilla, A., Moreno-Mendoza, D., Holleman, K., Cioppi, F., Algaba, F., Pybus, M., Friedrich, C., Wyrwoll, M.J., Casamonti, E., Pietroforte, S., Nagirnaja, L., Lopes, A.M., Kliesch, S., Pilatz, A., Carrell, D.T., Conrad, D.F., Ars, E., Ruiz-Castañé, E., Aston, K.I., Baarends, W.M.,

Tüttelmann, F., 2020. Genetic dissection of spermatogenic arrest through exome analysis: clinical implications for the management of azoospermic men. Genet. Med. 22, 1956–1966. https://doi.org/10.1038/s41436-020-0907-1

Kumar, P., Kumar, N., Thakur, D.S., Patidar, A., 2010. Male hypogonadism: Symptoms and treatment. J. Adv. Pharm. Technol. Res. 1, 297–301. https://doi.org/10.4103/0110-5558.72420

Kumar, S., Kang, H., Park, E., Park, H.-S., Lee, K., 2018. The expression of CKLFSF2B is regulated by GATA1 and CREB in the Leydig cells, which modulates testicular steroidogenesis. Biochim. Biophys. Acta - Gene Regul. Mech. 1861, 1063–1075. https://doi.org/10.1016/j.bbagrm.2018.10.002

Kupershmidt, I., Su, Q.J., Grewal, A., Sundaresh, S., Halperin, I., Flynn, J., Shekar, M., Wang, H., Park, J., Cui, W., Wall, G.D., Wisotzkey, R., Alag, S., Akhtari, S., Ronaghi, M., 2010. Ontology-based meta-analysis of global collections of high-throughput public data. PLoS One 5. https://doi.org/10.1371/journal.pone.0013066

Kuwatsuka, Y., Shimizu, K., Akiyama, Y., Koike, Y., Ogawa, F., Furue, M., Utani, A., 2014. Yusho patients show increased serum IL-17, IL-23, IL-1ß, and TNFα levels more than 40 years after accidental polychlorinated biphenyl poisoning. J. Immunotoxicol. 11, 246–249. https://doi.org/10.3109/1547691X.2013.835890

Labaronne, E., Pinteur, C., Vega, N., Pesenti, S., Julien, B., Meugnier-Fouilloux, E., Vidal, H., Naville, D., Le Magueresse-Battistoni, B., 2017. Low-dose pollutant mixture triggers metabolic disturbances in female mice leading to common and specific features as compared to a high-fat diet. J. Nutr. Biochem. 45, 83–93. https://doi.org/10.1016/j.jnutbio.2017.04.001

Lambrot, R., Muczynski, V., Lécureuil, C., Angenard, G., Coffigny, H., Pairault, C., Moison, D., Frydman, R., Habert, R., Rouiller-Fabre, V., 2009. Phthalates impair germ cell development in the human Fetal testis in vitro without change in testosterone production. Environ. Health Perspect. 117, 32–37. https://doi.org/10.1289/ehp.11146

Laplante, M., Sabatini, D.M., 2013. Regulation of mTORC1 and its impact on gene expression at a glance. J. Cell Sci. 126, 1713–1719. https://doi.org/10.1242/jcs.125773

Lea, R.G., Amezaga, M.R., Loup, B., Mandon-Pépin, B., Stefansdottir, A., Filis, P., Kyle, C., Zhang, Z., Allen, C., Purdie, L., Jouneau, L., Cotinot, C., Rhind, S.M., Sinclair, K.D., Fowler, P.A., 2016. The fetal ovary exhibits temporal sensitivity to a 'real-life' mixture of environmental chemicals. Sci. Rep. 6, 22279. https://doi.org/10.1038/srep22279

Lea, R.G., Mandon-Pépin, B., Loup, B., Poumerol, E., Jouneau, L., Egbowon, B.F., Bowden, A., Cotinot, C., Purdie, L., Zhang, Z., Fowler, P.A., Sinclair, K.D., 2022. Ovine fetal testis stage-specific sensitivity to environmental chemical mixtures. Reproduction 163, 119–131.

https://doi.org/10.1530/REP-21-0235

Lehraiki, A., Messiaen, S., Berges, R., Canivenc-Lavier, M.-C., Auger, J., Habert, R., Levacher, C., 2011. Antagonistic effects of gestational dietary exposure to low-dose vinclozolin and genistein on rat fetal germ cell development. Reprod. Toxicol. 31, 424–430. https://doi.org/10.1016/j.reprotox.2010.12.005

Levine, H., Jørgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Jolles, M., Pinotti, R., Swan, S.H., 2022. Temporal trends in sperm count: a systematic review and meta-regression analysis of samples collected globally in the 20th and 21st centuries. Hum. Reprod. Update 23, 646–659. https://doi.org/10.1093/humupd/dmac035

Levine, H., Jørgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Mindlis, I., Pinotti, R., Swan, S.H., 2017. Temporal trends in sperm count: a systematic review and meta-regression analysis. Hum. Reprod. Update 23, 646–659. https://doi.org/10.1093/humupd/dmx022

Li, X.-F., Mitch, W.A., 2018. Drinking Water Disinfection Byproducts (DBPs) and Human Health Effects: Multidisciplinary Challenges and Opportunities. Environ. Sci. Technol. 52, 1681–1689. https://doi.org/10.1021/acs.est.7b05440

Li, Y., Andersson, A., Xu, Y., Pineda, D., Nilsson, C.A., Lindh, C.H., Jakobsson, K., Fletcher, T., 2022. Determinants of serum half-lives for linear and branched perfluoroalkyl substances after long-term high exposure—A study in Ronneby, Sweden. Environ. Int. 163, 107198. https://doi.org/10.1016/j.envint.2022.107198

Lind, P.M., Gustafsson, M., Hermsen, S.A.B., Larsson, S., Kyle, C.E., Örberg, J., Rhind, S.M., 2009. Exposure to pastures fertilised with sewage sludge disrupts bone tissue homeostasis in sheep. Sci. Total Environ. 407, 2200–2208. https://doi.org/10.1016/j.scitotenv.2008.12.035

Liu, J., Goyer, R.A., Waalkes, M.P., 2008. Toxic Effects of Metals, in: Klaassen, C.D. (Ed.), Casarett & Doull's Toxicology: The Basic Science of Poisons. McGraw-Hill, pp. 931–979.

Liu, J., Tan, Y., Song, E., Song, Y., 2020. A Critical Review of Polychlorinated Biphenyls Metabolism, Metabolites, and Their Correlation with Oxidative Stress. Chem. Res. Toxicol. 33, 2022–2042. https://doi.org/10.1021/acs.chemrestox.0c00078

Lukowicz, C., Ellero-Simatos, S., Régnier, M., Polizzi, A., Lasserre, F., Montagner, A., Lippi, Y., Jamin, E.L., Martin, J.-F., Naylies, C., Canlet, C., Debrauwer, L., Bertrand-Michel, J., Al Saati, T., Théodorou, V., Loiseau, N., Mselli-Lakhal, L., Guillou, H., Gamet-Payrastre, L., 2018. Metabolic Effects of a Chronic Dietary Exposure to a Low-Dose Pesticide Cocktail in Mice: Sexual Dimorphism and Role of the Constitutive Androstane Receptor. Environ. Health Perspect. 126, 067007. https://doi.org/10.1289/EHP2877

Luo, J., Megee, S., Rathi, R., Dobrinski, I., 2006. Protein gene product 9.5 is a

spermatogonia-specific marker in the pig testis: Application to enrichment and culture of porcine spermatogonia. Mol. Reprod. Dev. 73, 1531–1540. https://doi.org/10.1002/mrd.20529

MacLeod, J., Gold, R.Z., 1951. The Male Factor in Fertility and Infertility. II. Spermatozoön Counts in 1000 Men of Known Fertility and in 1000 Cases of Infertile Marriage. J. Urol. 66, 436–449. https://doi.org/10.1016/S0022-5347(17)74358-3

Macleod, J., Wang, Y., 1979. Male Fertility Potential in Terms of Semen Quality: A Review of the Past a Study of the Present. Fertil. Steril. 31, 103–116. https://doi.org/10.1016/S0015-0282(16)43808-2

Manimaran, S., Selby, H.M., Okrah, K., Ruberman, C., Leek, J.T., Quackenbush, J., Haibe-Kains, B., Bravo, H.C., Johnson, W.E., 2016. BatchQC: interactive software for evaluating sample and batch effects in genomic data. Bioinformatics 32, 3836–3838. https://doi.org/10.1093/bioinformatics/btw538

Manna, P.R., Stetson, C.L., Slominski, A.T., Pruitt, K., 2016. Role of the steroidogenic acute regulatory protein in health and disease. Endocrine 51, 7–21. https://doi.org/10.1007/s12020-015-0715-6

Marcus, P.M., Vineis, P., Rothman, N., 2000. NAT2 slow acetylation and bladder cancer risk: a meta-analysis of 22 case???control studies conducted in the general population. Pharmacogenetics 10, 115–122. https://doi.org/10.1097/00008571-200003000-00003

Martin, O., Scholze, M., Ermler, S., McPhie, J., Bopp, S.K., Kienzler, A., Parissis, N., Kortenkamp, A., 2021. Ten years of research on synergisms and antagonisms in chemical mixtures: A systematic review and quantitative reappraisal of mixture studies. Environ. Int. 146, 106206. https://doi.org/10.1016/j.envint.2020.106206

Matlai, P., Beral, V., 1985. Trends in Congenital Malformations of External Genitalia. Lancet 325, 108. https://doi.org/10.1016/S0140-6736(85)91999-3

Matwiejczuk, N., Galicka, A., Brzóska, M.M., 2020. Review of the safety of application of cosmetic products containing parabens. J. Appl. Toxicol. 40, 176–210. https://doi.org/10.1002/jat.3917

Meeker, J.D., Johnson, P.I., Camann, D., Hauser, R., 2009. Science of the Total Environment Polybrominated diphenyl ether (PBDE) concentrations in house dust are related to hormone levels in men ☆. Sci. Total Environ. 407, 3425–3429. https://doi.org/10.1016/j.scitotenv.2009.01.030

Merhi, M., Demur, C., Racaud-Sultan, C., Bertrand, J., Canlet, C., Estrada, F.B.Y., Gamet-Payrastre, L., 2010. Gender-linked haematopoietic and metabolic disturbances induced by a pesticide mixture administered at low dose to mice. Toxicology 267, 80–90. https://doi.org/10.1016/j.tox.2009.10.024

Merzenich, H., Zeeb, H., Blettner, M., 2010. Decreasing sperm quality: A global

problem? BMC Public Health 10, 24. https://doi.org/10.1186/1471-2458-10-24

Mihaylova, M.M., Shaw, R.J., 2011. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. Nat. Cell Biol. 13, 1016–1023. https://doi.org/10.1038/ncb2329

Miyamoto, T., Bando, Y., Koh, E., Tsujimura, A., Miyagawa, Y., Iijima, M., Namiki, M., Shiina, M., Ogata, K., Matsumoto, N., Sengoku, K., 2016. A PLK4 mutation causing azoospermia in a man with Sertoli cell-only syndrome. Andrology 4, 75–81. https://doi.org/10.1111/andr.12113

Møller, H., 1998. Trends in sex-ratio, testicular cancer and male reproductive hazards: Are they connected? APMIS 106, 232–239. https://doi.org/10.1111/j.1699-0463.1998.tb01341.x

Moore-Ambriz, T.R., Acuña-Hernández, D.G., Ramos-Robles, B., Sánchez-Gutiérrez, M., Santacruz-Márquez, R., Sierra-Santoyo, A., Piña-Guzmán, B., Shibayama, M., Hernández-Ochoa, I., 2015. Exposure to bisphenol A in young adult mice does not alter ovulation but does alter the fertilization ability of oocytes. Toxicol. Appl. Pharmacol. 289, 507–514. https://doi.org/10.1016/j.taap.2015.10.010

Moreira, B.P., Oliveira, P.F., Alves, M.G., 2019. Molecular mechanisms controlled by mTOR in male reproductive system. Int. J. Mol. Sci. 20, 1633. https://doi.org/10.3390/ijms20071633

Morris, C.M., Savy, C., Judge, S.J., Blain, P.G., 2014. Acute Toxicity of Organophosphorus Compounds, in: Basic and Clinical Toxicology of Organophosphorus Compounds. Springer London, London, pp. 45–78. https://doi.org/10.1007/978-1-4471-5625-3_3

Mossa, A.-T.H., Refaie, A.A., Ramadan, A., 2011. Effect of Exposure to Mixture of Four Organophosphate Insecticides at No Observed Adverse Effect Level Dose on Rat Liver: The Protective Role of Vitamin C. Res. J. Environ. Toxicol. 5, 323–335. https://doi.org/10.3923/rjet.2011.323.335

Mruk, D.D., Cheng, C.Y., 2015. The Mammalian Blood-Testis Barrier: Its Biology and Regulation. Endocr. Rev. 36, 564–591. https://doi.org/10.1210/er.2014-1101

Narotsky, M.G., Best, D.S., Rogers, E.H., McDonald, A., Sey, Y.M., Simmons, J.E., 2008. Integrated disinfection by-products mixtures research: assessment of developmental toxicity in Sprague-Dawley rats exposed to concentrates of water disinfected by chlorination and ozonation/postchlorination. J. Toxicol. Environ. Health. A 71, 1216–21. https://doi.org/10.1080/15287390802182623

Narotsky, M.G., Klinefelter, G.R., Goldman, J.M., Best, D.S., McDonald, A., Strader, L.F., Suarez, J.D., Murr, A.S., Thillainadarajah, I., Hunter, E.S., Richardson, S.D., Speth, T.F., Miltner, R.J., Pressman, J.G., Teuschler, L.K., Rice, G.E., Moser, V.C., Luebke, R.W., Simmons, J.E., 2013. Comprehensive assessment of a chlorinated drinking water concentrate in a

rat multigenerational reproductive toxicity study. Environ. Sci. Technol. 47, 10653–10659. https://doi.org/10.1021/es402646c

Narotsky, M.G., Klinefelter, G.R., Goldman, J.M., DeAngelo, A.B., Best, D.S., McDonald, A., Strader, L.F., Murr, A.S., Suarez, J.D., George, M.H., Hunter, E.S., Simmons, J.E., 2015. Reproductive toxicity of a mixture of regulated drinking-water disinfection by-products in a multigenerational rat bioassay. Environ. Health Perspect. 123, 564–570. https://doi.org/10.1289/ehp.1408579

Narotsky, M.G., Pressman, J.G., Miltner, R.J., Speth, T.F., Teuschler, L.K., Rice, G.E., Richardson, S.D., Best, D.S., McDonald, A., Hunter, E.S., Simmons, J.E., 2012. Developmental Toxicity Evaluations of Whole Mixtures of Disinfection By-products using Concentrated Drinking Water in Rats: Gestational and Lactational Effects of Sulfate and Sodium. Birth Defects Res. Part B - Dev. Reprod. Toxicol. 95, 202–212. https://doi.org/10.1002/bdrb.21004

NAS, 2017a. Application of Systematic Review Methods in an Overall Strategy for Evaluating Low-Dose Toxicity from Endocrine Active Chemicals. National Academies Press, Washington, D.C. https://doi.org/10.17226/24758

NAS, 2017b. Using 21st Century Science to Improve Risk-Related Evaluations, Using 21st Century Science to Improve Risk-Related Evaluations. National Academies Press, Washington, D.C. https://doi.org/10.17226/24635

Naville, D., Gaillard, G., Julien, B., Vega, N., Pinteur, C., Chanon, S., Vidal, H., Le Magueresse-Battistoni, B., 2019. Chronic exposure to a pollutant mixture at low doses led to tissue-specific metabolic alterations in male mice fed standard and high-fat high-sucrose diet. Chemosphere 220, 1187–1199. https://doi.org/10.1016/j.chemosphere.2018.12.177

Naville, D., Labaronne, E., Vega, N., Pinteur, C., Canet-Soulas, E., Vidal, H., Le Magueresse-Battistoni, B., 2015. Metabolic Outcome of Female Mice Exposed to a Mixture of Low-Dose Pollutants in a Diet-Induced Obesity Model. PLoS One 10, e0124015. https://doi.org/10.1371/journal.pone.0124015

Naville, D., Pinteur, C., Vega, N., Menade, Y., Vigier, M., Le Bourdais, A., Labaronne, E., Debard, C., Luquain-Costaz, C., Bégeot, M., Vidal, H., Le Magueresse-Battistoni, B., 2013. Low-dose food contaminants trigger sex-specific, hepatic metabolic changes in the progeny of obese mice. FASEB J. 27, 3860–3870. https://doi.org/10.1096/fj.13-231670

Nelson, C.M.K., Bunge, R.G., 1974. Semen analysis: evidence for changing parameters of male fertility potential. Fertil. Steril. 25, 503–507. https://doi.org/10.1016/S0015-0282(16)40454-1

Newbold, R.R., Padilla Banks, E., Bullock, B., Jefferson, W.N., 2001. Uterine adenocarcinoma in mice treated neonatally with genistein. Cancer Res. 61, 4325–4328.

Nguyen, T.M.D., 2019. Role of AMPK in mammals reproduction: Specific controls and whole-body energy sensing. Comptes Rendus - Biol. 342, 1–6.

https://doi.org/10.1016/j.crvi.2018.10.003

Ni, F. Da, Hao, S.L., Yang, W.X., 2019. Multiple signaling pathways in Sertoli cells: recent findings in spermatogenesis. Cell Death Dis. 10. https://doi.org/10.1038/s41419-019-1782-z

Nistal, M., González-Peramato, P., Serrano, Á., 2017. Testicular Dysgenesis Syndrome (TDS), in: Clues in the Diagnosis of Non-Tumoral Testicular Pathology. Springer International Publishing, Cham, pp. 101–109. https://doi.org/10.1007/978-3-319-49364-0_13

O'Shaughnessy, P.J., Fowler, P.A., 2011. Endocrinology of the mammalian fetal testis. REPRODUCTION 141, 37–46. https://doi.org/10.1530/REP-10-0365

OECD, 2018. Considerations for Assessing the Risks of Combined Exposure to Multiple Chemicals, Series on Testing and Assessment No. 296. Environ. Heal. Saf. Div. Environ. Dir.

OECD, 2009. Endocrine disruption: Guidance for histopathologic evaluation. Part 2 - male reproductive system. OECD Environ. Heal. Saf. Publ. 27–56.

Ohoro, C.R., 2021. Polybrominated diphenyl ethers in the environmental systems : a review.

Ortega, M.E.M., Romero, D.M., Pato, A.M., Sosa-Holt, C.S., Ridolfi, A., Villaamil Lepori, E., Wolansky, M.J., 2018. Relationship between exposure, body burden and target tissue concentration after oral administration of a low-dose mixture of pyrethroid insecticides in young adult rats. Toxicology 409, 53–62. https://doi.org/10.1016/j.tox.2018.07.006

Paasch, U., Salzbrunn, A., Glander, H.J., Plambeck, K., Salzbrunn, H., Grunewald, S., Stucke, J., Skakkebæk, N.E., Jørgensen, N., 2008. Semen quality in sub-fertile range for a significant proportion of young men from the general German population: A co-ordinated, controlled study of 791 men from Hamburg and Leipzig. Int. J. Androl. 31, 93–102. https://doi.org/10.1111/j.1365-2605.2007.00860.x

Palladino, M.A., Pirlamarla, P.R., Mcnamara, J., Sottas, C.M., Korah, N., Hardy, M.P., Hales, D.B., Hermo, L., 2011. Normoxic expression of hypoxia-inducible factor 1 in rat Leydig cells in vivo and in vitro. J. Androl. 32, 307–323. https://doi.org/10.2164/jandrol.110.011494

Park, H.J., Lee, W.Y., Park, C., Hong, K., Song, H., 2019. CD14 is a unique membrane marker of porcine spermatogonial stem cells, regulating their differentiation. Sci. Rep. 9, 1–11. https://doi.org/10.1038/s41598-019-46000-6

Pascotto, V.M., Guerra, M.T., Franci, J.A.A., de Camargo, J.L. V., Kempinas, W.G., Franchi, C.A.S., 2015. Effects of a Mixture of Pesticides on the Adult Female Reproductive System of Sprague-Dawley, Wistar, and Lewis Rats. J. Toxicol. Environ. Heal. Part A 78, 602–616. https://doi.org/10.1080/15287394.2015.1010467

Patel, A.B., Shaikh, S., Jain, K.R., Desai, C., Madamwar, D., 2020. Polycyclic Aromatic Hydrocarbons: Sources, Toxicity, and Remediation Approaches. Front. Microbiol. 11. https://doi.org/10.3389/fmicb.2020.562813

Patel, S., Peretz, J., Pan, Y.-X., Helferich, W.G., Flaws, J.A., 2016. Genistein exposure inhibits growth and alters steroidogenesis in adult mouse antral follicles. Toxicol. Appl. Pharmacol. 293, 53–62. https://doi.org/10.1016/j.taap.2015.12.026

Paul, C., Rhind, S.M., Kyle, C.E., Scott, H., McKinnell, C., Sharpe, R.M., 2005. Cellular and hormonal disruption of fetal testis development in sheep reared on pasture treated with sewage sludge. Environ. Health Perspect. 113, 1580–1587. https://doi.org/10.1289/ehp.8028

Paul, C., Robaire, B., 2013. Impaired Function of the Blood-Testis Barrier during Aging Is Preceded by a Decline in Cell Adhesion Proteins and GTPases. PLoS One 8, e84354. https://doi.org/10.1371/journal.pone.0084354

Paulozzi, L.J., Erickson, J.D., Jackson, R.J., 1999. Hypospadias Trends in Two US Surveillance Systems. J. Urol. 161, 366–366. https://doi.org/10.1016/S0022-5347(01)62171-2

Pedrazzini, T., Pralong, F., Grouzmann, E., 2003. Neuropeptide Y: The universal soldier. Cell. Mol. Life Sci. 60, 350–377. https://doi.org/10.1007/s000180300029

Perez-Carreon, J.I., Dargent, C., Merhi, M., Fattel-Fazenda, S., Arce-Popoca, E., Villa-Treviño, S., Rouimi, P., 2009. Tumor promoting and co-carcinogenic effects in medium-term rat hepatocarcinogenesis are not modified by co-administration of 12 pesticides in mixture at acceptable daily intake. Food Chem. Toxicol. 47, 540–546. https://doi.org/10.1016/j.fct.2008.12.008

Perobelli, J.E., Martinez, M.F., da Silva Franchi, C.A., Fernandez, C.D.B., Camargo, J.L.V. de, Kempinas, W.D.G., 2010. Decreased Sperm Motility in Rats Orally Exposed to Single or Mixed Pesticides. J. Toxicol. Environ. Heal. Part A 73, 991–1002. https://doi.org/10.1080/15287391003751802

Pitetti, J.L., Calvel, P., Zimmermann, C., Conne, B., Papaioannou, M.D., Aubry, F., Cederroth, C.R., Urner, F., Fumel, B., Crausaz, M., Docquier, M., Herrera, P.L., Pralong, F., Germond, M., Guillou, F., Jégou, B., Nef, S., 2013. An essential role for insulin and IGF1 receptors in regulating sertoli cell proliferation, testis size, and FSH action in mice. Mol. Endocrinol. 27, 814–827. https://doi.org/10.1210/me.2012-1258

Porta, M., Zumeta, E., 2002. Implementing the Stockholm Treaty on Persistent Organic Pollutants. Occup. Environ. Med. 59, 651–652. https://doi.org/10.1136/oem.59.10.651

Porter, R.D., Wiemeyer, S.N., 1969. Dieldrin and DDT: Effects on Sparrow Hawk Eggshells and Reproduction. Science (80-.). 165, 199–200. https://doi.org/10.1126/science.165.3889.199

Qi, S., Fu, W., Wang, C., Liu, C., Quan, C., Kourouma, A., Yan, M., Yu, T., Duan,

P., Yang, K., 2014. BPA-induced apoptosis of rat Sertoli cells through Fas/FasL and JNKs/p38 MAPK pathways. Reprod. Toxicol. 50, 108–116. https://doi.org/10.1016/j.reprotox.2014.10.013

Quan, C., Wang, C., Duan, P., Huang, W., Chen, W., Tang, S., Yang, K., 2017. Bisphenol a induces autophagy and apoptosis concurrently involving the Akt/mTOR pathway in testes of pubertal SD rats. Environ. Toxicol. 32, 1977–1989. https://doi.org/10.1002/tox.22339

Rappaport, S.M., 2011. Implications of the exposome for exposure science. J. Expo. Sci. Environ. Epidemiol. 21, 5–9. https://doi.org/10.1038/jes.2010.50

Reddy, A.V.B., Moniruzzaman, M., Aminabhavi, T.M., 2019. Polychlorinated biphenyls (PCBs) in the environment: Recent updates on sampling, pretreatment, cleanup technologies and their analysis. Chem. Eng. J. 358, 1186–1207. https://doi.org/10.1016/j.cej.2018.09.205

Rehan, N.E., Sobrero, A.J., Fertig, J.W., 1975. The semen of fertile men: statistical analysis of 1300 men. Fertil. Steril. 26, 492–502. https://doi.org/10.1016/s0015-0282(16)41169-6

Ren, L., Huang, J., Wei, J., Zang, Y., Zhao, Y., Wu, S., Zhao, X., Zhou, X., Sun, Z., Lu, H., 2020. Maternal exposure to fine particle matters cause autophagy via UPR-mediated PI3K-mTOR pathway in testicular tissue of adult male mice in offspring. Ecotoxicol. Environ. Saf. 189, 109943. https://doi.org/10.1016/j.ecoenv.2019.109943

Repouskou, A., Stamatakis, A., Kitraki, E., 2021. In utero exposure to phthalates and reproductive toxicity in rodents. Best Pract. Res. Clin. Endocrinol. Metab. 35, 101512. https://doi.org/10.1016/j.beem.2021.101512

Rhind, S.M., Kyle, C.E., MacKie, C., McDonald, L., 2009. Accumulation of endocrine disrupting compounds in sheep fetal and maternal liver tissue following exposure to pastures treated with sewage sludge. J. Environ. Monit. 11, 1469–1476. https://doi.org/10.1039/b902085c

Rhind, S.M., Kyle, C.E., MacKie, C., McDonald, L., Zhang, Z., Duff, E.I., Bellingham, M., Amezaga, M.R., Mandon-Pepin, B., Loup, B., Cotinot, C., Evans, N.P., Sharpe, R.M., Fowler, P.A., 2010. Maternal and fetal tissue accumulation of selected endocrine disrupting compounds (EDCs) following exposure to sewage sludge-treated pastures before or after conception. J. Environ. Monit. 12, 1582–1593. https://doi.org/10.1039/c0em00009d

Rhind, S.M., Kyle, C.E., Telfer, G., Duff, E.I., Smith, A., 2005. Alkyl phenols and diethylhexyl phthalate in tissues of sheep grazing pastures fertilized with sewage sludge or inorganic fertilizer. Environ. Health Perspect. 113, 447–453. https://doi.org/10.1289/ehp.7469

Rhind, S.M., Smith, A., Kyle, C.E., Telfer, G., Martin, G., Duff, E., Mayes, R.W., 2002. Phthalate and alkyl phenol concentrations in soil following applications of inorganic fertiliser or sewage sludge to pasture and potential rates of ingestion by grazing ruminants. J. Environ. Monit. 4, 142–148. https://doi.org/10.1039/b107539j

Rhind, S.M.M., Kyle, C.E.E., Ruffie, H., Calmettes, E., Osprey, M., Zhang, Z.L.L., Hamilton, D., McKenzie, C., 2013. Short- and long-term temporal changes in soil concentrations of selected endocrine disrupting compounds (EDCs) following single or multiple applications of sewage sludge to pastures. Environ. Pollut. 181, 262–270. https://doi.org/10.1016/j.envpol.2013.06.011

Rhouma, K. Ben, Tébourbi, O., Krichah, R., Sakly, M., 2001. Reproductive toxicity of DDT in adult male rats. Hum. Exp. Toxicol. 20, 393–397. https://doi.org/10.1191/096032701682692946

Ribeiro, E., Ladeira, C., Viegas, S., 2017. EDCs mixtures: A stealthy hazard for human health? Toxics 5, 1–17. https://doi.org/10.3390/toxics5010005

Richburg, J.H., Boekelheide, K., 1996. Mono-(2-ethylhexyl) Phthalate Rapidly Alters both Sertoli Cell Vimentin Filaments and Germ Cell Apoptosis in Young Rat Testes. Toxicol. Appl. Pharmacol. 137, 42–50. https://doi.org/10.1006/taap.1996.0055

Rider, C. V., Dinse, G.E., Umbach, D.M., Simmons, J.E., Hertzberg, R.C., 2018. Predicting Mixture Toxicity with Models of Additivity, in: Chemical Mixtures and Combined Chemical and Nonchemical Stressors. Springer International Publishing, Cham, pp. 235–270. https://doi.org/10.1007/978-3-319-56234-6_9

Rider, C. V., Furr, J., Wilson, V.S., Gray, L.E., 2008. A mixture of seven antiandrogens induces reproductive malformations in rats. Int. J. Androl. 31, 249–262. https://doi.org/10.1111/j.1365-2605.2007.00859.x

Rider, C. V., Furr, J.R., Wilson, V.S., Gray Jr, L.E., 2010. Cumulative effects of in utero administration of mixtures of reproductive toxicants that disrupt common target tissues via diverse mechanisms of toxicity. Int. J. Androl. 33, 443–462. https://doi.org/10.1111/j.1365-2605.2009.01049.x

Rieke, S., Heise, T., Schmidt, F., Haider, W., Bednarz, H., Niehaus, K., Mentz, A., Kalinowski, J., Hirsch-Ernst, K.I., Steinberg, P., Niemann, L., Marx-Stoelting, P., 2017. Mixture effects of azole fungicides on the adrenal gland in a broad dose range. Toxicology 385, 28–37. https://doi.org/10.1016/j.tox.2017.04.012

Rigby, H., Dowding, A., Fernandes, A., Humphries, D., Jones, N.R., Lake, I., Petch, R.G., Reynolds, C.K., Rose, M., Smith, S.R., 2020. Concentrations of organic contaminants in industrial and municipal bioresources recycled in agriculture in the UK. Sci. Total Environ. 142787. https://doi.org/10.1016/j.scitotenv.2020.142787

Rochester, J.R., 2013. Bisphenol A and human health: A review of the literature. Reprod. Toxicol. 42, 132–155. https://doi.org/10.1016/j.reprotox.2013.08.008

Rochester, J.R., Bolden, A.L., 2015. Bisphenol S and F: A Systematic Review and Comparison of the Hormonal Activity of Bisphenol A Substitutes. Environ. Health Perspect. 123, 643–650. https://doi.org/10.1289/ehp.1408989

Rodprasert, W., Toppari, J., Virtanen, H.E., 2021a. Endocrine Disrupting Chemicals and Reproductive Health in Boys and Men. Front. Endocrinol. (Lausanne). 12, 833–847. https://doi.org/10.3389/fendo.2021.706532

Rodprasert, W., Toppari, J., Virtanen, H.E., 2021b. Endocrine Disrupting Chemicals and Reproductive Health in Boys and Men. Front. Endocrinol. (Lausanne). 12, 1–45. https://doi.org/10.3389/fendo.2021.706532

Rolland, M., Le Moal, J., Wagner, V., Royère, D., De Mouzon, J., 2013. Decline in semen concentration and morphology in a sample of 26 609 men close to general population between 1989 and 2005 in France. Hum. Reprod. 28, 462–470. https://doi.org/10.1093/humrep/des415

Rotter, S., Beronius, A., Boobis, A.R., Hanberg, A., van Klaveren, J., Luijten, M., Machera, K., Nikolopoulou, D., van der Voet, H., Zilliacus, J., Solecki, R., 2018. Overview on legislation and scientific approaches for risk assessment of combined exposure to multiple chemicals: the potential EuroMix contribution. Crit. Rev. Toxicol. 48, 796–814. https://doi.org/10.1080/10408444.2018.1541964

Ruel, M.V.M., Bos, A.F., Soechitram, S.D., Meijer, L., Sauer, P.J.J., Berghuis, S.A., 2019. Prenatal exposure to organohalogen compounds and children's mental and motor development at 18 and 30 months of age. Neurotoxicology 72, 6–14. https://doi.org/10.1016/j.neuro.2019.01.003

Saad, H.E.S., Meduri, G., Phrakonkham, P., Bergès, R., Vacher, S., Djallali, M., Auger, J., Canivenc-Lavier, M.C., Perrot-Applanat, M., 2011. Abnormal peripubertal development of the rat mammary gland following exposure in utero and during lactation to a mixture of genistein and the food contaminant vinclozolin. Reprod. Toxicol. 32, 15–25. https://doi.org/10.1016/j.reprotox.2011.03.001

Sabatini, D.M., 2017. Twenty-five years of mTOR: Uncovering the link from nutrients to growth. Proc. Natl. Acad. Sci. U. S. A. 114, 11818–11825. https://doi.org/10.1073/pnas.1716173114

Salian, S., Doshi, T., Vanage, G., 2011. Perinatal exposure of rats to Bisphenol A affects fertility of male offspring—An overview. Reprod. Toxicol. 31, 359–362. https://doi.org/10.1016/j.reprotox.2010.10.008

Salian, S., Doshi, T., Vanage, G., 2009. Neonatal exposure of male rats to Bisphenol A impairs fertility and expression of sertoli cell junctional proteins in the testis. Toxicology 265, 56–67. https://doi.org/10.1016/j.tox.2009.09.012

Sampieri, L., Di Giusto, P., Alvarez, C., 2019. CREB3 transcription factors: ER-golgi stress transducers as hubs for cellular homeostasis. Front. Cell Dev. Biol. 7, 1–13. https://doi.org/10.3389/fcell.2019.00123

Santiasih, I., Titah, H.S., Hermana, J., 2020. The effects of particulate matters inhalation exposures of prallethrin and d-phenothrin mixture in mice (Mus musculus) against exhaled carbon dioxide concentration. Toxicol. Res. 36, 59–67. https://doi.org/10.1007/s43188-019-00002-8

Sargent, K.M., Clopton, D.T., Lu, N., Pohlmeier, W.E., Cupp, A.S., 2016. VEGFA splicing: divergent isoforms regulate spermatogonial stem cell maintenance. Cell Tissue Res. 363, 31–45. https://doi.org/10.1007/s00441-015-2297-2

Sarigiannis, D.A., Karakitsios, S.P., 2018. Modeling Complex Exposures, in: Chemical Mixtures and Combined Chemical and Nonchemical Stressors. Springer International Publishing, Cham, pp. 81–125. https://doi.org/10.1007/978-3-319-56234-6_4

Satoh, T., Jokanović, M., 2014. Toxicity and Novel Biomarkers of OP Exposure, in: Basic and Clinical Toxicology of Organophosphorus Compounds. Springer London, London, pp. 119–139. https://doi.org/10.1007/978-1-4471-5625-3_5

SCHER/SCCS/SCENIHR, 2013. Toxicity and Assessment of Chemical Mixtures. Eur. Comm. https://doi.org/10.2772/21444

Schmidt, F., Marx-Stoelting, P., Haider, W., Heise, T., Kneuer, C., Ladwig, M., Banneke, S., Rieke, S., Niemann, L., 2016. Combination effects of azole fungicides in male rats in a broad dose range. Toxicology 355–356, 54–63. https://doi.org/10.1016/j.tox.2016.05.018

Schneider, S., Fussell, K.C., Melching-Kollmuss, S., Buesen, R., Gröters, S., Strauss, V., Jiang, X., van Ravenzwaay, B., 2017. Investigations on the dose-response relationship of combined exposure to low doses of three anti-androgens in Wistar rats. Arch. Toxicol. 91, 3961–3989. https://doi.org/10.1007/s00204-017-2053-3

Schneider, S., Fussell, K.C., Melching-Kollmuss, S., Gröters, S., Strauss, V., Siddeek, B., Benahmed, M., Frericks, M., van Ravenzwaay, B., 2015. Low Dose/Dose Response Relationship of Hormonally Active Substances and their Mixture – Testing Endocrine Disruptors in Classical and Molecular Endpoints at Human-Relevant Exposure Levels. Toxicol. Lett. 238, S52. https://doi.org/10.1016/j.toxlet.2015.08.145

Sergievich, A.A., Khoroshikh, P.P., Artemenko, A.F., Zakharenko, A.M., Chaika, V. V., Kodintsev, V. V., Stroeva, O.A., Lenda, E.G., Tsatsakis, A., Burykina, T.I., Agathokleous, E., Kostoff, R.N., Zlatian, O., Docea, A.O., Golokhvast, K.S., 2020. Behavioral impacts of a mixture of six pesticides on rats. Sci. Total Environ. 727, 138491. https://doi.org/10.1016/j.scitotenv.2020.138491

Serra, N.D., Velte, E.K., Niedenberger, B.A., Kirsanov, O., Geyer, C.B., 2017. Cell-autonomous requirement for mammalian target of rapamycin (Mtor) in spermatogonial proliferation and differentiation in the mouse. Biol. Reprod. 96, 816–828. https://doi.org/10.1093/biolre/iox022

Sharpe, R.M., 2010. Environmental/lifestyle effects on spermatogenesis. Philos. Trans. R. Soc. B Biol. Sci. 365, 1697–1712. https://doi.org/10.1098/rstb.2009.0206

Sharpe, R.M., 2001. Hormones and testis development and the possible adverse effects of environmental chemicals. Toxicol. Lett. 120, 221–232. https://doi.org/10.1016/S0378-4274(01)00298-3

Sharpe, R.M., Skakkebaek, N.E., 2008. Testicular dysgenesis syndrome: mechanistic insights and potential new downstream effects. Fertil. Steril. 89, e33–e38. https://doi.org/10.1016/j.fertnstert.2007.12.026

Sharpe, R.M., Skakkebaek, N.E., 1993. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 341, 1392–1396. https://doi.org/10.1016/0140-6736(93)90953-E

Siracusa, J.S., Yin, L., Measel, E., Liang, S., Yu, X., 2018. Effects of bisphenol A and its analogs on reproductive health: A mini review. Reprod. Toxicol. 79, 96–123. https://doi.org/10.1016/j.reprotox.2018.06.005

Sirotkin, A. V., Harrath, A.H., 2014. Phytoestrogens and their effects. Eur. J. Pharmacol. 741, 230–236. https://doi.org/10.1016/j.ejphar.2014.07.057

Skakkebæk, N.E., 2002. Endocrine Disrupters and Testicular Dysgenesis Syndrome. Horm. Res. Paediatr. 57, 43–43. https://doi.org/10.1159/000058100

Skakkebæk, N.E., Lindahl-Jacobsen, R., Levine, H., Andersson, A.-M., Jørgensen, N., Main, K.M., Lidegaard, Ø., Priskorn, L., Holmboe, S.A., Bräuner, E. V., Almstrup, K., Franca, L.R., Znaor, A., Kortenkamp, A., Hart, R.J., Juul, A., 2022. Environmental factors in declining human fertility. Nat. Rev. Endocrinol. 18, 139–157. https://doi.org/10.1038/s41574-021-00598-8

Skakkebæk, N.E., Rajpert-De Meyts, E., Main, K.M., 2001. Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. Hum. Reprod. 16, 972–978. https://doi.org/10.1093/humrep/16.5.972

Skakkebaek, N.E., Toppari, J., Söder, O., Gordon, C.M., Divall, S., Draznin, M., 2011. The exposure of fetuses and children to endocrine disrupting chemicals: A European Society for Paediatric Endocrinology (ESPE) and Pediatric Endocrine Society (PES) call to action statement. J. Clin. Endocrinol. Metab. 96, 3056–3058. https://doi.org/10.1210/jc.2011-1269

Smith, K., Brown, P., Barr, F., 2012a. A Survey of Congenital Reproductive Abnormalities in Rams in Abattoirs in South West England. Reprod. Domest. Anim. 47, 740–745. https://doi.org/10.1111/j.1439-0531.2011.01952.x

Smith, K., Brown, P., Barr, F., Parkinson, T., 2012b. Cryptorchidism in Sheep: A Clinical and Abattoir Survey in the United Kingdom. Open J. Vet. Med. 02, 281–284. https://doi.org/10.4236/ojvm.2012.24044

Snyder, E., Chukrallah, L., Seltzer, K., Goodwin, L., Braun, R.E., 2020. ADAD1 and ADAD2, testis-specific adenosine deaminase domain-containing proteins, are required for male fertility. Sci. Rep. 10, 11536. https://doi.org/10.1038/s41598-020-67834-5

Sobolewski, M., Conrad, K., Allen, J.L., Weston, H., Martin, K., Lawrence, B.P., Cory-Slechta, D.A., 2014. Sex-specific enhanced behavioral toxicity induced by maternal exposure to a mixture of low dose endocrine-disrupting chemicals. Neurotoxicology 45, 121–130.

https://doi.org/10.1016/j.neuro.2014.09.008

Soltaninejad, K., Shadnia, S., 2014. History of the Use and Epidemiology of Organophosphorus Poisoning, in: Basic and Clinical Toxicology of Organophosphorus Compounds. Springer London, London, pp. 25–43. https://doi.org/10.1007/978-1-4471-5625-3_2

Su, W., Wong, E.W.P., Mruk, D.D., Cheng, C.Y., 2012. The Scribble/Lgl/Dlg Polarity Protein Complex Is a Regulator of Blood-Testis Barrier Dynamics and Spermatid Polarity during Spermatogenesis. Endocrinology 153, 6041–6053. https://doi.org/10.1210/en.2012-1670

Sun, K., Song, Y., He, F., Jing, M., Tang, J., Liu, R., 2021. A review of human and animals exposure to polycyclic aromatic hydrocarbons: Health risk and adverse effects, photo-induced toxicity and regulating effect of microplastics. Sci. Total Environ. 773, 145403. https://doi.org/10.1016/j.scitotenv.2021.145403

Svechnikov, K., Spatafora, C., Svechnikova, I., Tringali, C., Söder, O., 2009. Effects of resveratrol analogs on steroidogenesis and mitochondrial function in rat Leydig cells in vitro. J. Appl. Toxicol. 29, 673–680. https://doi.org/10.1002/jat.1456

Svingen, T., Ramhøj, L., Mandrup, K., Christiansen, S., Axelstad, M., Vinggaard, A.M., Hass, U., 2018. Effects on metabolic parameters in young rats born with low birth weight after exposure to a mixture of pesticides. Sci. Rep. 8, 305. https://doi.org/10.1038/s41598-017-18626-x

Swan, S.H., Elkin, E.P., Fenster, L., 2000. The question of declining sperm density revisited: An analysis of 101 studies published 1934-1996. Environ. Health Perspect. 108, 961–966. https://doi.org/10.1289/ehp.00108961

Takahashi, N., Davy, P.M.C., Gardner, L.H., Mathews, J., Yamazaki, Y., Allsopp, R.C., 2016. Hypoxia inducible factor 1 alpha is expressed in germ cells throughout the murine life cycle. PLoS One 11, 1–14. https://doi.org/10.1371/journal.pone.0154309

Tang, M., Yin, S., Zhang, J., Chen, K., Jin, M., Liu, W., 2018. Prenatal exposure to polychlorinated biphenyl and umbilical cord hormones and birth outcomes in an island population. Environ. Pollut. 237, 581–591. https://doi.org/10.1016/j.envpol.2018.02.044

Tang, S., Wang, X., Li, W., Yang, X., Li, Z., Liu, W., Li, C., Zhu, Z., Wang, L., Wang, Jiaxiong, Zhang, L., Sun, X., Zhi, E., Wang, H., Li, H., Jin, L., Luo, Y., Wang, Jian, Yang, S., Zhang, F., 2017. Biallelic Mutations in CFAP43 and CFAP44 Cause Male Infertility with Multiple Morphological Abnormalities of the Sperm Flagella. Am. J. Hum. Genet. 100, 854–864. https://doi.org/10.1016/j.ajhg.2017.04.012

Tanida, T., Warita, K., Ishihara, K., Fukui, S., Mitsuhashi, T., Sugawara, T., Tabuchi, Y., Nanmori, T., Qi, W.-M., Inamoto, T., Yokoyama, T., Kitagawa, H., Hoshi, N., 2009. Fetal and neonatal exposure to three typical environmental chemicals with different mechanisms of action: Mixed

exposure to phenol, phthalate, and dioxin cancels the effects of sole exposure on mouse midbrain dopaminergic nuclei. Toxicol. Lett. 189, 40–47. https://doi.org/10.1016/j.toxlet.2009.04.005

Tarragoó-Castellanos, C.R., García-Lorenzana, C.M., Diaz-Sánchez, V., Velázquez-Moctezuma, J., 2006. Gonadotrophin levels and morphological testicular features in rats after different doses of the phytoestrogen coumestrol. Neuroendocrinol. Lett. 27, 487–492.

Temkin, C.L., 1941. Seven defences, the reply to certain calumniations of his enemies, in: Sigerist, H.E. (Ed.), Four Treatises of Theophrastus Von Hohenheim Called Paracelsus. The Johns Hopkins Press.

Terado, M., Nomura, M., Mineta, K., Fujimoto, N., Matsumoto, T., 2006. Expression of Neuropeptide Y gene in mouse testes during testicular development. Asian J. Androl. 8, 443–449. https://doi.org/10.1111/j.1745-7262.2006.00166.x

Tokuda, M., Kadokawa, Y., Kurahashi, H., Marunouchi, T., 2007. CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. Biol. Reprod. 76, 130–141. https://doi.org/10.1095/biolreprod.106.053181

Tong, N., Witherspoon, L., Dunne, C., Flannigan, R., 2022. Global decline of male fertility: Fact or fiction? B. C. Med. J. 64, 126–130.

Toppari, J., Virtanen, H.E., Main, K.M., Skakkebaek, N.E., 2010. Cryptorchidism and hypospadias as a sign of testicular dysgenesis syndrome (TDS): Environmental connection. Birth Defects Res. Part A Clin. Mol. Teratol. 88, 910–919. https://doi.org/10.1002/bdra.20707

Tsatsakis, A., Docea, A.O., Calina, D., Buga, A.M., Zlatian, O., Gutnikov, S., Kostoff, R.N., Aschner, M., 2019a. Hormetic Neurobehavioral effects of low dose toxic chemical mixtures in real-life risk simulation (RLRS) in rats. Food Chem. Toxicol. 125, 141–149. https://doi.org/10.1016/j.fct.2018.12.043

Tsatsakis, A., Docea, A.O., Constantin, C., Calina, D., Zlatian, O., Nikolouzakis, T.K., Stivaktakis, P.D., Kalogeraki, A., Liesivuori, J., Tzanakakis, G., Neagu, M., 2019b. Genotoxic, cytotoxic, and cytopathological effects in rats exposed for 18 months to a mixture of 13 chemicals in doses below NOAEL levels. Toxicol. Lett. 316, 154–170. https://doi.org/10.1016/j.toxlet.2019.09.004

Tsatsakis, A., Petrakis, D., Nikolouzakis, T.K., Docea, A.O., Calina, D., Vinceti, M., Goumenou, M., Kostoff, R.N., Mamoulakis, C., Aschner, M., Hernández, A.F., 2020. COVID-19, an opportunity to reevaluate the correlation between long-term effects of anthropogenic pollutants on viral epidemic/pandemic events and prevalence. Food Chem. Toxicol. 141, 111418. https://doi.org/10.1016/j.fct.2020.111418

Tsatsakis, A., Tyshko, N. V., Docea, A.O., Shestakova, S.I., Sidorova, Y.S., Petrov, N.A., Zlatian, O., Mach, M., Hartung, T., Tutelyan, V.A., 2019c. The effect of chronic vitamin deficiency and long term very low dose exposure to 6 pesticides mixture on neurological outcomes – A real-life risk simulation

approach. Toxicol. Lett. 315, 96–106. https://doi.org/10.1016/j.toxlet.2019.07.026

Tsatsakis, A.M., Docea, A.O., Tsitsimpikou, C., 2016. New challenges in risk assessment of chemicals when simulating real exposure scenarios; simultaneous multi-chemicals' low dose exposure. Food Chem. Toxicol. 96, 174–176. https://doi.org/10.1016/j.fct.2016.08.011

Tsatsakis, A.M., Kouretas, D., Tzatzarakis, M.N., Stivaktakis, P., Tsarouhas, K., Golokhvast, K.S., Rakitskii, V.N., Tutelyan, V.A., Hernandez, A.F., Rezaee, R., Chung, G., Fenga, C., Engin, A.B., Neagu, M., Arsene, A.L., Docea, A.O., Gofita, E., Calina, D., Taitzoglou, I., Liesivuori, J., Hayes, A.W., Gutnikov, S., Tsitsimpikou, C., 2017. Simulating real-life exposures to uncover possible risks to human health: A proposed consensus for a novel methodological approach. Hum. Exp. Toxicol. 36, 554–564. https://doi.org/10.1177/0960327116681652

Turyk, M.E., Persky, V.W., Imm, P., Knobeloch, L., Chatterton, R., Anderson, H.A., 2008. Hormone Disruption by PBDEs in Adult Male Sport Fish Consumers. Environ. Health Perspect. 116, 1635–1641. https://doi.org/10.1289/ehp.11707

U.S. EPA, 2022. TSCA Chemical Substance Inventory.

U.S. EPA, 2016. Chemical Data Reporting Data.

U.S. EPA, 2007. Concepts, Methods, and Data Sources for Cumulative Health Risk Assessment of Multiple Chemicals, Exposures and Effects: A Resource Document (Final Report). U.S. Environ. Prot. Agency, Natl. Cent. Environ. Assessment, Cincinnati, OH EPA/600/R-.

U.S. EPA, 1999. 40 CFR Part 503: Standards for the use or disposal of sewage sludge, Federal Register. USA.

U.S. HHS, 2019. Fourth National Report on Human Exposure to Environmental Chemicals. Updated Tables, January 2019, Volume One. Fourth Natl. Rep. Hum. Expo. to Environ. Chem. 1–529.

Uguz, C., Iscan, M., Togan, İ., 2009. Alkylphenols in the Environment and Their Adverse Effects on Living Organisms. Kocatepe Vet. J. 2, 49–58.

Van den Berg, M., Birnbaum, L.S., Denison, M., De Vito, M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N., Peterson, R.E., 2006. The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol. Sci. 93, 223–241. https://doi.org/10.1093/toxsci/kfl055

Venkatesan, A.K., Halden, R.U., 2014a. Wastewater treatment plants as chemical observatories to forecast ecological and human health risks of manmade chemicals. Sci. Rep. 4, 4–10. https://doi.org/10.1038/srep03731

Venkatesan, A.K., Halden, R.U., 2014b. Contribution of polybrominated dibenzo-p-dioxins and dibenzofurans (PBDD/Fs) to the toxic equivalency of dioxin-like compounds in archived biosolids from the U.S. EPA's 2001 national sewage sludge survey. Environ. Sci. Technol. 48, 10843–10849. https://doi.org/10.1021/es503110j

Vigezzi, L., Bosquiazzo, V.L., Kass, L., Ramos, J.G., Muñoz-de-Toro, M., Luque, E.H., 2015. Developmental exposure to bisphenol A alters the differentiation and functional response of the adult rat uterus to estrogen treatment. Reprod. Toxicol. 52, 83–92. https://doi.org/10.1016/j.reprotox.2015.01.011

Vom Saal, F.S., Cooke, P.S., Buchanan, D.L., Palanza, P., Thayer, K.A., Nagel, S.C., Parmigiani, S., Welshons, W. V., 1998. A Physiologically Based Approach To the Study of Bisphenol a and Other Estrogenic Chemicals On the Size of Reproductive Organs, Daily Sperm Production, and Behavior. Toxicol. Ind. Health 14, 239–260. https://doi.org/10.1177/074823379801400115

Von Schönfeldt, V., Wistuba, J., Schlatt, S., 2004. Notch-1, c-kit and GFRα-1 are developmentally regulated markers for premeiotic germ cells. Cytogenet. Genome Res. 105, 235–239. https://doi.org/10.1159/000078194

Wade, M.G., Foster, W.G., Younglai, E. V., McMahon, A., Leingartner, K., Yagminas, A., Blakey, D., Fournier, M., Desaulniers, D., Hughes, C.L., 2002a. Effects of Subchronic Exposure to a Complex Mixture of Persistent Contaminants in Male Rats: Systemic, Immune, and Reproductive Effects. Toxicol. Sci. 67, 131–143. https://doi.org/10.1093/toxsci/67.1.131

Wade, M.G., Parent, S., Finnson, K.W., Foster, W., Younglai, E., McMahon, A., Cyr, D.G., Hughes, C., 2002b. Thyroid Toxicity Due to Subchronic Exposure to a Complex Mixture of 16 Organochlorines, Lead, and Cadmium. Toxicol. Sci. 67, 207–218. https://doi.org/10.1093/toxsci/67.2.207

Wang, A., Padula, A., Sirota, M., Woodruff, T.J., 2016. Environmental influences on reproductive health: the importance of chemical exposures. Fertil. Steril. 106, 905–929. https://doi.org/10.1016/j.fertnstert.2016.07.1076

Wang, L., Bu, T., Li, L., Wu, X., Wong, C.K.C., Perrotta, A., Silvestrini, B., Sun, F., Cheng, C.Y., 2022. Planar cell polarity (PCP) proteins support spermatogenesis through cytoskeletal organization in the testis. Semin. Cell Dev. Biol. 121, 99–113. https://doi.org/10.1016/j.semcdb.2021.04.008

Wang, T., Han, J., Duan, X., Xiong, B., Cui, X.-S., Kim, N.-H., Liu, H.-L., Sun, S.-C., 2016. The toxic effects and possible mechanisms of Bisphenol A on oocyte maturation of porcine in vitro. Oncotarget 7, 32554–32565. https://doi.org/10.18632/oncotarget.8689

Wang, X., Zou, Z., Yang, Z., Jiang, S., Lu, Y., Wang, D., Dong, Z., Xu, S., Zhu, L., 2019. HIF 1 inhibits STAR transcription and testosterone synthesis in murine Leydig cells. J. Mol. Endocrinol. 62, 1–13. https://doi.org/10.1530/JME-18-0148

WHO, 2012. State of the Science of Endocrine Disrupting Chemicals 2012, State of the Science of Disrupting Endocrine Chemicals. Geneva, Switzerland.

WHO, 2009. Assessment of Combined Exposures to Multiple Chemicals: Report of a WHO/IPCS International Workshop. IPCS Harmon. Proj. Doc. no. 7.

Wohlfahrt-Veje, C., Main, K.M., Skakkebaek, N.E., 2009. Testicular dysgenesis syndrome: foetal origin of adult reproductive problems. Clin. Endocrinol. (Oxf). 71, 459–465. https://doi.org/10.1111/j.1365-2265.2009.03545.x

Wolansky, M.J., Gennings, C., DeVito, M.J., Crofton, K.M., 2009. Evidence for dose-additive effects of pyrethroids on motor activity in rats. Environ. Health Perspect. 117, 1563–1570. https://doi.org/10.1289/ehp.0900667

Xia, M., Huang, R., Sun, Y., Semenza, G.L., Aldred, S.F., Witt, K.L., Inglese, J., Tice, R.R., Austin, C.P., 2009. Identification of chemical compounds that induce HIF-1α activity. Toxicol. Sci. 112, 153–163. https://doi.org/10.1093/toxsci/kfp123

Xie, R., Lin, Xiaolin, Du, T., Xu, K., Shen, H., Wei, F., Hao, W., Lin, T., Lin, Xia, Qin, Y., Wang, H., Chen, L., Yang, S., Yang, J., Rong, X., Yao, K., Xiao, D., Jia, J., Sun, Y., 2016. Targeted disruption of miR-17-92 impairs mouse spermatogenesis by activating mTOR signaling pathway. Med. (United States) 95, e2713. https://doi.org/10.1097/MD.0000000000002713

Xing, J.-S., Bai, Z.-M., 2018. Is testicular dysgenesis syndrome a genetic, endocrine, or environmental disease, or an unexplained reproductive disorder? Life Sci. 194, 120–129. https://doi.org/10.1016/j.lfs.2017.11.039

Xiong, M., Ferder, I.C., Ohguchi, Y., Wang, N., 2015. Quantitative analysis of male germline stem cell differentiation reveals a role for the p53-mTORC1 pathway in spermatogonial maintenance. Cell Cycle 14, 2905–2913. https://doi.org/10.1080/15384101.2015.1069928

Xu, Y., Fletcher, T., Pineda, D., Lindh, C.H., Nilsson, C., Glynn, A., Vogs, C., Norström, K., Lilja, K., Jakobsson, K., Li, Y., 2020. Serum Half-Lives for Short- and Long-Chain Perfluoroalkyl Acids after Ceasing Exposure from Drinking Water Contaminated by Firefighting Foam. Environ. Health Perspect. 128, 077004. https://doi.org/10.1289/EHP6785

Yang, J., Cao, J., Sun, X., Feng, Z., Hao, D., Zhao, X., Sun, C., 2012. Effects of long-term exposure to low levels of organophosphorous pesticides and their mixture on altered antioxidative defense mechanisms and lipid peroxidation in rat liver. Cell Biochem. Funct. 30, 122–128. https://doi.org/10.1002/cbf.1825

Yoon, M.S., 2017. The role of mammalian target of rapamycin (mTOR) in insulin signaling. Nutrients 9. https://doi.org/10.3390/nu9111176

Yuan, G., Liu, Y., Liu, G., Wei, L., Wen, Y., Huang, S., Guo, Y., Zou, F., Cheng, J., 2019. Associations between semen phytoestrogens concentrations and semen quality in Chinese men. Environ. Int. 129, 136–144. https://doi.org/10.1016/j.envint.2019.04.076

Yuan, W., Leisner, T.M., McFadden, A.W., Clark, S., Hiller, S., Maeda, N., O'Brien, D.A., Parise, L. V., 2006. CIB1 Is Essential for Mouse Spermatogenesis. Mol. Cell. Biol. 26, 8507–8514. https://doi.org/10.1128/MCB.01488-06

Zhang, K., Shen, X., Wu, J., Sakaki, K., Saunders, T., Rutkowski, D.T., Back, S.H., Kaufman, R.J., 2006. Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response. Cell 124, 587–599. https://doi.org/10.1016/j.cell.2005.11.040

Zhang, Z., Le Velly, M., Rhind, S.M., Kyle, C.E., Hough, R.L., Duff, E.I., McKenzie, C., 2015. A study on temporal trends and estimates of fate of Bisphenol A in agricultural soils after sewage sludge amendment. Sci. Total Environ. 515–516, 1–11. https://doi.org/10.1016/j.scitotenv.2015.01.053

Zhao, S., Fernald, R.D., 2005. Comprehensive algorithm for quantitative real-time polymerase chain reaction. J. Comput. Biol. 12, 1047–1064. https://doi.org/10.1089/cmb.2005.12.1047

Zheng, Y., He, Y., An, J., Qin, J., Wang, Y., Zhang, Y., Tian, X., Zeng, W., 2014. THY1 is a surface marker of porcine gonocytes. Reprod. Fertil. Dev. 26, 533–539. https://doi.org/10.1071/RD13075

Component Based Methodologies						
Mixture	Relevant group(s) &	Exposure and duration	Number, species, route	Significant Mixture Findings	Comments	Reference
Antiandrogen Mixtures						
4 Pesticides and 3 Phthalates (Vinclozolin, procymidone, linuron, prochloraz, BBP, DBP, and DEHP)	≤ 0.5x NOAEL ≤ 1x NOAEL	3.75 - 37.5 mg/kg/d (≤ 0.5x NOAEL), 7.5 - 75 mg.kg/d (≤ 1x NOAEL), GD14-18, daily dosing of corn oil dose solution, as a mixture only.	n = 2-3 pregnant dams per group, Rat (♂ Pup Sprague-Dawley), Parental P.O.	Increased numer of areolae at 1x NOAEL. Logistically regressed ED50s for hypospadias, undescended testes, and gubernacular agenesis significantly lower than additivity model predictions (DA, TEF, RA, IAd).	NOAELs derived from historical / new unpublished data for reproductive tract malformations.	Rider et al., 2008
3 Pesticides and 1 Phthalate (Vinclozolin, finasteride, prochloraz, and DEHP)	1x NOAEL	0.01 - 5 mg/kg/d, GD7 - PND16, daily dosing of corn oil dose solution, individually and as a mixture.	n = 5-16 pregnant dams per group, Rat (♂ Pup Wistar), Parental P.O.	Reduced AGD and increased NR at 1x NOAEL. logistically regressed ED50 values for AGD shortening, cleft phallus, and malformations in general significantly lower than additivity model predictions (DA, IA).	NOAELs derived from preliminary investigation. NOAELs for NR were used as the most sensitive indicator of disruption.	Christiansen et al., 2009
4 Pesticides and 6 Phthalates (Vinclozolin, procymidone, linuron, prochloraz, BBP, DBP, and DEHP, DiBP, DiHP, and DPP)	≤ 0.5x NOAEL ≤ 1x NOAEL	3 - 15 mg/kg/d (≤ 0.5x NOAEL), 6 - 30 mg.kg/d (≤ 1x NOAEL), GD14-18, daily dosing of corn oil dose solution, as a mixture only.	n = 4 - 6 pregnant dams per group, Rat (♂ Pup Sprague-Dawley), Parental P.O.	NR in 1x NOAEL F1 males. DA model accurately predicted reductions in all endpoints except for ventral prostate weight, with an under-prediction of effect magnitude.	NOAELs derived from historical / new unpublished data for reproductive tract malformations.	Rider et al., 2010
6 Phthalates (DMP, DEP, DBP, BBP, DEHP, and DnOP)	0.1x NOAEL 1x NOAEL	1.6 mg/kg/d (0.1x NOAEL), 16 mg/kg/d (1x NOAEL), 15 week daily dosing of 0.5% CMC dose solution, as a mixture only	n = 10 per group, Rat (♂ Juvenile Sragues-Dawley), P.O.	Reduced body weight at 0.1x and 1x NOAEL. Reduced testicular fat, perirenal fat, and epididymis weights at 1x NOAEL, liver and testicular fat weights at 0.1x and 1x NOAEL, and kidney weight at 0.1x NOAEL. Increased spleen and heart weights at 1x NOAEL. Decreased serum testosterone and increased luteinizing hormone at 0.1x and 1x NOAEL. Increased incidents of deciduous spermatids at 0.1x and 1x NOAEL. Reduced testicular protein levels of total p53 at 0.1x NOAEL, Bcl-2, Cdc2, Chk1 at 0.1x and 1x NOAEL, and Cdk6, and Star at 1x NOAEL.	NOAELs taken from regulatory / advisory panel determinations.	Gao et al., 2017
1 Antiandrogen Pharmaceutical and 2 Pesticides (Flutamide, prochloraz, vinclozolin)	1x TDI 1x NOAEL	0.00025 - 0.01 mg/kg (1x ADI), 0.025 - 5 mg/kg (1x NOAEL), GD6 - PND30 / PND82 (±2), daily dosing of corn oil dose solution, individually and as a mixture	n = 25 pregnant dams per group, Rat (♀ Dam Wistar, and pups), Parental and Direct P.O.	Increased gestation, pup mortality and cannibalism at 1x NOAEL. At PNG21: Decreased adrenal gland weights at 1x TDI (females) and decreased pituitary gland weights at 1x NOAEL (males). At PNG83: Decreased corticosterone at 1x TDI (males), and decreased bulbo-urethral gland and absolute cauda epididymis weights, and progesterone at 1x NOAEL (males). In parental dams at PND30: Decreased estradiol at 1x NOAEL. No effects attributed to exposure. Greater than additive effects for decreased male anogenital index and delayed preputial separation when the chemicals were administered at LOAEL.	NOAELs derived from previously published data for signs of antiandrogenic action.	Schneider et al., 2017
3 Phthalate , 1 Alkylphenol and 1 Alkylbenzene (DEHP, DBP, BBP, NP, and 4-tert-octylphenol)	1x TDI	0.05 - 0.3 mg/kg/d, GD0.5 - PND60, via drinking water, individually and as a mixture	n ≥ 3 pregnant mice per group, Mouse (♂ C57BL/6J), Parental and Direct P.O.	Increased body weight, reduced relative testis weight, and seminiferous tubules diameter. Increased proportion of seminiferous tubules with exfoliated germ cells, without lumen, and with an undetermined stage of spermatogenesis. Increased caspase-3 positive cells, and pyknotic cells per seminiferous tubule. Reduced estradiol and estradiol: testosterone ratio in intratesticular fluid. Increased testicular expression of Cyp11a1, Cyp19a1, Sp1, and Hsd3b1 reduced, and Cyp17a1 and Star.	TDIs from regulatory determinations.	Buñay et al., 2018
Mixture	Relevant group(s) &	Exposure and duration	Number, species, route	Significant Mixture Findings	Comments	Reference
Antiandrogen + Xenoestrogen Mixtures						
1 Bisphenol, 1 Phthalate, and 1 Pollutant (BPA, DEHP, and TCDD)	1x NOAEL	1 - 5 mg/kg/d (DEHP and BPA)+ 1x 8ng/kg (TCDD), GD 8–17 and PD 3–7, daily (single for TCDD) dosing of sesame oil dose solution, individually and as a mixture	n = 3-6 pregnant mice per group, Mouse (Dam ICR and ♂ pups), Parental P.O.	Increased brain weight when exposed to mixture or components individually. Decreased body weights (PND14) and several additional histopathological findings (PND14 and PND42) when exposed to individual components, but not seen when exposed to mixture. No adverse effects attributed to mixture exposure, but suggested counteraction measures occuring from co-expsoure.	Literature derived NOAELs for reproductive toxicity	Tanida et al., 2009
1 Phytoestrogen and 1 Fungicide (Genistein and vinclozolin)	1xTDI / <NOAEL	1 mg/kg/d, GD1 - PND80, daily dosing of corn oil dose solution, individually and as a mixture	n = 10 pregnant dams per group, Rat (♂ Wistar), Parental and direct P.O.	Reduced litter size, increased post-implantation loss, increased pup birth weight. Reduced anogenital distances and increased immature penile development at PND25. Reduced epididymis and seminal vesicle weights at PND80. Reduced epididymal sperm counts, motility, and velocity at PND80. Increased plasma FSH and reduced plasma estradiol at PND80. Differential testicular gene expression correlation with individual exposure to a 30x dose of vinclozolin. KEGG pathway analysis identified enrichment in insulin signalling, fructose/mannose metabolism, neuroactive ligand/receptor interaction, ribosome, and GnRH signalling pathways.	NOAEL from regulatory determinations and dietary level accepted as TDI.	Eustache et al., 2009
1 Phytoestrogen and 1 Fungicide (Genistein and vinclozolin)	1xTDI / <NOAEL	1 mg/kg/d, GD1 - PND21, daily dosing of corn oil dose solution, individually and as a mixture	n = 15 pregnant dams per group, Rat (♀ Juvenile Wistar), Parental P.O.	Earlier vaginal opening. Higher proportion of large and very large terminal end buds of mammary ducts and increased epithelial branching at PND35. Increased proliferation of terminal end buds and expression of Ki67 at PND35. Increase in hyperplasic ducts and mammary gland thickening at PND35. Increased total mammary gland area, hyperplastic alveolar structures, and acini at PND50.	NOAEL from regulatory determinations and dietary level accepted as TDI.	Saad et al., 2011
1 Phytoestrogen and 1 Fungicide (Genistein and vinclozolin)	1xTDI / <NOAEL	1 mg/kg/d, GD0.5 - PND3, daily dosing of corn oil dose solution, individually and as a mixture	n = 12 - 28 per group, Rat (♂ Pup Wistar), Parental P.O.	Reduced ex vivo testicular testosterone secretion upon stimulation.	NOAEL from regulatory determinations and dietary level accepted as TDI.	Lehraiki et al., 2011
1 Phytoestrogen and 1 Fungicide (Genistein and vinclozolin)	1xTDI / <NOAEL	1 mg/kg/d, GD1 - PND21, daily dosing of corn oil dose solution, individually and as a mixture	n = 15 pregnant dams per group, Rat (♀ Pup Wistar), Parental P.O.	Reduced number of striated submandibular salivary ducts, and increased area of striated salivary ducts. Decreased proliferation in submandibular salivary glands. Decreased expression of growth factors EGF, NGF, and TGFα in submandibular salivary glands.	NOAEL from regulatory determinations and dietary level accepted as TDI.	Kouidhi et al., 2012
1 Bisphenol, 1 Phytoestrogen and 1 Fungicide (BPA, genistein, and vinclozolin)	1xTDI / <NOAEL	5µg - 1 mg/kg/d, F1♂: GD0-PND110, daily (F0) or alternate daily (F1) dosing of corn oil dose solution, as various mixtures only	n = 20 per group, Rat (Adult Wistar), Parental or Grand Parental P.O.	Altered forepaw 2nd and 4th digit lengths and length ratios in F1 and F2 (non-directly exposed) males, towards a more feminine digit length ratio.	NOAELs from regulatory determinations and dietary level accepted as TDI.	Auger et al., 2013
1 Bisphenol, 1 Phytoestrogen and 1 Fungicide (BPA, genistein, and vinclozolin)	Sub-NOAEL (0.9-0.01x NOAEL) Far-NOAEL (0.0009-0.05x NOAEL)	1 mg/kg/d (without BPA) - Sub-NOAEL, 5µg - 1 mg/kg/d (with BPA) - Far-NOAEL, GD1 - PND30 / 100, daily dosing of corn oil dose solution, individually and as various mixtures	n = 10 pregnant dams per group, Rat (Pup and Juvenile Wistar), Parental (F1) or Grand-Parental (F2) P.O.	Reduced average lengths of vertebrae v5-v8 at D30, and increased inter transverse apophysis widths of vertebrae v5-v8 at D110 at far-NOAEL doses in F1 females. Variances seen in proliferative and hypertrophic zone thicknesses in vertebral growth plates for sub-NOAEL females and far-NOAEL males at D30. No effects in F2 generation.	NOAELs from regulatory determinations and dietary level accepted as TDI.	Auxietre et al., 2014

Component Based Methodologies						
Mixture	Relevant group(s) & POD(s) used	Exposure and duration	Number, species, route	Significant Mixture Findings	Comments	Reference
Neurotoxic Mixtures						
11 Pyrethroid Pesticides (S-bioallethrin, permethrin, cypermethrin, deltamethrin, esfenvalerate, β-cyfluthrin, fenpropathrin, tefluthrin, λ-cyhalothrin, bifenthrin, resmethrin)	0.84% - 84% NOAEL	0.004 - 0.976 mg/kg; 1.52 mg/kg total (0.84%) to 0.4 - 97.6 mg/kg; 152.4 mg/kg total (84%), single dose of corn oil dosing solution as a mixture only	n = 4 - 12 per group, Rat (♂ Adult Long-Evans), P.O.	Mild whole-body tremors at 84% NOAEL. Decline in motor activity at 43% and 84% NOAEL (1-8 hours post-dose). When dosed simultaneously effects on decreased activity matched predictions by dose addition model. When dosed in three sets to align effect maxima of individual chemicals (at 1, 2 or 4hours), effects appeared to be greater than additive (mixture threshold dose 3.7x lower than predicted), but did not reach statistical significance (p=0.07).	NOAELs from previously published determinations for neurotoxicity	Wolansky et al., 2009
4 Organophosphorus Pesticides (Diazinon, chlorpyrifos, malathion, and profenofos)	1x NOAEL	0.06 - 29 mg/kg/d, 28 day, daily dosing of aqueous dose solution, as a mixture only	n = 6 per group, Rat (♂ Adult Wistar), P.O.	Reduced bodyweight, increased liver weight. Reduced serum total protein, increased serum ALT, AST, ALP, LDH, and MDA activity. Increased incidents of dilated and congested hepatic central veins and sinusoids, congested hepatic portal veins, dilated cystic bile ducts, and degenerated hepatocytes.	Literature derived NOAELs for unknown end points.	Mossa et al., 2011
4 Organophosphorus Pesticides (Dichlorvos, dimethoate, acephate, and phorate)	1x NOAEL	0.04 - 2.4 mg/kg, 24 week, via drinking water, individually and as a mixture	n = 10 per group, Rat (♂ Adult Wistar), P.O.	No effects on anti-oxidative defence mechanisms or lipid peroxidation.	Regulatory derived NOAELs for unknown end points.	Yang et al., 2012
4 Organophosphorus Pesticides (Dichlorvos, dimethoate, acephate, and phorate)	1x NOAEL	0.04 - 2.4 mg/kg, 24 week, via drinking water, as a mixture only	n = 5 per group, Rat (♂ Adult Wistar),P.O.	Increased TG, HDL, and LDL from 12 weeks, and total cholesterol from 16 weeks. Renal tubular epithelial cell swelling and granular degeneration from 12 weeks.	Literature derived NOAELs for unknown end points.	Du et al., 2014
4 Pyrethroid Pesticides (Tefluthrin, bifenthrin, cypermethrin, and deltamethrin)	0.3x Threshold Dose	0.09 - 0.341 mg/kg, single dose of corn oil dosing solution, as a mixture only	n = 4 - 6 per group, Rat (Juvenile Hsd:WI Wistar), P.O.	No effects on body temperature.	Threshold doses from previously published determinations for neurotoxicity or unpublished pilot data	Ortega et al., 2018
2 Pyrethroid Pesticides (Prallethrin and phenothrin)	1x NOAEL	0.114 µg/L and 0.104 mg/L, 60 day, whole body exposure chamber, 4 hours per day, as a mixture only	n = 9 per group, Mouse (♂ Juvenile BALB/c), Inhalation	No effects on exhaled carbon dioxide seen	Regulatory derived NOAELs for unknown end points.	Santiasih et al., 2020
Mixture	Relevant group(s) &	Exposure and duration	Number, species, route	Significant Mixture Findings	Comments	Reference
Mixed EDC Mixtures						
5 Fungicides (Procymidone, mancozeb, epoxyconazole, tebuconazole, and prochloraz)	0.25x NOAEL, 0.5x NOAEL, 0.75 NOAEL, 1x NOAEL	3.75 - 12.5 mg/kg/d (0.25x NOAEL), 7.5 - 25 mg/kg/d (0.5x NOAEL), 11.25 - 37.5 mg/kg/d (0.75 NOAEL), 15 - 50 mg/kg/d (1x NOAEL), GD7 - 20, daily dosing of corn oil dose solution, as a mixture only	n = 8 pregnant dams per group, Rat (Pup Wistar), Parental P.O.	Increased gestation at 0.25x - 1x NOAEL, and perinatal pup mortality at 0.5x - 1x NOAEL. Complications in birthing caused 0.75x and 1x NOAEL groups to discontinue. In remaining groups: reduced birth and liver weight at 0.5x NOAEL (females). Reduced prostate and epididymis weights at 0.25x and 0.5x NOAEL and liver weight at 0.5x NOAEL (males). Increased (female) and decreased (male) birth anogenital index at 0.25x and 0.5x NOAEL. Increased nipple retention, hypospadias, and genital dysgenesis at 0.25x and 0.5x NOAEL (males) at PND13.	NOAELs derived from unpublished data for gestation length.	Jacobsen et al., 2010
5 Fungicides (Procymidone, mancozeb, epoxyconazole, tebuconazole, and prochloraz)	0.083x NOAEL, 0.17x NOAEL, 0.25x NOAEL	1.25 - 4.17 mg/kg/d (0.083x NOAEL), 2.5 - 8.33 mg/kg/d (0.17x NOAEL), 3.75 - 12.5 mg/kg/d (0.25x NOAEL), GD7 - PND16, daily dosing of corn oil dose solution, individually and as a mixture	n = 4 - 17 pregnant dams per group, Rat (Pup Wistar), Parental P.O.	Same study as Jacobsen et al, 2012. Increased gestation at 0.17x and 0.25x NOAEL. At birth: increased anogenital index (females), and number of areola (males), at 0.17x and 0.25x NOAEL. Dose dependant increase in nipple retention by PND13 at all doses (males). Increased accumulative genital malformations at 0.25x NOAEL (males) throughout whole life period.	NOAELs from previously published determinations for study endpoints	Hass et al, 2012
				Same study as Hass et al, 2012. Males only. Reduced testis (0.17x and 0.25x NOAEL), epididymis (0.083x, 0.17x and 0.25x NOAEL), prostate (0.17x and 0.25x NOAEL), and seminal vesicle (0.25x NOAEL) weights at PND16. Reduced prostate and levator ani/bulbocavernosus muscle weights, and lower sperm counts at PND260-280 at 0.25x NOAEL. Higher atypical hyperplasia and cribriform pattern scores in ventral prostate at PND260-28 at 0.25x NOAEL adults. Increased total swim lengths and latency in Morris maze performance tests at PND260-28 at 0.25x NOAEL.		Jacobsen et al., 2012
3 Pesticides (Atrazine, chlorpyrifos, and endosulfan)	1x TDI	0.01 - 0.044 ppm, GD0 - PND98, via dietary, individually and as a mixture	n = 15 pregnant mice per group, Mouse (Juvenile C57BL/6J), Parental and direct P.O.	Decreased body weight at PND98 (both sexes). Distinguishable metabolic profiles between groups and sexes at PND21 and 98. Metabolomic analysis at PND21 showed increased plasma concentrations of glucose (both sexes) and taurine (males), and decreased choline, glutamate, lysine, proline (both sexes), valine, methionine (females), creatine, alanine, glycine, and lipids (males). Metabolomic analysis at PND98 showed increased plasma concentrations of glucose (females), glutamine, dimethylamine, valine, and leucine/isoleucine (males), and decreased choline (females), and lactate was increased in males and decreased in females. Increased haemoglobin (females), and reticulocytes and progenitor colony number (bone marrow ex vivo culture) (both sexes) at PND98. Decreased β-actin, aMAP2, and Pyk2-pTyr881 (males) and increased vinculin, caspase 8 and caspase 4 (females) and bone marrow protein levels (both sexes) at PND98.	TDI extrapolated from regulatory determined human TDI.	Demur et al., 2013
3 Plasticisers and 1 Pollutant (TCDD, PCB153, DEHP, and BPA)	1x TDI	2 pg - 50 µg/kg/d, 5 week parental exposure + gestation + 12 week, via dietary in high-fat-high-sucrose diet, as a mixture only	n = 9 - 41 per group, Mouse (juvenile C57Bl6), Parental and direct P.O.	Reduced pup survival. Deterioration of glucose tolerance (females). Reduced hepatic expression of LXRβ (females), and increased expression of Ahr, Pparα, and Lxrα (males). Decreased hepatic expression of FA metabolism related genes SrebF1 and Dgat2 (females). Increased hepatic expression of cholesterol metabolism related genes SrebF2, Hmgcr, Cd36, Acaca, and Cyp7a1 (males). Reduced hepatic expression of estrogen receptor, Nqo1 and Ugt1a1, and increased Sult1e1 (females).	TDIs from regulatory determinations.	Naville et al., 2013

Component Based Methodologies						
Mixture	Relevant group(s) & POD(s) used	Exposure and duration	Number, species, route	Significant Mixture Findings	Comments	Reference
Mixed EDC Mixtures Continued						
1 Herbicide, 1 Plasticiser, 1 Pollutant, and 1 Surfactant (Atrazine, PFOA, BPA, TCDD)	1xTDI / <NOAEL	0.25 µg - 10 mg/kg, GD7 -PD2, via dietary or weekly peanut oil dose solution (TCDD), individually or as a mixture	n = 6 - 11 pregnant dams per group, ≤ 2 per sex per dam, Mouse (Pup C57BL/6J), Parental P.O.	At PND60: Reduced environmental habituation upon repeated testing (both sexes). Reduced exploratory behaviour, short-term memory, and attention to task or impaired motivation (males).	TDIs extrapolated from regulatory determined human TDI. Literature derived NOAELs for unknown endpoints.	Sobolewski et al., 2014
9 Water disinfection by-products (DBPs): 4 trihalomethanes (THM) and 5 haloacetic acids (HAA) (Chloroform, bromodichloromethane, chlorodibromomethane, bromoform, chloroacetic acid, dichloroacetic acid, trichloroacetic acid, bromoacetic acid, and dibromoacetic acid)	500x MCL (< 0.5x NOAEL), 1000x MCL (< 1x NOAEL)	0.34 - 22.39 mg/L (500x), 0.68 - 44.77 mg/L (1000x), F1 GD0 - F2 PND6, via drinking water, as a mixture only.	n = 24 - 25 pregnant dams per group, Rat (♀ Dam Sprague-Dawley, and F1 and F2 pups), Parental P.O.	Water consumption reduced in dams; intermittently during gestation and consistently throughout lactation at 1000x. F1 female water consumption reduced at 500x and 1000x. F1 pup weight reductions in males at PND26 and in females at PNDs 21 and 26. Onset of F1 male and female puberty delayed at 1000x. No effects seen in F2 generation.	Proportions relative to drinking water. Doses below lowest NOAELs from regulatory studies.	Narotsky et al., 2015
3 Plasticisers and 1 Pollutant (TCDD, PCB153, DEHP, and BPA)	1x TDI	2 pg - 50 µg/kg/d, 5 week parental exposure + gestation + 7 or 12 week, via dietary in high-fat-high-sucrose diet, as a mixture only	n = 5 - 11 pregnant mice per group, Mouse (♀ juvenile C57Bl6),P.O.	Increased glucose tolerance at 7 weeks, but decreased at 12 weeks. Increased hepatic expression at 7 weeks of Ahr, Pxr, Pparα, and Ppary, and Pparα-regulated genes Acadm, Acox, Cpt1a, and Hsl. Reduced oestrogen receptor α protein in the liver at 7 weeks. Reduced hepatic expression of Igf1, Sepp1, Nqo1 and Ugt1a1 at 12 weeks. Reduced visceral and subcutaneous fat, increased lean mass, and increased insulin induced Akt/Pkb phosphorylation in muscle at 7 weeks. Decreased subcutaneous fat expression of Tnfa, Ccl5, and IL1b at 7 weeks, and increased Ccl5, IL1b, and IL6 at 12 weeks.	TDIs from regulatory determinations.	Naville et al., 2015
3 Azole Fungicides (Cyproconazole, epoxiconazole, and prochloraz)	0.01x NOAEL 1x NOAEL	0.9 - 1 ppm (0.01x NOAEL), 90 - 100 ppm (1x NOAEL), 28 day, via dietary, individually and as a mixture. Mix-1 without prochloraz, Mix-2 with prochloraz.	n = 5 and 10 per group, Rat (♂ Juvenile Wistar), P.O.	No effects at doses equal to or lower than NOAEL.	NOAELs from regulatory determinations.	Schmidt et al., 2016
3 Azole Fungicides (Cyproconazole, epoxiconazole, and prochloraz)	0.01x NOAEL 1x NOAEL	0.9 - 1 ppm (0.01x NOAEL), 90 - 100 ppm (1x NOAEL), 28 day, via dietary, individually and as a mixture. Mix-1 without prochloraz, Mix-2 with prochloraz.	n = 10 per group, Rat (♂ Juvenile Wistar), P.O.	Reduced aldosterone, progesterone, and absolute adrenal weights at 0.01x NOAEL Mix-1. Effects not attributed to exposure.	NOAELs from regulatory determinations.	Rieke et al., 2017
3 Plasticisers and 1 Pollutant (TCDD, PCB153, DEHP, and BPA)	1x TDI	2 pg - 50 µg/kg/d, 5 week (parental dam exposure) + gestation + 12 week, via dietary, as a mixture only	n ≥5 - ≥10 per group, Mouse (♀ Juvenile C57Bl6), P.O.	Decreased plasma cholesteryl esters. Increased hepatic TG. Microarray identification of 251 differentially expressed genes in the liver. KEGG analysis indicated enrichment in drug and xenobiotic metabolism, steroid biosynthesis, fatty acid metabolism, and circadian rhythm pathways. Decreased hepatic expression of Cyp1a2, Sqle, Cyp51, Soat2, Fasn, and Arntl, and increased expression of Cyp4a10, Cidec, and Per1.	TDIs from regulatory determinations.	Labaronne et al., 2017
3 Plasticisers and 1 Pollutant (TCDD, PCB153, DEHP, and BPA)	1x TDI	2 pg - 50 µg/kg/d, 5 week parental exposure + gestation + 12 week, via dietary in high-fat-high-sucrose diet, as a mixture only	n = 7 - 9 per group, Mouse (♀ complete and ovariectomized juvenile C57Bl6), P.O.	Lower bodyweights, lower fasting insulin levels, higher insulin production following glucose administration, lower HOMA-IR, and lower liver TG in ovariectomized mice. Aggravation of glucose intolerance in complete mice, and an alleviation of glucose tolerance in ovariectomized mice. Pro-inflammatory Ccl2 gene expression in visceral adipose tissue was reduced due to exposure in ovariectomized mice, but not to non-ovariectomized levels. Hepatic oestrogen receptor 1 gene expression was increased due to exposure in ovariectomized mice.	TDIs from regulatory determinations.	Julien et al., 2018
3 Plasticisers and 1 Pollutant (TCDD, PCB153, DEHP, and BPA)	1x TDI	2 pg - 50 µg/kg/d, 15 week, via dietary in high-fat-high-sucrose diet, as a mixture only	n = 6 - 8 per group, Mouse (♀ ovariectomized juvenile C57Bl6, with and without estrogen replacement implant), P.O.	Increased expression of Cd36 and Shp in ovariectomized only, and LXRα in ovariectomized with and without oestrogen replacement, in the liver. Reduced hepatic expression of Cyp7a1 in ovariectomized with oestrogen replacement only. Increased subcutaneous adipose tissue expression of ERα in ovariectomized only, ERβ in ovariectomized with oestrogen replacement only, and ERα:AR expression ratios in both. Increased visceral adipose expression of ERβ and IL12a expression in ovariectomized with oestrogen replacement, and ERα:AR expression ratio in ovariectomized only.	TDIs from regulatory determinations.	Julien et al., 2019
3 Plasticisers and 1 Pollutant (TCDD, PCB153, DEHP, and BPA)	1x TDI	2 pg - 50 µg/kg/d, 15 week, via dietary in standard or high-fat-high-sucrose diet, as a mixture only	n = 8 per group, Mouse (♂ juvenile C57Bl6), P.O.	With standard diet: Reduced plasma TG and FFA, and increased adiponectin: leptin ratios. Increased hepatic expression of Apoc3, and decreased Acaca and Cpy2b10. Increased jejunal expression of Cpy3a11. Increased subcutaneous adipose tissue expression of IL10, Cidec and Lipe. Reduced perigonadal adipose tissue expression of IL10. With high-fat-high-sucrose diet: Increased perigonadal adipose tissue weight. Increased hepatic expression of Cyp7a1. Increased subcutaneous adipose tissue expression of Ppary. Increased perigonadal adipose tissue expression of IL1b. Jejunal expression of IL10 increased in standard diet but decreased in high-fat-high-sucrose diet. Jejunal expression of Slc27a4 (decreased) and Mttp (increase) between standard and high-fat-high-sucrose diet was only observed in exposed groups. Expression of ER1 was increased in the liver, but decreased in perigonadal adipose tissue due to high-fat-high-sucrose diet only in exposed animals, and increased in subcutaneous adipose tissue of standard diet fed, but unchanged in high-fat-high-sucrose diet fed.	TDIs from regulatory determinations.	Naville et al., 2019

Component Based Methodologies						
Mixture	Relevant group(s) & POD(s) used	Exposure and duration	Number, species, route	Significant Mixture Findings	Comments	Reference
Mixed MOA Mixtures						
5 Pesticides (Chlorpyrifos, alphacypermethrin, bromopropylate, carbendazim and mancozeb)	Chlorpyrifos: 0.04x NOAEL 0.2x NOAEL 1x NOAEL Remaining: 1x NOAEL	0.15 - 45 mg/kg/d, 28 day, via dietary, chlorpyrifos individually (at 1x NOAEL) and at various doses as a mixture with remaining components at NOAEL.	n = 8 per group, Rat (Juvenile Wistar), P.O.	Increased relative liver and thyroid weights at all doses (both sexes) and decreased relative thymus weight at 0.04x, 0.2x (both sexes), and 1x NOAEL (males). Reduced plasma acetylcholinesterase at 1x NOAEL (males). Decreased haematocrit and haemoglobin at 0.04x, 0.2x (males) and 1x NOAEL (both sexes), and red blood count at 1x NOAEL (both sexes).	Regulatory derived NOAELs for end points across many organs.	Jacobsen et al., 2004
6 Pesticides (Alachlor, captan, diazinon, endosulfan, maneb, and mancozeb)	1x TDI	0.5 - 100 µg/kg/d, 4 week, thrice weekly of DMSO dose solution, as a mixture only	n = 5 per group, Mouse (Juvenile C57BL/6J), P.O.	Reduced relative liver weights (males), and increased relative spleen weights (females). Distinguishable metabolomic profiles (both sexes). Increased glutamine, alanine, valine, acetate, and AMP/ATP resonances decreased taurine and inosine (males). Increased glutamine, glycogen, glutathione, and glucose resonances, decreased alanine, valine, acetate, inosine, lactate, and uridine (females). Increased platelets (males), white blood cells, and neutrophils (females). Ex vivo bone marrow cultures showed reduced total colonies, and CFU-GM specific counts (females), and increased CFU-M counts (males). Alternate staining showed increased granulocytes (females), and reduced macrophages (females), and increased macrophages (males). Bone marrow from treated animals showed increased c-Myc (females), Cyclin D1, and phosphorylation of GSK3β (both sexes), and decreased PKD/Akt (females), Pky2-pTyr, and phosphorylation of PKB/Akt (both sexes).	TDIs from regulatory determinations.	Merhi et al., 2010
5 Pesticides (Dicofol, dichlorvos, permethrin, endosulfan, and dieldrin)	1x NOAEL	0.025 - 5 mg/kg/d, 8 week, via dietary, individually and as a mixture	n = 6 - 12 per group, Rat (♂ Juvenile Lewis), P.O.	Reduced motile (type A) and increased immotile (type C) sperm.	Regulatory derived NOAELs for unknown end points.	Perobelli et al., 2010
5 Pesticides (Dicofol, dichlorvos, permethrin, endosulfan, and dieldrin)	1x NOAEL	0.025 - 5 mg/kg/d, 10 week, via dietary, as a mixture only	n = 10 per group, Rat (♀ Juvenile Sprague-Dawley, Wistar, and Lewis), P.O.	Decreased oestrous cycle and dioestrus period lengths (Lewis). Increased serum FSH (Sprague-Dawley), and decreased progesterone in (Lewis). Decreased ovarian primordial and primary follicles, antral follicles, and corpora lutea (Sprague-Dawley).	Regulatory derived NOAELs from chronic assays with unknown end points.	Pascotto et al., 2015
6 Pesticides (Cyromazine, MCPB, pirimicarb, quinoclamine, thiram, and ziram)	0.05x BMD5, 0.16x BMD5, 0.375x BMD5 *Main study only	0.5 - 17.5 mg/kg/d (0.05x BMD5), 1.9 - 64.8 mg/kg/d (0.16x BMD5), 3.75 - 131.25 mg/kg/d (0.375x BMD5), GD7 - PND16, daily dosing of corn oil dose solution, as a mixture only	n = 22 pregnant dams per group, Rat (Pup Wistar (HanTac:WH)), Parental P.O.	Same study as Svingen et al., 2018. Reduced gestational weight gain at 0.16x and 0.375x BMD5, and increased weight gain from PND1 -24 at 0.375x BMD5 (dams). Dose-dependent decrease in birth weights, pup body weight at PND6 at 0.16x and 0.375x BMD5, and pup body weight at PND14 and PND27 at 0.375x BMD5.	Benchmark doses from regulatory Draft Assessment Reports for 5% reduction in birthweight.	Hass et al, 2017
				Same study as Hass et al, 2017. Reduced body weights at 0.375x BMD5 at PND16 (both sexes). Reduced relative retroperitoneal fat pad weights at 0.375x BMD5 (both sexes), and relative liver weights at 0.16x and 0.375x BMD5 at PND16 (males). Decreased hepatic glycogen accumulation (males), and increased plasma leptin (females) at 0.375x BMD5 at 4 months.		Svingen et al., 2018
6 Pesticides (Ziram, chlorpyrifos, thiacloprid, boscalid, thiofanate, and captan)	1x TDI	0.006 - 0.1 mg/kg/d, 12 month, via dietary, as a mixture only	n = 18, Mouse (♀ adult WT and Car KO C57Bl/6J), P.O.	WT: Increases in body weight from week 20, overall body weight gain, and terminal relative epididymal and subcutaneous white adipose tissue weights (males). Reduced fasting blood glucose levels from 36 weeks (both sexes), and decreased glucose tolerance from 16 weeks (males), and 48 weeks (females). Increased hepatic steatosis, TG, and ALT (males). Increased reduced GSH, decreased oxidised GSH, and decreased reduced: oxidised GSH ratios in the liver (females). 5 clusters of differentially regulated lipid species identified in livers at 12 months (both sexes). Increased hepatic expression of 536 (males) and 670 (females) genes; 43 common to both sexes. Decreased hepatic expression of 511 (males) and 853 (females) genes; 38 common to both sexes. Pathway enrichment analysis indicated enrichment in DNA replication, extracellular matrix, cytoskeleton constituent, ER membrane, oxidoreductase, and nucleotide binding pathways (males), and peroxisome, mitochondrion, FA β-oxidation, isomerase, translation, and Acyl-CoA metabolism pathways (females). Car KO: Increased body weight and reduced survival (females). Restored fasted blood glucose levels (males). Reduced hepatic expression of Cyp2b9, Cpy4a10 (males), Cyp4a10, Cpt1, and Acox1 (females). Restored glutathione redox states (both sexes).	TDIs from regulatory determinations.	Lukowicz et al., 2018
1 Heavy Metal and 1 Flame Retardant (DecaBDE and lead)	< 0.017x TDI / RfD	6 and 60 ng/kg/d, GD9.5 - PND21, via osmotic pump, individually and as a mixture	n = 12 pregnant mice per group, Mouse (♂ Pup C57Bl6), Parental S.C.	Reduced relative brain weights. Decreased neuronal cells in hippocampal CA1 and CA3 sub-regions. Increased serum cytokine IL4, IL6, IL10, IL17A, TNFα, and IFNγ levels.Increased repetitive stereotyped behaviours. Reduced spatial learning ability.	TDI and RfD from regulatory determinations.	Chen et al., 2019
6 Pesticides (Diquat, imazamox, bentazone, imazethapyr, tepraloxydim, and acifluorfen)	0.01x NOAEL (1x TDI)	0.002 - 10.68 mg/kg/d, 9 month, via water, as a mixture only, with either 100% or 25% RDI of vitamins	n = 50 per group, Rat (♂ Juvenile Wistar), P.O.	Reduced locomotor activity in vitamin deficient controls and vitamin sufficient treated, but increased in vitamin deficient treated. Increased spatial orientation activity in vitamin deficient treated. Increased anxiety in both vitamin deficient groups, treatment caused reductions in anxiety compared to respective groups. Reduced long-term memory in vitamin deficient treated.	Regulatory derived NOAELs for unknown end points.	Tsatsakis et al., 2019a
6 Pesticides (Diquat, imazamox, bentazone, imazethapyr, tepraloxydim, and acifluorfen)	0.25x NOAEL, 1x NOAEL	0.0475 - 267 mg/kg/d (0.25x NOAEL), 0.19 - 1068 mg/kg/d (1x NOAEL), 12 month, via drinking water, as a mixture only	n = 13 per group, Rat (Juvenile outbred albino), P.O.	Decreased problem solving at 3 and 6 months at 0.25x and 1x NOAEL, and at 12 months at 1x NOAEL. Improved problem solving from 3 months to 12 in 2/3 trails at 0.25x NOAEL. Decreased researching activity at 3 and 6 months in 0.25x and 1x NOAEL dose groups, by 12 months both groups showed mixed results of increased and decreased research activity. Decreased anxiety related behaviour at 3 months at 0.25x and 1x NOAEL, and mixed results of increased and decreased anxiety at 6 and 12 months. Anxiety increased with repeated testing in controls at 3, 6, and 12 months, whereas repeated testing produced reduced anxiety at 0.25x and 1x NOAEL.	Regulatory derived NOAELs for unknown end points.	Sergievich et al., 2020

Component Based Methodologies						
Mixture	Relevant group(s) & POD(s) used	Exposure and duration	Number, species, route	Significant Mixture Findings	Comments	Reference
Complex Mixtures (≥10 components)						
16 Organochloride Pesticides, and 2 Heavy Metals (DDT, DDE, HCB, TCDD, PCBs, methoxychlor, endosulfan, heptachlor, HCH, dieldrin, aldrin, mirex, chlorinated benzenes, lead, and cadmium)	1x TDI, RfD, MRL, or NOAEL	0.1 ng - 2.3 µg/kg/d, 70 day, daily dosing of corn oil dose solution, as a mixture only	n = 9 - 10 per group, Rat (♂ Juvenile Sprague Dawley), P.O.	Same study as Wade et al., 2002b. No effects noted at this dose level.	PODs from regulatory determinations.	Wade, et al., 2002a
				Same study as Wade et al., 2002a. Increased circulating TSH. Increased median thyroid follicle area. Reduced hepatic thyroxine outer-ring deiodinase activity.		Wade, et al., 2002b
12 Pesticides (Alachlor, atrazine, carbofuran, chlorpyrifos, diazinon, dicofol, endosulfan, iprodione, mancozeb, maneb, procymidone and rotenone)	1x TDI	0.25 - 150 µg/kg/d, 8 week, daily dosing of 1% CMC dose solution, as a mixture only, with a Solt–Farber model of hepatocellular carcinoma (diethylnitrosamine (200 I.P.), 2-acetylaminofluorene (P.O.), and a 70% partial hepatectomy)	n = 5 - 9 per group, Rat (♂ Adult F344), P.O.	No effects of mixture on carcinogenesis seen.	TDIs from regulatory determinations.	Perez-Carreon et al., 2009
3 Plasticisers, 6 Pesticides, 2 Pollutants, 1 Preservative, and 1 Analgesic (DBP, DEHP, vinclozolin, prochloraz, procymidone, linuron, epoxiconazole, OMC, 4-MBP, DDE, BPA, butylparaben, and APAP)	1x NOAEL / <LOAEL (Based on PODI calculations)	0.09 - 18 mg/kg/d + 120 mg/kg/d, GD7 -20 (GD13 - 19 APAP), daily dosing of corn oil dose solution, as a mixture only	n = 14 pregnant dams per group, Rat (Pup Wistar), Parental P.O.	Increased number of nipples at birth and nipple retention at PND13 (males).	Literature derived NOAELs for developmental / reproductive toxicity	Christiansen et al., 2012
8 Heavy Metals, 4 Pollutants, 3 Pesticides, 2 Food-Derived Carcinogens, 2 Plasticisers, 2 Preservatives, 2 Surfactants, 1 disinfectant, 1 Food Additive, 1 Photostabilizer, and 1 Polycyclic Musk (Acrylamide, BP-3, BPA, triclosan, OPP, trans-nonachlor, DDE, 2.4.6-TCP, 3-PBA, arsenic, barium, cadmium, cesium, cobalt, lead, mercury, thallium, PFOS, PFNA, MBP, AHTN, PCB153,TCDD, BaP, PhIP, and MeIQX)	< 1xTDI (Based on comparison to measured concentrations in human samples)	0.01 - 20 µg/kg/d, 14 week, daily dosing of corn oil solution, as a mixture only	n = 10 per group, Rat (♂ Juvenile Wistar (HanTac:WH)), P.O.	Increased relative liver weight. Hepatic macrovascular changes and vacuolisation. Decreased hepatic expression of Osmr and Car3, and increased Cyp1a2, Avpr1a, Nqo1, Rhbg, Abcc3, and Serpine1. Separation of metabolomic profile of plasma lipids at 30, 60 and 90 days. Individual lipid quantification analysis identified one, unidentified lipid, increased at 30 days.	Exposure at levels calculated to best reflect levels detected in biomonitoring of human exposure.	Hadrup et al., 2016
5 Pesticides, 3 Pharmaceuticals, 9 Phthalates, and 1 Pollutant (Linuron, prochloraz, procymidone, pyrifluquinazon, vinclozolin, finasteride, flutamide, simvastatin, DPP, DCHP, DEHP, DBP, BBP, DIBP, DIHP, DnHP, DHPP, and DDE)	≤0.125x NOAEL, ≤0.25x NOAEL, ≤1x NOAEL	0.000325 - 5 mg/kd/d (≤ 0.125x NOAEL), 0.00075 - 10 mg/kd/d (≤ 0.25x NOAEL), 0.0015 - 20 mg/kd/d (≤ 0.5x NOAEL), 0.003 - 50 mg/kd/d (≤ 1x NOAEL), GD14 - 18, daily dosing of corn oil dose solution, as mixtures only.	n = 5 - 11 pregnant dams per group, Rat (♂ Pup Sprague-Dawley), Parental P.O.	Reduced testes, epididymus, and levator ani plus bulbocavernosus muscles (LABC) weights (PND21) from 0.125x NOAEL, reduced AGD (PND2) and glans penis weights (PND21) from 0.25x NOAEL, and increased NR (PND13), seminal vesicle weights and totl malformation rates (PND21) at 0.5x and 1x NOAEL. Differential expression of genes in fetal testis related to testosterone synthesis, cholesterol uptake and transport, conversion of cholesterol to pregnenolone, and the adrenal enzymes; 14 genes at 1x NOAEL, 3 genes at 0.5x NOAEL, and 2 genes at 0.25x NOAEL.	Components in proportion to regulatory LOAEL values.	Conley et al., 2018

Component Based Methodologies						
Mixture	Relevant group(s) & POD(s) used	Exposure and duration	Number, species, route	Significant Mixture Findings	Comments	Reference
Complex Mixtures (≥10 components) Continued						
4 Preservatives, 6 Pesticides, 1 Plasticiser, 1 Food Additive, and 1 Chelating Agent (Carbaryl, dimethoate, glyphosate, methomyl, methyl parathion, triadimefon, aspartame, sodium benzoate, EDTA, ethylparaben, butylparaben, BPA, acacia gum)	0.25x TDI, 1x TDI, 5x TDI	0.00025 - 8.5 mg/kg/d (0.25x TDI), 0.001 - 34 mg/kg/d (1x TDI), 0.005 - 170 mg/kg/d (5x TDI), 18 month, via drinking water, as a mixture only Docea et al., 2018 - 6 month Docea et al., 2019 - 12 month Tsatsakis et al., 2019a - 12 month Fountoucidou et al. 2019 - 12/18 month Tsatsakis et al., 2019b - 18 month	n = 5 per group, Rat (Sprague Dawley), P.O.	Increased body weight gain at all doses (males). Decreased food consumption at 1x (both sexes) and 5x TDI (females). Reduced water consumption at 0.25x (females), 1x (both sexes), and 5x TDI (females). Decreased serum cholesterol at 0.25x and 5x TDI, and increased ALT at 0.25x and 1x TDI, ALP at 0.25x TDI, and bilirubin at 5x TDI (males). Decreased serum protein carbonyls at 0.25x (females), 1x, and 5x TDI (both sexes), total antioxidant capacity at 1x and 5x TDI (both sexes). Increased serum catalase at 0.25x TDI (both sexes), but decreased at 1x (males) and 5x TDI (both sexes).	TDIs from regulatory determinations.	Docea et al., 2018
				Increased body weight at 0.25x (both sexes), 1x (males), and 5x TDI (both sexes). Male growth rate coefficient increased at 1xTDI). Female growth rate coefficient decreased at 0.25x and 5x TDI. Increased age dependent reduction in food consumption at 0.25x (both sexes), 1x (males), and 5x TDI (both sexes). Increased age dependent reduction in water consumption at all doses (both sexes). Increased serum ALP, AST and ALT at 0.25x TDI, and Na+ at 5xTDI, and decreased urea nitrogen at 0.25x TDI (males). Increased serum ALP at 0.25x and 5x TDI, ALT at 0.25x TDI, and TG, Cl-, and Na+ at 1x TDI, and decreased CRE at 0.25x TDI, and TP at 5x TDI (females). Serum pseudocholinesterase was decreased at 0.25x and 1x TDI in males, but increased at 0.25x TDI in females and 5x TDI in males.		Docea et al., 2019
				At 12 months in blood: Dose dependent increase in GSH at all doses (both sexes), and an increase in catalase activity (CAT) at 0.25x (females) and 1x (both sexes) TDI. Decreases in protein carbonyls (Crbnls) at 1x TDI (females), thiobarbituric acid reactive substances (TBARS) at 0.25x (males), 1x (both sexes), and 5x TDI (females), and total antioxidant capacity (TAC) at 1x and 5x TDI (both sexes). At 18 months: In blood: Dose dependent decrease in CAT in 1x and 5x TDI (both sexes), an increase in Crbnls at 5x TDI (both sexes), and decreases in GSH at 0.25x and 1x TDI (females), and TBARS at 1x and 5x TDI (both sexes). In muscle: Dose dependent increase in GSH at all doses (both sexes), increased TBARS at 0.25x (females) and 1x TDI (males) but reduced at 5x TDI (males). Decreased TAC at 5x TDI (males). In liver: Reduced GSH at 0.25x (female) and 5x TDI (both sexes), and increased CAT in 0.25x (females), 1x, and 5x TDI (both sexes), and Crbnls at 0.25x, 1x (males), and 5x TDI (both sexes). In heart: Increased CAT at 1x TDI (males), Crbnls at 5x TDI (both sexes), TBARS at 1x (females) and 5x TDI (both sexes). In kidney: increased GSH at 1x and 5x TDI (both sexes), Crbnls at 1x TDI (both sexes), and TAC at 1x TDI (males). In brain: Increased GSH at 0.25x (females), 1x, and 5x TDI (both sexes), CAT at 0.25x TDI (males), Crbnls at 1x and 5x TDI (females), and TAC at 1x TDI (females). In pancreas: Increased GSH at 0.25x (males) and 1x TDI (females), CAT at 5x TDI (females), TBARS at 5x TDI (both sexes) and Crbnls at 0.25x but reduced at 1x and 5x TDI (both sexes). In lung: Increased GSH at 0.25x (females), 1x (both sexes), and 5x TDI (females), and decreased CAT at all doses (females), and TAC 0.25x (females), 1x (males), and 5x TDI (females). In spleen: Increased GSH at 0.25x, 1x (females), and 5x TDI (both sexes), and Crbnls at all doses (females), and decreased CAT at all doses (females), TBARS at 0.25x (males), and TAC at 0.25x (males), and 1x TDI (females). In stomach: Increased GSH at all doses (both sexes), Crbnls at 0.25x (females), 1x, and 5x TDI (both sexes), and TBARS at 0.25x (males) and 1x TDI (females), and decreased CAT at 5x TDI (females).		Fountoucidou et al., 2019
				Increased exploratory behaviour in open field exploratory test by three metrics at 0.25x TDI, and by one metric at 1x TDI.		Tsatsakis et al., 2019b
				Increased frequency of micronuclei in bone marrow at 5xTDI (females). Increased marrow cytotoxicity at all doses (both sexes), as evaluated by a decreased proportion of immature to total erythrocytes. Dose-dependent worsening of histopathological observations in testis, kidneys, lungs, and brains at all doses, and in the liver and stomach at 1x and 5x TDI (both sexes).		Tsatsakis et al., 2019c

Whole Mixture Methodologies			
Number, strain, route, duration	Exposure	Significant Mixture Findings	Reference
Biosolid Treated Pasture Sheep Model			
n = 10 per group, Texel-cross (5 Month lambs), Parental and direct P.O..Ewes: 2.5 - 5 years prior to mating, lambs: gestation + 5 months	Undetermined	Increased birth weights (both sexes). Increased vocalisation and lower maximal activity levels while restrained within weigh crate (both sexes). Increased exploratory behaviour (males).	Erhand and Rhind, 2004
n = 12 per group, Strain not mentioned (♂ fetuses at GD110), Parental P.O., Ewes and rams: 5 years prior to mating	Undetermined	Reduced foetal body and relative testis weights, and reductions in testicular cell numbers (Sertoli cells, Leydig cells, and gonocytes). Decrease in serum testosterone and inhibin A levels. Lower immunostaining for androgen receptor in interstitial and peritubular cells, and α-smooth muscle actin in peritubular cells.	Paul et al., 2005
n = 8 - 15 per group, Texel (♀ fetuses at GD110), Parental P.O. Ewes and rams: 5 years prior to mating	Undetermined	Reduced foetal body weight. Decreased serum prolactin levels. Reduced total oocyte and type 1 MCL1-positive oocyte densities, and oocyte ratios. Reduced numbers of GDF9 positive oocytes, and induced numbers of MCL1 positive oocytes. Increased BAX and decreased SOD2 protein levels in ovaries. Ovarian proteomic identification of differential expression in pathways relating to cytoskeleton and its regulation, gene expression, transcription and processing, protein synthesis, and protein phosphorylation and receptor activity.	Fowler et al., 2008
n = 7 - 8 ewes and n = 12 - 17 fetuses per group, Strain not mentioned (Ewes and fetuses at GD110), Parental P.O. Ewes and rams: 5 years prior to mating	Undetermined	Reduced expression of KiSS-1 in all regions of the foetal hypothalamus and pituitary, and reduced expression of kisspeptin in foetal pituitary LHβ- and ERα-positive cells. Reduced total foetal pituitary LHβ- and ERα-positive cells.	Bellingham et al., 2009
n = 12 per group, Texel (18 Month Yearlings), Parental and direct P.O., Gestation + 18 months	Undetermined (30 components quantified in biosolids, spread at 2.25 tonnes/ha, twice annually: PCBs, PBDEs, and PAHs at 2 - 2171 µg/kg dry mass)	Increased trabecular and diaphysicular total bone mineral content, and cortical thickness, and reduced trabecular and cortical total cross-sectional areas, periosteal and endosteal circumferences, and marrow cavities in femurs (males).	Lind et al., 2009
n = 12 per group, Texel (♂ 19 Month Yearlings), Parental and direct P.O. Ewes and rams: whole of breeding lives, Lambs: gestation + 7 months	Quantification of 31 components in the liver (PAHs, PBDEs, and PCBs at 0.015 - 216 µg/kg dry mass)	High occurrence of seminiferous tubules which consisted of Sertoli cells only. Reduced total germ cell number and volume, and germ cell number and volume relative to Sertoli cells, in a subset of treated animals (5 of 12).	Bellingham et al., 2012
n = 9 - 16 per group, Texel (fetuses at GD110), Parental P.O. Rams: whole of breeding lives. Ewes: prior breeding lives + continuing through gestation (TT), pre-conception only (TC), and post-conception only (CT)	Quantification of 31 components in soil (PAHs, PBDEs, PCBs, and DEHP at 1.07 - 3409 µg/kg dry mass) (Rhind et al., 2010)^relative quantification, but not absolute, reported for maternal and fetal liver load	Same study as Bellingham et al., 2013 and 2016. Increased relative thyroid weights in males from TC ewes. Reduced plasma free tetraiodothyronine in TC and CT ewes, and reduced plasma free triiodothyronine in CT ewes only. Reduced area of small blood vessels in thyroids of fetuses of TC and TT ewes. Reduced thyroid follicle number and size in males from TC and CT ewes. Increase in the percentage of medium sized follicles for all treatment groups (when both sexes combined). Increased thyroid cell proliferation in females from TC and CT ewes, and in males from all treatment regimes.	Hombach-Klonisch et al., 2013
		Same study as Hombach-Klonisch et al., 2013 and Bellingham et al., 2016. Female fetuses only. Increased ovary weight and reduced plasma luteinising hormone in TC ewes. Higher progesterone in TC and TT ewes. Increased ovary weight and plasma inhibin A in fetuses from TT ewes. Increased ovarian levels of proteins involved in stress response, oocyte maturation, and apoptosis in all treatment groups. Reduced type 1a ovary follicle density in fetuses from CT ewes, and type 1a follicles accounted for a lower percent of total follicles fetuses from TC and CT ewes. Fetuses from CT ewes had increased numbers of unhealthy type 0 follicles, and fewer unhealthy type 2 follicles. Proteomic analysis revealed 90 differentially expressed proteins within 5 major molecular/cellular functional categories: small molecule biochemistry, post-translational modification, protein folding, drug metabolism, and lipid metabolism. Pathway analysis identified two functional networks: cancer, gastrointestinal disease, and cellular movement, and cancer, genetic disorder, and respiratory disease. Expression of 14 differentially expressed proteins was confirmed at the mRNA level.	Bellingham et al., 2013
		Same study as Hombach-Klonisch et al., 2013 and Bellingham et al., 2013. Increased luteinising hormone in males from TC and CT ewes, and increased oestradiol in males from TT ewes. Decreased oestradiol in females from TT ewes. Increased expression of GnRH in the hypothalamic preoptic area of females from CT and TT exposed ewes. Reduced expression of GnRH in the hypothalamic preoptic area of males from TT ewes, and reduced in the hypothalamic arcuate nucleus of males from ewes in all treatment groups. Decreased pituitary GnRHR expression in females from CT and TT exposed ewes, and in males from CT ewes. Increased expression of KISS1 in hypothalamic preoptic area of males from TC and CT ewes, and reduced in males from TT ewes. Increased expression of KISS1 in hypothalamic arcuate nucleus of males from CT ewes, and reduced in males from TT ewes. Reduced expression of KISS1R in hypothalamic preoptic area of males from TT ewes. Increased expression of ERα in hypothalamic preoptic area of females from TT ewe, and decreased in the pituitary of females from all treatment groups. Decreased expression of ERα in hypothalamic arcuate nucleus of males from TC and TT ewes. Increased expression of AhR in hypothalamic arcuate nucleus of males from CT ewes, and decreased in the pituitary of females from all treatment groups.	Bellingham et al., 2016

Biosolid Treated Pasture Sheep Model Continued			
Number, strain, route, duration	Exposure	Significant Mixture Findings	Reference
Biosolid Treated Pasture Sheep Model Continued			
n = 8 - 11 per group, Texel (♀ fetuses at GD140), Parental P.O., GD0-80 (early), GD30-110 (mid), GD60-140 (late), GD0-140 (continuous).	Quantification of 31 components in soil (PAHs, PBDEs, PCBs, and DEHP at 0.14 - 3162 µg/kg dry mass). Relative quantification, but not absolute, reported for maternal and fetal liver load	Fetal body weight reduced in early, mid, and late exposure groups. Increased normalised fetal anogenital distances in mid and late exposure groups. Reduced relative fetal liver weight in continuously exposure group. Increased relative liver, thyroid, and uterine weight in late exposure group. Fetal plasma testosterone was increased in the mid exposure group. Reduced free triiodothyronine and increased free tetraiodothyronine in the late exposure group. Reduced proportion of healthy type 1a ovarian follicles in all exposure groups, and increased proportion of atretic type 1a follicles in early, mid, and late exposure groups. Transcriptome analysis produced 256 differentially expressed genes (60 up- and 196 down-regulated), with little overlap between groups; 22 differentially expressed genes in common across all exposure groups. Pathway analysis highlighted enrichment within cellular growth and differentiation, cell cycle regulation, cell death, cellular development, and cell movement functions. ERK/MAPK and PI3K/AKT signalling, growth hormone signalling, and actin cytoskeleton signalling pathways were the most effected. Proteomic analysis produced 64 differentially expressed proteins (53 up- and 11 down-regulated), with little overlap between groups; 2 differentially expressed proteins in common across all exposure groups. Pathway analysis highlighted enrichment in free radical scavenging, cell-to-cell signalling and interaction, small molecule biochemistry, drug metabolism, and protein synthesis functions.	Lea et al., 2016
n = 10 - 12 per group, Texel (19 Month Yearlings), Parental and direct P.O., Gestation + 7 months (♂) or 19 months (♀)	Quantification of 31 components in the liver (PAHs, PBDEs, PCBs, and DEHP at 0.51 ng - 144 µg/kg dry mass)	Same study as Bellingham et al., 2012. Increased hepatic expression of CYP1A1 (females), AhR, and OATP8 (males), and decreased expression of CYP1B1 (females). Increased hepatic protein levels of AFP (females) and PCK1 (males). Proteomic analysis of livers revealed 185 (females; 98 up- and 87 down-regulated) and 97 (males; 57 up- and 40 down-regulated) differentially expressed proteins. 35 of the 49 most expressed of differentially expressed proteins were identified as 26 unique proteins, many involved in detoxification and fatty-acid β-oxidation. Pathway analysis predicted dysregulation of cancer-related pathways, increased production of reactive oxygen species, and decreased concentration of lipids (females), and reduction and dysregulation of cancer-related pathways, increased lipid synthesis and increased fatty acid metabolism (males). Decreased total hepatic lipids (females), and increased hepatic albumin and transferrin (males). Increase of less sialylated transferrin, and decrease of more sialylated transferrin (females).	Filis et al., 2019
Number, species, route, duration	Exposure	Significant Mixture Findings	Reference
Drinking water disinfection by-products			
n = 19 - 20 pregnant dams per group, GD 6-16, via drinking water, as a mixture only Rat (♀ Dam Sprague-Dawley, and pups), Parental P.O., GD 6-16, via drinking water, as a mixture only	Concentrated ozonated/postchlorinated and chlorinated water. Approx. 130x higher concentrations of DBPs from ozonated/postchlorinated and chlorinated water.	Increased water consumption in both treatment groups. Reduced gestation length with concentrated ozonated/postchlorinated water. No adverse developmental effects from any treatment.	Narotsky et al., 2008
n = 11 - 36 pregnant dams per group, Rat (♀ Dam Sprague-Dawley and F344, and pups), Parental P.O., GD1, GD4, or GD5 - PND21, via drinking water, as a mixture only	Concentrated chlorinated water. Approx 120x higher concentrations of DBPs from chlorinated water by two concentration methods.	For water chlorinated and then concentrated: maternal diarrhea and polyuria in both strains - concluded most likely due to sodium and sulphate levels by dose-range finding study. In F344 rats; lower maternal body weight at GD20 - PND6, increased gestation length, and reduced pup weight at PND6. In Sprague-Dawley rats; Fewer live pups at PND6 with increased perinatal loss/mortality, and reduced pup weight at PND1 and PND6. No effects for water concentrated and then chlorinated.	Narotsky et al., 2012
n = 40 - 60 pregnant dams per group, Rat (♀ Dam Sprague-Dawley, and F1 and F2 pups), Parental P.O., F1 GD2 - F2, via drinking water, as a mixture only	Concentrated chlorinated water. Approx. 130x higher concentrations of DBPs from chlorinated water.	Delayed puberty in females, reduced caput sperm counts in adult males, and thyroid follicular cell hypertrophy in adult females. Increased birthweights in F2 offspring.	Narotsky et al., 2013