Pollutant Impact on the Postnatal Ovine Testis: Utilising a Sheep Model to Explore Multi-generational Effects

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Elizabeth Proctor

20235674

Supervisors:

Professor Kevin Sinclair

and

Professor Richard Lea

School of Bioscience, Faculty of Science

University of Nottingham

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Abstract

In-utero exposure to environmental chemicals is frequently associated with reproductive disorders and reduced fertility. Due to the complexity of real-life mixtures of environmental chemicals, studies utilising models with environmentally relevant concentrations of chemicals are vital.

This study employed immunohistochemistry and qPCR to examine the effects of exposure to a mixture of environmental chemicals present in biosolids at the gross, cellular and molecular levels in 8-week-old male lambs, across the two subsequent generations (i.e., F1 and F2) when exposure was limited to the F0 ewes' pregnancy ensuring all exposure was only in-utero. In exposed F1 male lambs, testis weight and body weight were both significantly reduced. F2 lambs sired by F1 exposed lambs had greater testis weight and body weights. Histologically, in the F1s there was no significant difference in mean Sertoli cell numbers and though there was a noticeable decline in the staining of Leydig cell marker CYP17A1 this was not statistically significant. In the F2 generation no change in Sertoli cell number was observed, however there was a notable decrease in CYP17A1 staining in the Biosolids group indicating a reduction in Leydig cell population. At the molecular level, in the F1 testis, there was a significant increase in the relative expression of Hexokinase-1 (*HK1*), an kinase which phosphorylates glucose in the first step of glycolysis, and a significant decrease in the relative expression of Calcium and Integrin Binding protein 1 (*CIB1*) a binding protein thought to be involved in the proliferation and survival of germ cells. Whilst in the F2 lamb testis, only the relative expression of CUG-BP, Elav-like family 1 (*CELF1*) was altered.

Reductions in Sertoli cell and Leydig cell populations have previously been observed at the foetal level. The lack of significant differences seen in somatic testicular cell populations in this study in 8-week-old F1 lambs and in other studies in adult rams suggests that during the first 8-weeks of post-natal development there is a degree of compensation. Decreased CYP17A1 staining in the F2 testis potentially highlights a multigenerational effect of gestational exposure to the biosolids mixture. Overall, this study's findings suggest the effects of low-level gestational exposure to environmental chemicals on somatic testicular cells may be corrected in the pre-pubescent period in the F1 generation. However, some effects may persist into the F2 generation potentially causing adverse outcomes on adult fertility.

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List of Abbreviations

Abbreviations	Definition
17β-HSD	17-β hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
4NP	4-nonylphenol
ABC	Avidin-Biotin Complex
ABP	Androgen Binding Protein
ACTB	Beta-ACTIN
AhRs	Aryl hydrocarbon receptors
AMH	Anti-mullerian Hormone
AMHR	Antimullerian Hormone Receptor
AMPK	AMP-activated protein kinase
AR	Androgen Receptor
ARE	Androgen Response Element
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
ARRIVE	Animal Research: Reporting of In Vivo Experiments
AWERB	Animal Welfare and Ethical Review Board
B2M	Beta-2-Microglobulin
BCL11A	BCL11 Transcription Factor A
BPA	Bisphenol A
BTB	Blood-Testis-Barrier
cAMP	Cyclic adenosine monophosphate
CELF1	CUG-BP, Elav-like family 1
CIB1	Calcium and Integrin Binding protein 1
CREB1	CAMP responsive element binding protein 1
CREB3L3	CAMP Responsive Element Binding Protein 3 Like 3
CYP11A1	Cytochrome P450 Family 11 Subfamily A Member 1
CYP17A1	Cytochrome P450 Family 17 Subfamily A Member 1
CYP19A1	Cytochrome P450 Family 19 Subfamily A Member 1
CYP1B1	Cytochrome P450 Family 1 Subfamily B Member 1

DAB	Diaminobenzidine
DBP	Dibutyl phthalate
DDT	Dichlorodiphenyl-dichloroethylene
DEFRA	Department for Environment, Food and Rural Affairs
DEGs	Differentially Expressed Genes
DEHP	Di-2-ethylhexyl Phthalate
DEP	Diethyl phthalate
MBP	Monobutyl phthalate
DES	Diethylstilbesterol
DHEA	Dehydroepiandrosteron
DHH	Desert Hedgehog
DHT	Dihydrotestosterone
DPX	Dibutylphthalate Polystyrene Xylene
EMC:	Extracellular matrix
EPA	Environment Protection Agency
EPAS1	Endothelial PAS Domain Protein 1
ErbB	Erb-B2 Receptor Tyrosine Kinase 2
FASN	Fatty Acid Synthase
FGF9	Fibroblast Growth Factor 9
FLC	Foetal Leydig cells
FOSL1	FOS Like 1, AP-1 Transcription Factor Subunit
FOXA1	Forkhead Box A1
FOXK1	Forkhead Box K1
FOXP2	Forkhead Box P2
FSH	Follicle Stimulating Hormone
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GD	Gestation Day
GnRH:	Gonadotropin Releasing Hormone
GO	Gene Ontology
Gsk3	Glycogen synthase kinase 3
hCG:	human Chorionic Gonadotropin
HIF1α:	Hypoxia Inducible Factor 1 Subunit Alpha

HK1:	Hexokinase-1
HPG axis:	Hypothalamic Pituitary Gonadal axis
HRP	Horseradish peroxidase
IDT	Integrated DNA Technology
IHC	Immunohistochemistry
IMS	Industrial Methylated Spirits
INSL3:	Insulin-like Hormone 3
JUND	JunD Proto-Oncogene, AP-1 Transcription Factor Subunit
LH:	Luteinising Hormone
MEHP	Mono-2-ethylhelyl phthalate
MIH:	Mullerian Inhibitory Hormone
MMP2	Matrix metallopeptidase 2
MPW:	Masculine Programming Window
mTOR:	Mechanistic Target of Rapamycin Kinase
mTORC	mTOR Complex
NRF1	Nuclear Respiratory Factor 1
PAH:	Polycyclic Aromatic Hydrocarbons
PBB	Polybrominated biphenyl
PBDE	Polybrominated Diphenyl Ethers
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline and Tween
PCB	Polychlorinated Biphenyls
PDPK1	3-Phosphoinositide Dependent Protein Kinase 1
PGD2	Prostaglandin D2
PIK3CG	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Gamma
PKA	Protein Kinase A
PMC	Peritubular Myoid Cells
PND	Post-Natal Day
PPAR	Peroxisome Proliferator Activated Receptor
PPRC1	PPARG Related Coactivator 1
qPCR	quantitative Polymerase Chain Reaction

RBM23	RNA Binding Motif Protein 23
Rheb	Ras Homolog, MTORC1 Binding
RICTOR	Rapamycin-insensitive companion of mTOR
rpS6	Ribosomal Protein S6
SCO	Sertoli cell only tubules
SF-1	Steroidogenic Factor 1
SOX9	SRY-Box Transcription Factor 9
SRY	Sex determining region Y
STAR	Steroidogenic Acute Regulatory protein
TDF	Testis-Determining Factor
TDS	Testicular Dysgenesis Syndrome
TF	Transcription Factor
TGCT	Testicular Germ Cell Tumours
TSC	Tuberous Sclerosis Complex
TSPO	Translocator Proteins
USEPA	United States Environmental Protection Agency
VEGFA	Vascular Endothelial Growth Factor A
WHO	World Health Organization
Wnt4	Wingless-related MMTV integration site 4
β-ME	β-mercaptoethanol

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1. Introduction

In recent years there has been a rising concern over falling fertility rates in humans with particular attention being drawn to male infertility (Mann et al., 2020, Aitken, 2022). Sperm count and sperm concentration are largely considered to be declining at an increasing rate in recent years. Issues with subfertility have been documented globally with sperm concentration decreasing by approximately 52% between 1973 and 2011 in Europe, North America, Australia and New Zealand (Levine et al., 2017). Declines in the percentage of sperm with normal morphology and motility have also been observed across the globe (Adiga et al., 2008, Morbeck et al., 2011, Geoffroy-Siraudin et al., 2012, Sugihara et al., 2021). Due to the temporal nature of these studies, some suggest that the decline seen may arise or be impacted by human error and differing methodologies for parameters such as sperm count (Pacey, 2013) and sperm morphology (Levine et al., 2023).

There has also been an increase in the incidence of testicular dysgenesis syndrome (TDS), a combination of reproductive tract malformations including, hypospadias, cryptorchidism, testicular cancer and impaired/reduced spermatogenesis (Sharpe, 2010a, Ferguson and Agoulnik, 2013, Skakkebaek et al., 2016). The shared prenatal origin of these symptoms has led to investigations into potential disruptions to the normal development of the reproductive system in the foetus (Skakkebaek et al., 2016).

The development of the reproductive system relies on the expression of several developmental genes, transcription factors and hormonal signalling (Biason-Lauber, 2010, Svingen and Koopman, 2013, Moses and Behringer, 2019). Disruption to any of these factors can greatly impact the function of the reproductive system during the male reproductive life. The hormones produced by Leydig cells, particularly testosterone, are vital for the masculinisation of the reproductive tract and the descent of the testes (Svingen and Koopman, 2013, Grinspon et al., 2020). A lack of sufficient testosterone signalling during the masculinisation programming window, a key foetal developmental period in which sufficient androgen signalling is required for normal reproductive development in males, can lead to the development of cryptorchidism and hypospadias (Vinggaard et al., 2000, Sharpe, 2020).

Due to the rapid decline in male fertility, environmental chemicals, particularly those classified as endocrine disrupting chemicals (EDCs), have been identified as a potential factor (Mann et al., 2020). EDCs can have oestrogen-like and anti-androgenic effects, and thus in-utero exposure can lead to insufficient testosterone signalling during the key masculinisation programming window (Sharpe, 2020). This results in malformations of the reproductive tract and the development of TDS symptoms (Vinggaard et al., 2000, Sharpe, 2020). Rodent and human studies have shown that in-utero exposure to a range of individual environmental chemicals such as phthalates, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated Bisphenyls (PCB) can cause reduced testosterone levels, and anogenital distances (AGD), as well as increased incidence of cryptorchidism (Mylchreest et al., 1998, Gray et al., 2000, Moore et al., 2001, Carruthers and Foster, 2005, Culty et al., 2008, Hu et al., 2009, MacLeod et al., 2010, Ferguson and Agoulnik, 2013, Li et al., 2022, Montano et al., 2022). Many of these studies use high concentrations of individual EDCs and are carried out on rodents. This, therefore, may not reflect effects on humans exposed to mixtures of chemicals at environmentally relevant concentrations.

Some studies (Paul et al., 2005, Bellingham et al., 2012, Elcombe et al., 2021, Elcombe et al., 2022, Lea et al., 2022, Elcombe et al., 2023, Evans et al., 2023, Tanner, 2023) with sheep have utilised treated human sewage sludge (biosolids) to mimic a more realistic combination of environmental chemicals at more relevant real-life concentrations. This mixture of chemicals

was applied to forage and fed to pregnant ewes throughout gestation to assess the effects of exposure on the resulting male lambs. Studies have examined multiple ages with some concentrating on the foetus, the neonate, the pre-pubescent lamb and adult rams. Significant differences have been noted in key testicular cell populations including germ cells, Sertoli cells and Leydig cells, however this varies with age (Paul et al., 2005, Bellingham et al., 2012, Elcombe et al., 2021, Elcombe et al., 2022, Lea et al., 2022, Elcombe et al., 2023). Somatic cell numbers are reduced in the foetus but there appears to be no difference in adult rams (Paul et al., 2005, Bellingham et al., 2012, Lea et al., 2022).

A previous study has identified differentially expressed genes and highlighted several enriched GO terms including spermatid development and the mTOR signalling pathway, which is known to be involved in spermatogenesis. Quantification of downstream mTOR genes identified *FASN, HK1, PDPK1* and *VEGFA*, of which three (VEGFA, HK1 and PDPK1) are transcribed via HIF1α activation (Elcombe et al., 2022).

With the foregoing discussion in mind, the current series of experiments sought to identify potential differences in parameters not included in the previous analysis of 8-week-old prepubescent lambs. This involved investigating differences in testis weight, body weight and variation in testicular somatic cell numbers. Molecular analysis included the quantification of the relative expression of steroidogenic markers, genes previously identified through RNAseq to enrich the GO term 'spermatid development' and to confirm the increased expression of downstream mTOR genes. Furthermore, this study was the first to examine the effects of in-utero exposure to environmental chemicals on second generation offspring.

2. Background and Context

2.1. Male Infertility

Infertility is defined by the World Health Organisation (WHO) as an inability to conceive following a minimum of 12 months regular unprotected sexual intercourse (WHO, 2024). It is a growing global concern with 8-12% of heterosexual couples affected by infertility (Agarwal et al., 2021). It is estimated that in the UK, 14% of heterosexual couples are affected by infertility (Wilkes et al., 2009, NICE, 2013). Male infertility contributes to roughly 50% of infertility in couples; with 20-30% of cases being due solely to the male partner's infertility and an additional 20% of cases in which both partners are part of the problem (Agarwal et al., 2021).

The topic of male infertility has been highly debated for decades with various groups publishing evidence to support a decline in semen concentrations (Carlsen et al., 1992, Centola et al., 2016, Levine et al., 2017, Levine et al., 2023) whilst others have found no evidence of a significant decline (Sugihara et al., 2021, Cipriani et al., 2023).

Large meta-regression studies have found evidence of a decline in both total sperm count and sperm concentrations. In men who were previously unaware of their fertility (i.e. general health screening) in North America, Europe, Australia, and New Zealand between 1973 and 2011, Levine et al. identified a significant overall temporal decline in sperm concentration of 52.4% (or -1.4% per annum) and of 59.3% in total sperm count (-1.58% per annum) (Levine et al., 2017). Levine et al. later expanded on their previous study identifying a decline in sperm concentration in men from South/Central America, Asia, and Africa, highlighting a notable increase in percentage decline of sperm concentration and total semen count from post-1972 (1.16% and 1.40% per annum respectively) to post-2000 (2.64% and 2.48% per annum respectively). This continuous and accelerated decline in sperm parameters is a

significant health concern, especially with the increased rate of decline since the year 2000 (Levine et al., 2023).

Although the meta-analyses above focused on sperm concentration and total sperm count, there is also evidence of a decline in other semen parameters such as percentage normal sperm morphology and motility (Adiga et al., 2008, Morbeck et al., 2011, Geoffroy-Siraudin et al., 2012, Sugihara et al., 2021). A study in South India examining semen parameters identified not only a decline of 30.31% in sperm count, but also a decline in sperm motility, and morphology by 22.92% and 51.25% respectively, over 13 years (Kumar and Singh, 2015). Geoffroy-Siraudin and colleagues examined the semen parameters of 10,932 men from infertile couples as well as several samples selected for their normal total sperm count between 1988 and 2007. They identified the rate of decrease per year for multiple semen parameters such as sperm concentration (1.5%), total sperm count (1.6%), total mobility (0.4%) and rapid motility (5.5%). Between 1988 and 2002 the percentage of normally formed sperm declined at a rate of 2.2% per annum from 43.1% to 34.8% with abnormalities appearing in all regions of the sperm. They also identified an increase in the mean number of immature germ cells from 2.1% to 8.5. This study was limited to Marseille a heavily polluted and industrialised region of France (Geoffroy-Siraudin et al., 2012).

The temporal nature of the various studies makes it difficult to directly compare the sperm parameters of interest as methods have varied across the last 50 years and, in older papers, some parameters such as motility and morphology were unavailable (Levine et al., 2017, Levine et al., 2023). As Pacey highlights, the WHO only introduced a standardised methodology in 1980, with multiple revisions up until 2010, to refine semen analysis. Temporal studies which include data pre-dating and throughout this period of refinement, could be impacted (Pacey, 2013). It is estimated that less than 5% of laboratories in the UK and less than 60% in the US comply with the WHO Laboratory manual method for examining and processing human semen. This makes it difficult to compare the results of various studies in real time (Agarwal et al., 2021). How effectively these methodologies are implemented is also up for debate. Training and experience have led to less variable results. As training courses relating to sperm count are relatively new, it is unclear as to the impact this may have had on earlier and even current studies (Pacey, 2013).

Guidance for quality assurance and quality control of andrology was only introduced into the 1999 WHO manual due to rising concerns over significant variation between laboratories examining the same samples (Pacey, 2013). Sperm morphology data can be variable as not only are there multiple assessment systems in use (i.e., Tygerberg strict criteria, and the WHO 2nd and 3rd criteria), but there are also variations within the same lab (Morbeck et al., 2011).

Along with declines in semen parameters previously discussed, there are also rising trends in malformations in the male reproductive system. A combination of some of these symptoms is referred to as testicular dysgenesis syndrome.

2.1.1. Testicular Dysgenesis Syndrome

The term testicular dysgenesis syndrome (TDS) commonly refers to a combination of reproductive problems, namely cryptorchidism, hypospadias, reduced/impaired spermatogenesis, and testicular cancer (Sharpe, 2010a, Ferguson and Agoulnik, 2013, Skakkebaek et al., 2016). Though its likely for an individual to present with one or two symptoms, it is extremely rare for them to have three or all the symptoms of TDS (Ferguson and Agoulnik, 2013). These conditions are commonly grouped due to a suggested shared prenatal origin (Skakkebaek et al., 2016) but the exact mechanisms behind TDS are unclear. It has been linked to disruptions in the hypothalamus pituitary gonadal axis and in the dysfunction of Leydig and Sertoli cells within the foetus (Elcombe et al., 2021).

Links have been made between reproductive disorders such as cryptorchidism and the risk of developing testicular germ cell tumours (TGCT) later in life, with around 10% of TGCT cases occurring in men who have a history of cryptorchidism (Ferguson and Agoulnik, 2013, HooKim and McCue, 2020). It is suggested that this association may be due to a common underlying cause (MacLeod et al., 2010) such as disruption to normal androgen signalling during foetal development (Ferguson and Agoulnik, 2013, Sharpe, 2020). There is evidence that the incidence rates of TGCTs have increased in multiple Western countries including Denmark, Norway, the UK, Iceland, New Zealand, and Australia (Skakkebaek et al., 2016). The rapid increase in incidence suggests an environmental cause as opposed to a genetic factor. Immigration studies have highlighted that men migrating from countries with high TGCT incident rates to countries with low TGCT incidence rates have a comparable risk of TGCT with their home countries (Sharpe, 2010a). However, their male children born abroad will have the same risk of developing TGCT as is present in the host country supporting the likelihood of a foetal origin affected by environmental factors (Skakkebaek et al., 2016).

It is estimated that, globally, 2-4% of boys are diagnosed with unilateral or bilateral cryptorchidism (Ferguson and Agoulnik, 2013). Bilateral cryptorchidism has been linked with infertility and impaired spermatogenesis (Fatma et al., 2015). Animal models have demonstrated that the elevated associated temperatures interfere with spermatogenesis through the inhibition of spermatogonia differentiation, depletion of germ cells, reduction in seminiferous tubule size and fibrosis (Ferguson and Agoulnik, 2013).

It was initially hypothesised that the underlying cause of TDS may be due to disruption to the developing reproductive system in the foetus, specifically the development and differentiation of the Leydig and Sertoli cells (Bay et al., 2006). Disruption to the Leydig cells could result in a lack of expression of Insulin-like hormone 3 (INSL3) which is involved in the transabdominal phase of testicular descent and a lack of androgen production which is needed for the inguinoscrotal phase of testicular descent, causing cryptorchidism. A lack of androgen production can result in the incomplete development of the prostate and external genitalia, such as hypospadias (the abnormal formation of the external urinal orifice) (Bay et al., 2006, Hu et al., 2009, White et al., 2018, Li et al., 2022).

As the evidence suggests a foetal origin of TDS, it is important to understand the development of the male reproductive system.

2.2. The Development of the Male Reproductive Tract

For the purpose of this section, the following information and timeline will focus primarily on the development of the human male reproductive system. However, it is important to emphasise that the fundamental processes will be the same across most mammalian species.

2.2.1. Testis development

In humans, during the fifth week of gestation urogenital ridges form from the intermediate mesoderm near the midline of the posterior abdominal wall (Rey et al., 2009, Pawlina and Ross, 2020, Bastawros, 2024). Primordial germ cells migrate from the yolk sac to these genital ridges (HooKim and McCue, 2020). At the genital ridge there are a minimum of three bipotential precursor cells, supporting cells (which can differentiate into Sertoli cells or granulosa cells), steroidogenic precursors (which can differentiate into Leydig cells or theca cells) and germ cells (which could differentiate into spermatogonia or oogonia) (Svingen and Koopman, 2013).

Following the migration of germ cells, between weeks six and seven, two pairs of undifferentiated accessory ducts form from the mesonephros called the Müllerian (also called paramesonephric) and wolffian (mesonephric) ducts, the embryonic origins of the female and male reproductive tracts respectively (Mitchell and Sharma, 2009, Biason-Lauber, 2010, Bastawros, 2024). Between weeks eight and thirteen (Grinspon et al., 2020), the Müllerian (paramesonephric) duct regresses in the male embryo (Figure 1) as Sertoli cells are stimulated by the transcription factor *SOX9* and steroidogenic factor 1 (*SF-1)* which causes the expression of anti-müllerian hormone (AMH) (Biason-Lauber, 2010). Knockout studies suggest that the SOX9 activates AMH transcription and SF-1 regulates the expression levels of AMH (Moses and Behringer, 2019).

SOX9 and *SF-1* are just two of several developmental genes controlling the determination of sex in the embryo (Val et al., 2003, Rey and Grinspon, 2011). The *Sry* (sex determining region Y) gene, found on the Y chromosome, initiates male sex determination. Activation of this gene in cells from the supporting cell lineage leads to the expression of the transcription factor, testis-determining factor (TDF) or SRY protein. SRY stimulation causes the expression of SOX9. These SOX9-positive cells are the precursors of Sertoli cells (Svingen and Koopman, 2013).

Sertoli cell precursors are believed to be the first cell type to appear in the differentiated male gonads (Bellingham et al., 2012, Svingen and Koopman, 2013). The number of Sertoli cell precursors must reach a critical threshold to recruit additional cell types for the developing testis (Svingen and Koopman, 2013, She and Yang, 2017).

SRY expression increases the rate of cell proliferation in SOX9-positive cells (Svingen and Koopman, 2013). SOX9 activates several feedforward loops including fibroblast growth factor 9 (FGF9) and prostaglandin D2 (PGD2) signalling, this allows for the amplification and maintenance of SOX9 expression (Kim et al., 2006, Moniot et al., 2009, Biason-Lauber, 2010) and the recruitment of other cells of the same lineage to become SOX9-positive cells (Svingen and Koopman, 2013). FGF9 is also involved in the repression of Wnt4 (wingless-related MMTV integration site 4) expression, a factor in female sex determination (Kim et al., 2006, Svingen and Koopman, 2013, Sánchez and Chaouiya, 2016). SOX9 expression is further upregulated by the binding of SRY and the transcription factor SF-1 to its promotor region (Biason-Lauber, 2010, Sánchez and Chaouiya, 2016).

Sertoli cell precursors start to loosely cluster around germ cells and adhere to one-another encasing the germ cells. Endothelial cells migrate from the mesonephros to surround and partition these groups of Sertoli cells, separating the groups into primitive cord structures (Svingen and Koopman, 2013). These primitive cords will grow into the intermediate mesoderm (Pawlina and Ross, 2020). As the Sertoli cell precursors proliferate and further adhere to one another and germ cells, the cords elongate (Svingen and Koopman, 2013). At around 8 weeks, the seminiferous cords are visible (Biason-Lauber, 2010, Himelreich-Perić et

al., 2023) with peritubular myoid cells (PMCs) and an extracellular matrix (ECM) forming around the seminiferous cords at week 12 (Svingen and Koopman, 2013).

Follicle stimulating hormone (FSH) stimulation induces the replication of Sertoli cells and production of AMH from mid-gestation. Sertoli cells proliferate throughout gestation and in the pre-pubertal period to a fixed population of cells (O'Donnell and McLachlan, 2012). The final fixed number of Sertoli cells will affect the quality and quantity of spermatogenesis (Bellingham et al., 2012, Wang et al., 2022).

Foetal Leydig cells (FLCs) differentiate from an unknown progenitor, the origin of FLC is highly debated (Svingen and Koopman, 2013, Li et al., 2023). It is proposed that the DHH (desert hedgehog), a Sertoli cell marker, may be a trigger for the differentiation of FLC populations (Svingen and Koopman, 2013). Human chorionic gonadotropin (hCG), produced by the placenta, stimulates the differentiation of FLC and the production of testosterone by week 8 (Figure 1) (Luetjens and Weinbauer, 2012, Lanciotti et al., 2018, Bizzarri and Cappa, 2020).

Foetal Leydig cells don't express the enzyme 17-β hydroxysteroid dehydrogenase (17β-HSD), required for testosterone synthesis, this is instead expressed by foetal Sertoli cells (Shima et al., 2013, Sararols et al., 2021).

Testosterone is important for the development of the mesonephric duct (Svingen and Koopman, 2013) and the masculinisation of the reproductive tract, including the differentiation of accessory organs and external genitalia (Svingen and Koopman, 2013, Grinspon et al., 2020, HooKim and McCue, 2020, Li et al., 2023). The production of steroidogenic enzymes needed for testosterone synthesis is stimulated by SF-1 (Biason-Lauber, 2010, Pawlina and Ross, 2020). Leydig cells are also important, as they are vital for normal male development and descent of the testis in the foetus (Svingen and Koopman, 2013). Testosterone levels peak between week 11-14 and begin to decline after week 20 (Lanciotti et al., 2018, Bizzarri and Cappa, 2020).

The "Masculinisation Programming Window (MPW)" is a critical period in the development of the male reproductive tract. Insufficient androgen action during the MPW can lead to the development of TDS symptoms including cryptorchidism and hypospadias (Vinggaard et al., 2000, Sharpe, 2020). In humans, this period is thought to be in the first trimester, possibly between weeks 8 and 14 (Figure 1) (MacLeod et al., 2010, Sharpe, 2020).

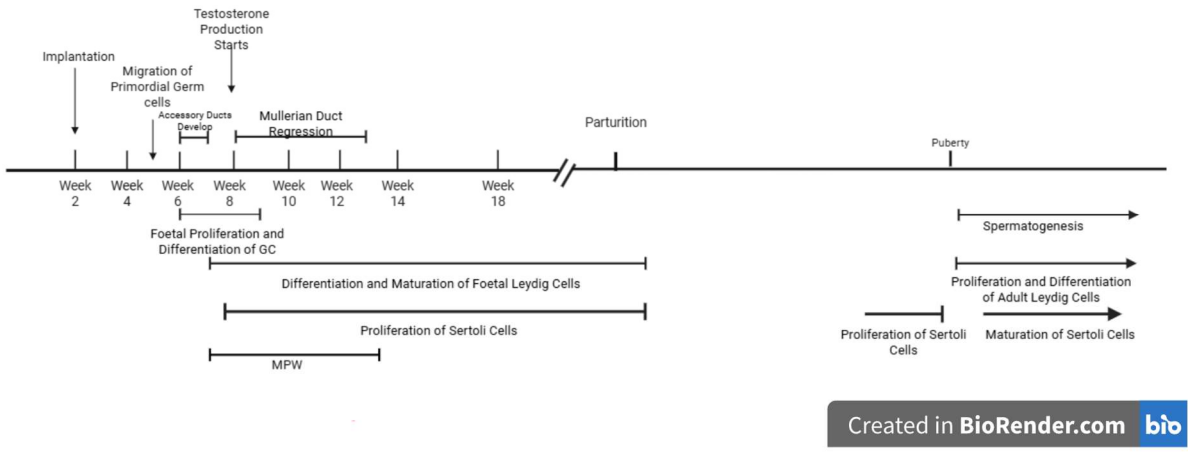

Figure 1. Timeline of the Development of the Male Reproductive Tract in Humans. Masculinisation Programming Window (MPW), Germ Cells (GC). Created in BioRender.

2.2.2. Development of the HPG axis

Gonadotrophin releasing hormone (GnRH) producing neurones migrate to the foetal hypothalamus at approximately 40 days of gestation. Gonadotrophin production is GnRH independent and remains so until week 30 of gestation, after which the release of LH and FSH becomes GnRH induced (Lanciotti et al., 2018, Bizzarri and Cappa, 2020).

The pituitary gland begins producing the gonadotropins, luteinising hormone (LH) and follicle stimulating hormone (FSH), at 9 weeks of gestation. LH slowly replaces human Chorionic Gonadotropin in regulating Leydig cell function. Gonadotropin levels peak at mid-gestation, which coincides with high levels of testosterone and the maturation of the first seminiferous cord (Bizzarri and Cappa, 2020).

Following these peaks in LH and FSH, the levels of the gonadotrophins gradually decline and are suppressed at birth, likely by increased levels of placental oestrogens produced during late gestation (Bizzarri and Cappa, 2020).

2.2.3. Post-Natal and Prepubertal Development

Following birth, LH levels spike, followed by a rise in testosterone levels lasting for approximately 12 hours (Lanciotti et al., 2018). The lack of placental oestrogens removes the suppression on the HPG axis thus allowing for a rise in LH and FSH levels between 6-10 days of life. LH levels increase to pubertal levels and peak between weeks 2-10, following this, levels drop by month 4-6 and settle at pre-pubertal levels (Lanciotti et al., 2018, Becker and Hesse, 2020).

Foetal Leydig cell numbers significantly increase up until month 3 until numbers fall due to progressive apoptosis (Lanciotti et al., 2018). Testosterone secretion follows a similar pattern to LH production and Leydig cell proliferation with levels gradually rising to peak between months 1-3, before declining to pre-pubertal levels after 6-9 months (Rey et al., 2009, Lanciotti et al., 2018, Becker and Hesse, 2020).

FSH levels are lower in males compared to females and will peak between week 1 and month 3 before declining to pre-pubertal levels by month 4 (Lanciotti et al., 2018). Following birth Sertoli cells proliferate under the stimulation of FSH and continue to secrete AMH which peaks at 3 months of age before declining to stable levels until puberty. Immature Sertoli cells will continue to increase in number up until puberty at which point they will terminally differentiate and be unable to proliferate (Figure 1) (Sharpe, 2010b, Becker and Hesse, 2020).

Puberty is triggered by the release of GnRH from the hypothalamus which in turn causes the pulsatile secretion of LH. LH stimulation leads to the proliferation and differentiation of adult Leydig cells which in turn increases testosterone levels (Sharpe, 2010b).

During puberty Sertoli cell maturation will occur, with Sertoli cells beginning to express androgen receptors, and express less AMH (Sharpe, 2010b, Becker and Hesse, 2020).

Whilst most studies suggest that it is the initial developmental period of the male reproductive system that is most affected by exposure to environmental chemicals, it is also important to appreciate that testosterone and spermatozoa are produced throughout adult life. Interference in the regulation, function and populations of cell types within the male reproductive system may also lead to sub-fertility problems. Thus, an understanding of the regulation and cellular components of the adult male is also needed.

2.3. The Hypothalamic-Pituitary-Gonadal (HPG) Axis in Adults

GnRH is produced in pulses by the hypothalamus, in turn causing the production and secretion of the gonadotropins, FSH and LH from the anterior pituitary gland which stimulates Sertoli and Leydig cells respectively (Jones, 1997, White et al., 2018). Removal or damage to the pituitary gland results in a loss of spermatozoa production, a decrease in Leydig cell functionality and therefore a drop in testosterone levels (Jones, 1997).

LH stimulation promotes the production of testosterone which is involved in a negative feedback loop and will inhibit the production of GnRH, LH and to a lesser extent FSH (White et al., 2018). At puberty LH stimulation of Leydig cells causes the secretion of testosterone which initiates spermatogenesis in the primitive germ cells. Stimulation of the germ cells and Sertoli cells with FSH further drives spermatogenesis (HooKim and McCue, 2020)

Mature Sertoli cells have both FSH receptors and androgen receptors (AR). The FSH receptor in Sertoli cells is also coupled to a Gs-cAMP-PKA pathway (Ni et al., 2020). Stimulation with FSH results in increased expression of proteins required for normal germ cell maturation, such as androgen binding protein (ABP). ABP binds to androgens and increases their concentration within the seminiferous tubules (White et al., 2018, Lowe et al., 2019). FSH binding also causes the production of inhibin which inhibits the production of FSH in the pituitary (White et al., 2018, Lowe et al., 2019). Inhibin secretion is pulsatile and coincides with pulses in testosterone. Rats administered recombinant human inhibin A showed disruptions in the frequency, amplitude of FSH pulses and peak FSH levels, with no effect on LH pulsatility (Luisi et al., 2005).

2.4. Testicular Cells

2.4.1. Leydig Cells

Leydig cells are steroidogenic interstitial cells which secrete hormones important to the development of the foetus and the reproductive system. There are sequential populations of Leydig cells, with foetal Leydig cells differentiation in the foetus and adult Leydig cells forming shortly after birth (White et al., 2018). It is hypothesised that these two cell types differentiate from completely separate populations of progenitor cells, rather than adult cells differentiating from foetal Leydig cells (Svingen and Koopman, 2013).

Unlike foetal Leydig cells, LH signalling is indispensable for the differentiation of adult Leydig cells and testosterone production (Sararols et al., 2021). LH-mediated stimulation of adult Leydig cells triggers a cascade of intracellular events through cAMP signalling (Hu et al., 2009).

LH binding is involved in promoting the growth and proliferation of Leydig cells (White et al., 2018). LH stimulation also results in increased expression and activation of steroidogenic proteins and enzymes involved in testosterone synthesis (Hu et al., 2009, Zirkin and Papadopoulos, 2018).

Binding of LH to its receptor causes the hydrolysis of cholesterol esters and an increase in the expression of steroidogenic enzymes and the transporter protein Steroidogenic Acute Regulatory protein (STAR). Binding of LH to its G-protein coupled receptor activates adenylate cyclase which increases the level of intracellular cyclin adenosine monophosphate (cAMP) (Althumairy et al., 2020). cAMP activates protein kinase A (PKA) which phosphorylates STAR (Wang et al., 2017a, White et al., 2018, Zirkin and Papadopoulos, 2018).

Leydig cells also secrete oxytocin, which stimulates the PMCs surrounding the seminiferous tubules to contract helping to move the spermatozoa through the tubules (Pawlina and Ross, 2020).

2.4.1.1. Testosterone

In the adult, testosterone is needed for the production of sperm and the maintenance of secondary sex characteristics (White et al., 2018). It's believed that the human testes produce 6-7mg of testosterone daily (Luetjens and Weinbauer, 2012, Pawlina and Ross, 2020).

Testosterone binds to the androgen receptor (AR) in the cytoplasm causing nuclear translocation of the hormone-receptor complex. The complex forms a dimer and binds to an androgen-response element (ARE) and recruits coregulatory proteins to specific gene promotors (White et al., 2018).

Testosterone can be further metabolised into oestrogen by the steroidogenic enzyme cyp19a1, or into dihydrotestosterone (DHT) by 5α-reductase-2 (Luetjens and Weinbauer, 2012, White et al., 2018). DHT is required for the development of the prostate gland and the external genitalia (Jones, 1997, White et al., 2018).

2.4.1.1.1. Biosynthesis of Testosterone

All steroid hormones, including testosterone, are synthesised from cholesterol. Intracellular lipid droplets act as the primary source of cholesterol for testosterone synthesis (Hinson et al., 2022)

The transporter protein STAR, along with translocator proteins (TSPO), form a transduceosome complex which mediates the transportation of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, the site of steroidogenesis. This is thought to be the rate limiting step of steroidogenesis (Tsuchiya et al., 2003, Midzak et al., 2011, Mendoza-Villarroel et al., 2014, Manna et al., 2016).

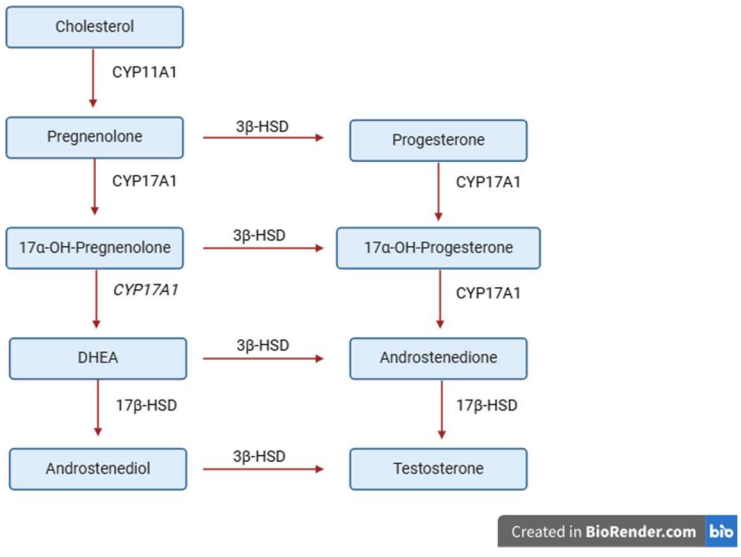

Figure 2. Simplified diagram of steroidogenesis; the biosynthesis of testosterone from cholesterol showing the enzymes which catalyse each step of the process. CYP11A1 (cholesterol side chain cleaver enzyme), CYP17A1 (p450c17$_{17\text{-}\alpha\ hydroxylase/17,\ 20\ lyase}$), 3β-HSD (3β-hydroxysteroid dehydrogenase), 17β-HSD (17β-hydroxysteroid dehydrogenase), DHEA (dehydroepiandrosterone). Created with BioRender.com.

Once imported into the mitochondria cholesterol is converted into pregnenolone by cleaving the side chain between carbon 20 and carbon 22. This is catabolised by a steroidogenic enzyme from the cytochrome p450 family, CYP11A1, also called p450scc (cholesterol side chain cleaver enzyme) (Figure 2). The pregnenolone is transported to the smooth endoplasmic reticulum for further enzymatic activity (Zirkin and Papadopoulos, 2018, White, 2023).

Another member of cytochrome p450 family is *p450c17*$_{17\text{-}\alpha\ hydroxylase/17,\ 20\ lyase}$, also known as *CYP17A1*. *CYP17A1* is bifunctional, meaning it has two distinct roles, it can catabolise the 17-α hydroxylation of pregnenolone to 17α-hydroxy-pregnenolone (17-OH pregnenolone) and the hydroxylation of progesterone to 17α-hydroxyprogesterone (17-OH progesterone) (Figure 2) and also catabolising the removal of a side chain through the cleavage of the c17, 20 bonds in 17-OH pregnenolone and 17-OH progesterone to form dehydroepiandrosterone (DHEA) and androstenedione respectively (Figure 2) (Liu et al., 2005, White et al., 2018, White, 2023).

The enzymes *HSD3b2* or *3β-HSD* (3β-hydroxysteroid dehydrogenase) and *17β-HSD* (17β hydroxysteroid dehydrogenase) are also important for the synthesis of testosterone. *3β-HSD* is a membrane bound enzyme found in both the endoplasmic reticulum (Liu et al., 2005, Hinson et al., 2022).

3β-HSD catalyses multiple conversions within the steroidogenic pathway, including the conversion of pregnenolone into progesterone, of 17-OH pregnenolone into 17-OH progesterone, of DHEA into androstenedione and finally of androstenediol into testosterone (Figure 2) (White et al., 2018, White, 2023).

17β-HSD catabolises the conversion of DHEA into androstenediol as well as the conversion of androstenedione to testosterone (Figure 2) (White et al., 2018, White, 2023).

2.4.2. Sertoli Cells

In the adult, Sertoli cells are non-dividing epithelial cells which typically appear at the basement membrane of the seminiferous tubules (Bastawros, 2024). The position and shape of the nucleus can vary, sometimes appearing close to the base of the cell or closer to the lumen of the seminiferous tubule. Its shape can vary from flattered, to ovoid to triangular (Pawlina and Ross, 2020).

Following proliferation, Sertoli cells form tight junctions with other Sertoli cells which forms the blood-testis barrier. It essentially creates a microenvironment within the tubules with a basal and luminal compartment (O'Donnell and McLachlan, 2012, Bastawros, 2024). The spermatogonia and early primary spermatocytes reside within the basal compartment, they must pass through to the luminal compartment as they proceed through meiosis (Pawlina and Ross, 2020). To do so Sertoli cells form new junctions behind the spermatocytes and break down the junctions in front. By keeping the differentiating spermatogenic cells within the luminal compartment and behind the blood-testis barrier it prevents the immune system from attacking the haploid cells which would be seen as foreign to the body (Pawlina and Ross, 2020, Bastawros, 2024).

Their cytoskeleton contains microtubules which may be responsible for the repositioning of elongated spermatids embedded in Sertoli cell cytoplasm. It is also made up of intermediate filaments, primarily vimentin which forms a peri-nuclear sheath which extend to the Sertoli cell-to-Sertoli cell junctions (Pawlina and Ross, 2020).

Sertoli cells are responsible for the nurturing of spermatozoa as they develop. They provide metabolic substrates and remove waste from around the developing spermatozoa (Alves et al., 2013, Crisóstomo et al., 2018, Bastawros, 2024).

Testosterone and FSH mediated stimulation of Sertoli cells is important for the maintenance and self-renewal of spermatogonia stem cells, the initiation of and continuation of spermatogenesis and the release of spermatozoa into the lumen (Roberts and Chauvin, 2019, Pawlina and Ross, 2020, Wang et al., 2022). Testosterone is also required for the maintenance of the blood-testis barrier and interactions between Sertoli cells and germ cells (Roberts and Chauvin, 2019).

The majority of Sertoli cell specific gene transcripts upregulated by the presence of functioning ARs, encoded proteins involved in calcium, cytoskeletal protein and actin binding which are associated with the maintenance of the blood-testis barrier and Sertoli-germ cell interactions (Roberts and Chauvin, 2019). Stimulation with FSH results in increased expression of proteins required for normal germ cell maturation, such as androgen binding protein (ABP) (White et al., 2018, Lowe et al., 2019). ABPs bind to testosterone in the luminal compartment to increase testosterone concentration to the needed level for normal spermatogenesis. Other examples of Sertoli cell secretions include the glycoproteins anti-mullerian hormone and inhibin (Pawlina and Ross, 2020, Bastawros, 2024).

2.4.2.1. Anti-Müllerian Hormone (AMH)

Anti-Müllerian hormone (AMH) or Müllerian inhibiting hormone (MIH) is a glycoprotein expressed only by Sertoli cells through foetal life and through childhood, although serum AMH levels do vary during post-natal life (White et al., 2018). It is responsible for the regression of the Müllerian ducts, which would otherwise become the oviducts, uterus, cervix, and upper vagina (Mullen and Behringer, 2014, White et al., 2018).

AMH acts through a coreceptor complex, namely with AMH receptor type II (AMHR-II) and AMH receptor type I (AMHR-I) (White et al., 2018). These receptors are expressed in the Müllerian duct adjacent mesenchymal cells. AMH first binds to AMHR-II which forms a heteromeric complex with AMHR-I which it then phosphorylates and activates (Mullen and Behringer, 2014). This coreceptor complex has serine-threonine kinase activity which causes the phosphorylation of R-SMAD (Mullen and Behringer, 2014, White et al., 2018). This transduces the extracellular binding of AMH through supposedly a R-SMAD/SMAD-4 complex to the nucleus where it activates target genes (Mullen and Behringer, 2014). Examples of possible target genes include matrix metallopeptidase 2 (MMP2), Osterix, and ß-catenin (Visser, 2003, Moses and Behringer, 2019). AMH levels reduce at puberty as AHM gene expression is inhibited by testosterone (Lowe et al., 2019).

2.4.3. Germ Cells

Spermatogenesis is the development and maturation of undifferentiated spermatogonia stem cells into mature haploid sperm cells. This process begins just before puberty and will continue throughout reproductive life (Pawlina and Ross, 2020). Testosterone and FSH are responsible for the initiation of spermatogenesis (Pawlina and Ross, 2020, Wang et al., 2022)

Spermatogonia undergo mitosis to replenish their numbers and create a population of differentiated spermatogonia which are committed to becoming spermatozoa (Pawlina and Ross, 2020). These committed spermatogonia differentiate into primary spermatocytes, which then replicate their DNA before entering meiosis I to form haploid secondary spermatocytes. These cells immediately enter meiosis II which produces round spermatids (Pawlina and Ross, 2020, Wang et al., 2022). These cells undergo considerable cytodifferentiation in which they elongate, the DNA condenses down, and they form an acrosome cap and a flagellum (Holstein et al., 2003). An excess cytoplasm is removed as residue bodies which are phagocytosed by Sertoli cells (Pawlina and Ross, 2020). The release of spermatids from the Sertoli cells is referred to as Spermiation which involves the breakdown of Sertoli cell and spermatid

junctions (Holstein et al., 2003, Pawlina and Ross, 2020). The spermatids are released into the lumen of the seminiferous tubules and referred to as spermatozoa (Holstein et al., 2003, Pawlina and Ross, 2020, Wang et al., 2022). There is evidence that these various cell types can be affected in multiple ways by variety of chemicals which are present in the environment. Disrupting these cell type's functions and development can lead to sub-fertility issues.

2.5. Environmental Chemicals and their Effects on the Male Reproductive System

Various groups have suggested that the geographical variation and rapid nature of declining sperm count and linked reproductive and developmental pathologies, may be due to environmental factors, rather than being primarily genetic (Acerini and Hughes, 2006, Mann et al., 2020, Aitken, 2022). Examples of these factors include diet, disease, drugs and exposure to environmental chemicals (Mann et al., 2020).

There are a wide variety of environmental chemicals that have been linked to infertility. Examples include bisphenol A (BPA), phthalates, polychlorinated biphenyl (PCB) and polycyclic aromatic hydrocarbons (PAHs). These chemicals are present in multiple different products used in everyday life (Table 1).

Table 1. List of common Environmental Chemical classes, examples of chemicals and their uses and sources.

Chemical Class	Example	Uses/Sources	References
Environmental Phenols	Bisphenol A	Used in packaging for food and drinks. Can be used in plastic toys and goods.	(Cannarella et al., 2023)
Phthalates	Di-2-ethylhexyl Phthalate (DEHP)	Food packaging, children's toys, furniture upholstery, mattresses, and cosmetics as well as medical equipment including intravenous bags and tubes, umbilical artery catheters and blood bags.	(Rowdhwal and Chen, 2018)
Pesticides	Dichlorodiphenyl-dichloroethylene (DDT)	Pesticides – used to control malaria vectors	(Mima et al., 2018)
Polybrominated Diphenyl Ethers (PBDEs)	PentaBDE OctaBDE DecaBDE	Used as a flame retardant in products such as textiles, furniture, plastics, and construction materials.	(Ohoro et al., 2021, Lan et al., 2024)
Polybrominated biphenyl (PBB)	Hexabromobiphenyl PBB mixture	Flame retardants used in textiles, electrical devices (e.g. computers, televisions) and plastic foams.	(CDC, 2015)
Polycyclic Aromatic Hydrocarbons (PAH)	Naphthalene Fluorene Phenanthrene Pyrene Benzo[a]anthracene Benzo[a] pyrene	Can be released naturally but primary emission sources include industrial (power production, iron and steel manufacture), domestic (residential heating), and agricultural (biomass burining). PVC	(Patel et al., 2020, Beduk et al., 2023)

		products used daily, including toys, bike handles and mouse pads also contain PAHs	
Polychlorinated Biphenyls (PCB)	Aroclor mixture	Previously used as a coolant in electrical equipment including transformers and capacitors. Also been used in paints, dyes, plastics, rubber and carbonless copy-paper.	(Cannarella et al., 2023)
Heavy metals	Cadmium	Can be found in dyes, plastics, cigarettes and fertilisers.	(Mima et al., 2018)

Although some endocrine disrupting chemicals are no longer being used in manufacturing there are some EDCs such as polycyclic aromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) which have a half-life of over 10 years (Rhind et al., 2010).

Some of these chemicals are classed as Endocrine Disrupting Chemicals (EDCs), or chemicals which have an effect by altering normal endocrine homeostasis through a variety of mechanisms including disrupting hormone production, metabolism or function (Corpuz-Hilsabeck and Culty, 2023). These EDCs may affect the reproductive system through targeting the hypothalamic-pituitary gonadal axis and/or testicular hormones (Kakavandi et al., 2023). They can have oestrogen-like and anti-androgenic effects on reproductive tissue (Ferguson and Agoulnik, 2013).

Some EDCs can supress the function of Leydig cells and as such the production of testosterone and INSL3 (Ferguson and Agoulnik, 2013), others bind to androgen receptors (ARs), inhibiting their function, this has damaging effects on the in-utero differentiation and development of the male reproductive system. Exposure during the first trimester could result in insufficient androgen signalling in the MPW, leading to the development of TDS symptoms later in life (Vinggaard et al., 2000, Sharpe, 2020). Metabolites of several fungicides and insecticides have been identified as potent antagonists of ARs in humans (Vinggaard et al., 2000).

Epidemiological studies suggest links between the presence of phthalates pregnant women's serum and urine with TDS symptoms including neonatal cryptorchidism, hypospadias and reduced anogenital distance (AGD) in male children (Hu et al., 2009, Ferguson and Agoulnik, 2013, Li et al., 2022).

Even at low concentrations EDCs can affect various physiological systems and are suggested to have synergistic effects which can make their effects unpredictable, and that the additive effects of these chemical mixtures can result in physiological effects despite individual chemical concentrations being too low to have an effect by themselves (Rhind et al., 2010).

2.5.1. Bisphenol A

Bisphenol A (BPA) is an oestrogen mimic/xenoestrogen which can imitate the effects of endogenous oestrogen, this can change the testosterone: oestrogen hormone balance. It is commonly used in the manufacturing of products such as baby bottles, plastic containers, dental sealant, and epoxy resin (Mima et al., 2018, Park et al., 2021).

Various epidemiological studies have examined BPA exposure on semen parameters such as sperm concentration, sperm motility, sperm morphology.

A study examining occupational exposure to BPA identified a significant correlation between increasing urinary BPA and declining sperm concentration, total sperm count, vitality, and motility. They also stated that individuals with detectable urinary BPA have more than four times the risk of a lower sperm count and more than 3 times the risk of lower sperm vitality and concentration than those with undetectable urinary BPA (Li et al., 2011).

Results from studies conducted on infertility patients are variable, possibly due to differences in measurements, methodology and geographical location. Some studies utilise urinary BPA levels (Li et al., 2011, Benson et al., 2021, Chen et al., 2022) and others seminal plasma (Vitku et al., 2015, Vitku et al., 2016, Davis et al., 2024) and blood plasma BPA levels (Vitku et al., 2015). Some groups have found urinary BPA to be highly variable and suggest taking multiple measurements to acquire an accurate marker for BPA exposure (Lassen et al., 2014, Chen et al., 2022), whilst others argue a single measurement is enough to quantify long-term exposure (Adoamnei et al., 2018). Some studies did not follow WHO criteria when examining particular sperm parameters (Li et al., 2011).

In a study of 984 Chinese men BPA exposure was associated with reduced sperm quality and BPA's metabolites with reduced sperm motility and a greater percentage of abnormal sperm head morphology (Chen et al., 2022). However, other studies have found no association (Benson et al., 2021)

2.5.2. Phthalates

Phthalates are a relatively large group of plasticisers used in a variety of plastic products (Table 1). These chemicals can leach into their environment and are taken into the body (Cannarella et al., 2023).

Phthalates have been identified as antiandrogens due to their similarities with other antiandrogens such as linuron and vinclozolin (Martinez-Arguelles et al., 2013). Pregnant rats exposed to high doses of DEHP and dibutyl phthalate (DBP) for varying periods of time yet throughout the rat MPW (gestational day (GD)) 15.5-18.5 (MacLeod et al., 2010)) had male pups with reduced AGD, hypospadias, absent epididymis and prostate gland and testicular atrophy and reduced testis weight (Mylchreest et al., 1998, Gray et al., 2000, Moore et al., 2001, MacLeod et al., 2010). One study shows that DBP exposure limited to only 2 GD during the MPW can still significantly reduce AGD (GD 15-16, and GD 18-19), epididymis weight and testis weight (GD 16-17) (Carruthers and Foster, 2005). Phthalate has age and dose dependent effects with foetal and prepubescent animals having a greater sensitivity than adults (Hu et al., 2009).

Research suggests phthalates have a negative effect on male fertility with some papers stating that in pregnant rodents they can reduce litter size and induce cryptorchidism and testicular atrophy. They are suggested to have effects on both Sertoli and Leydig cells (Culty et al., 2008).

Studies have demonstrated that phthalate exposure (between 500-750mg/kg/day) in rats lowers testosterone production and INSL3 expression and reducing foetal Leydig cell numbers (Parks, 2000, Wilson et al., 2004, McKinnell et al., 2005, Lin et al., 2008). Thus, phthalate exposure can induce cryptorchidism and hypospadias in rats (Fisher et al., 2003, Hu et al., 2009). If phthalates effect on FLC is linked to testicular cancer and reduced fertility is unclear as foetal Leydig cells involute during childhood (Hu et al., 2009, Ivell and Anand-Ivell, 2009).

The exact mechanism behind Leydig cell toxicity is unknown. It has been proposed that phthalates may affect Leydig cells through peroxisome proliferator-activated receptors (PPAR) and through aryl hydrocarbon receptors (AhRs) (Hu et al., 2009). Phthalates have been found to induce the actions of the two PPARs expressed by rat foetal Leydig cells, PPARα and PPARγ (Latini et al., 2008, Hu et al., 2009). Exposure to phthalates in foetal testis led to an increase in expression AhRs and cyp1b1, a downstream gene of the AhR signalling pathway (Hu et al., 2009).

2.5.2.1. Di(2-EthylHexyl) Phthalate (DEHP)

DEHP or Di(2-ethylhexyl) phthalate is a plasticiser, and the most common phthalate used (Rowdhwal and Chen, 2018, Xing and Bai, 2018). DEHP has multiple metabolites but its primary metabolite it mono-2-ethylhelyl phthalate (MEHP) (Martinez-Arguelles et al., 2009). DEHP is present in various everyday items detailed in Table 1. Epidemiological studies identified DEHP in food products including meat (i.e. poultry), dairy products, and bread (Serrano et al., 2014). The Environment Protection Agency (EPA) reported that a dosage of 20 µg/kg/day may pose a risk of enlarged liver and the European Food Safety authority state that a dosage of 50 µg/kg/day can lead to testicular toxicity (Rowdhwal and Chen, 2018). Serrano and colleagues reported the intake of DEHP in a typical infant (between 1-2 years) diet to be at 42.1 µg/kg/day which more than exceeds the EPA's references dose. They also highlighted that if the diet was high in dairy and meat this would be over four times the recommended threshold (Serrano et al., 2014). DEHP has also been identified in human amniotic fluid, umbilical cord blood and breast milk, suggesting exposure to neonates and in-utero exposure of foetuses (Martinez-Arguelles et al., 2009).

Di-(n)-Butyl phthalates and MEHP have been suggested to be linked to the apoptosis of germ cells through altering vimentin, the structural intermediate filaments of Sertoli cells (Richburg and Boekelheide, 1996, Alam et al., 2010). Vimentin filaments surround the cell nucleus and reach the periphery of the cell. They are involved in the formation of tight junctions with other Sertoli cells (Alam et al., 2010).

One study examining phthalate levels in infertile men from China found associations between monobutyl phthalate (MBP) and reduced sperm concentration and sperm count (Wang et al., 2015). Radke and colleagues (Radke et al., 2018) conducted a systematic review of epidemiological studies on EDC exposure and male reproductive outcomes in humans and found moderate evidence that DEHP was negatively associated with AGD. Meta-studies conducted on human data identified association between increased DEHP metabolites levels in maternal urine and reduced AGD in their male infant sons (Dorman et al., 2018, Zarean et al., 2019).

Rats treated with DEHP in-utero at increasing levels between gestational day 14 and birth were shown to have reduced testosterone levels in both the foetus and the adult male offspring (Culty et al., 2008). It was suggested that this was due to reduction in the expression of enzymes involved in steroidogenesis in the foetus, however, in the adult the number of Leydig cells, the transportation of cholesterol across the mitochondrial membrane and the steroidogenic enzymes were all normal suggesting an alternative pathway of affect. DEHP would have to affect the adult Leydig cell population in-utero to continue to affect testosterone levels in the adult offspring as DEHP is removed after birth. The research group suggested that DEHP may be targeting the adult Leydig stem cells in-utero which would then influence testosterone levels in the adult (Culty et al., 2008, Martinez-Arguelles et al., 2013).

2.5.3. Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic pollutants which are produced through incomplete combustion of fossil oil, coal, timber and tobacco (Table 1) (Wang et al., 2017a, Beduk et al., 2023). Exposure can occur through occupation, through inhalation of vehicle emissions and tobacco smoke as well as through ingestion of contaminated water and food (Bolden et al., 2017, Patel et al., 2020). Based on concentration, toxicity, exposure and resistance to degradation or composure the United States Environmental Protection Agency (USEPA) named 16 PAHs as priority pollutants, these include naphthalene, fluorene, phenanthrene, pyrene, benzo[a]anthracene, and benzo[a] pyrene (Patel et al., 2020, Beduk et al., 2023).

Exposure to naphthalene, benzo[a]anthracene and benzo[a] pyrene are thought to have caused embryo toxicity (Patel et al., 2020). It is suggested that these PAHs bind to oestrogen and androgen receptors exhibiting anti-oestrogenic and/or anti-androgenic effects (Bolden et al., 2017, Patel et al., 2020). PAHs such as benzo[a] pyrene, fluoranthene and benz[a] anthracene have been identified as having an anti-androgenic effect (Bolden et al., 2017). Exposure to benzo[a] pyrene reduced testosterone production and the expression of the steroidogenic genes STAR and 3β-HSD. Also observed was a significant increase in CYP11A1 expression though this is believed to be in compensation for the downregulation of STAR (Chung et al., 2011).

2.5.4. Polychlorinated Bisphenyls (PCBs)

PCBs are chemicals known to have carcinogenic and mutagenic effects on humans as well as disrupting the endocrine system (Beduk et al., 2023). They are oestrogen mimics which accumulate in the environment and in water supplies. They are lipophilic in nature and tend to accumulate in fatty tissue (Cannarella et al., 2023).

Use of PCBs in electrical capacitors and transformers ended in the 1970s (Cannarella et al., 2023) however some of these are still in use (Beduk et al., 2023), and they are resistant to both chemical and thermal degradation meaning they are classified as a persistent organic pollutant (POP) (Montano et al., 2022). One study linked the consumption of high levels of PCB with higher rates of cryptorchidism (Ferguson and Agoulnik, 2013). PCBs have also been associated with reduced AGDs and affects sperm factors including poor sperm motility and morphology, reduced sperm concentrations and altered sperm DNA integrity (Meeker and Hauser, 2010, Sheinberg et al., 2020, Montano et al., 2022). It has been suggested that PCBs may directly affect spermatogenesis as they are able to cross the blood-testis barrier (Montano et al., 2022).

2.5.5. Transgenerational Studies

Research also suggests that exposure to EDCs may have an effect on future generations which were not exposed to the chemicals (Rhind et al., 2010). Alternations in DNA methylation and histone methylation patterns in the testis has been observed in rodents exposed to various EDCs. These epigenetic alterations can be passed onto future generations (Sweeney et al., 2015) or may not appear until later generations (Thacharodi et al., 2023).

In-utero BPA exposure in rats showed significant alterations in the expression profiles of steroid receptor coregulators and significant decreases in sperm count and motility in the F1, F2 and F3 generations through the paternal germ cell lineage (Salian et al., 2009a, Salian et al., 2009b). Exposure to Diethylstilbesterol (DES) in-utero passed through maternal germ cell lineage led to an increased incidence of tumours and proliferative lesions on the rete testis in F2 male mice (Newbold et al., 2006).

Mice exposed to DEHP between gestational days 12.5-18.5 showed increased levels of DNA methylation and expression of DNA methyltransferases Dnmt1 on gestational day 19 and post-natal day 3. Expression of the de-novo DNE methyltransferases Dnmt3a and Dnmt3b remained significantly higher at post-natal day 21 (Wu et al., 2010).

Another study examining the transgenerational effect of in-utero exposure to DEHP in mice found F1, F2 and F3 generations presented with abnormalities in the seminiferous tubules including loss of germ cell organisation and bulk germ cell loss, considerable cell sloughing and lacked lumen. These mice also had reduced sperm counts and poor sperm motility in generations F1-F4 (Doyle et al., 2013).

The rodent studies discussed in this section have had a huge impact on the current field of research, allowing for the examination of exposure effects and mechanisms of actions of various chemicals at controlled dosages. Within many of these previous studies rodent models have demonstrated what various concentrations of individual environmental chemicals can disrupt within both the endocrine and reproductive system (Viguié et al., 2020). However, most of the current research utilising rodent models use supra-environmental concentrations of individual chemicals (Bellingham et al., 2012), which is not necessarily reflective of real-life exposure and few studies have addressed the effects of mixtures of chemicals at real-life concentrations. Additionally, though studies in rodents are important they may not reflect possible effects in humans. This then raises the reality of what is a suitable animal model.

2.6. Animal Models

Rodent models have many advantages, particularly in study duration, with their short gestation periods and short generation intervals producing 4-9 pups in a single litter (Phifer-Rixey and Nachman, 2015, Viguié et al., 2020). However, it would be irresponsible to base all findings on non-human species and assume this directly correlates with the effects of environmental chemicals on human health. Especially when examining in-utero exposure as there are numerous differences between the reproductive physiology and endocrinology of rodents and humans (Viguié et al., 2020). Studies have suggested that there may be species-specific differences between environmental chemicals such as BPA on rodents and humans raising concerns about extrapolating findings in rodents to the possible risk in humans (N'Tumba-Byn et al., 2012).

2.6.1. Sheep as a Model

Sheep have been used as a model for the study of human physiology and the mechanisms of various diseases for many years now, in multiple body systems including nervous, cardiovascular, gastro-intestinal and endocrine systems (Banstola and Reynolds, 2022). They have additionally been used in research on pregnancy and foetal development and physiology (Wallace, 2011, Abi-Nader et al., 2012). To better represent the heterogeneity of the human population, sheep function as an outbred model species, compared to the more homogenous rodent models (Bellingham et al., 2012, Elcombe et al., 2022, Murray and Mitchell, 2022). Their relatively long lifespan of between 9-12 years (Murray and Mitchell, 2022) means they can be utilised in longitudinal studies. Sheep typically give birth to twins or single lambs, which is more similar to humans than the litter size of rodents. Additionally, pregnancies in sheep can be limited to a single lamb (Wallace, 2011).

The development of the foetus is very similar in the sheep and human (Abi-Nader et al., 2012) with all organ systems having similar ontogeny (Wallace, 2011). The hypothalamus ontogeny is similar in both species (Padmanabhan and Veiga-Lopez, 2013). Ewes and human mothers are of a similar adiposity and general size (Wallace, 2011), with the birthweight of lambs and

human babies being relatively similar (3.5kg-4.5kg) (Murray and Mitchell, 2022). Most of the major organs of the body and bodily regulatory systems develop pre-natal and perinatal in both the sheep and in humans, unlike in rodents in which this occurs postnatally. The human temporal development of the brain, lungs, cardiovascular system, and activation of the foetal hypothalamic pituitary adrenal axis is much more similar to that in sheep than in rodents (Morrison et al., 2018).

Sheep have a relatively long and consistent gestation period typically between 145-147 days and although this differs from humans (280 days) the events of gestation occur at comparable times (Wallace, 2011, Abi-Nader et al., 2012, Padmanabhan and Veiga-Lopez, 2013, Banstola and Reynolds, 2022). Gonadal differentiation and the start of meiosis occur at day 35 and 55 respectively in the sheep and in the humans at days 50 and 90 respectively. Relative to the species gestation length these processes occur at 20% and 37% of gestation in the sheep and 18% and 32% of gestation in humans (Padmanabhan and Veiga-Lopez, 2013, Banstola and Reynolds, 2022).

Sheep models have the advantage of being large enough to be able to take multiple samples and measurements across their longer gestation period from the same animal. This can be particularly useful when examining the developmental origins of health and disease (Morrison et al., 2018). Being able to take multiple measurements from the same animal reduces the biological variance which would come from the use of smaller species like mice and rats. In these species an entire animal, if not a whole litter would often need to be used for a measurement at a particular time (Morrison et al., 2018). Though similar work could be done in non-human primates, sheep make for a much more cost-effective and ethical alternative (Padmanabhan and Veiga-Lopez, 2013, Morrison et al., 2018).

Sheep do however require specialised facilities and husbandry with dedicated space allotted for general maintenance (Elcombe et al., 2022, Murray and Mitchell, 2022). The availability of reagents such as antibodies is also limited within this species compared to rodents. Although their gestation does make them more like humans, this also means a longer generation interval which increases the amount of time needed to gather necessary data (Murray and Mitchell, 2022), especially when monitoring intergenerational and transgenerational effects.

Sheep have been implemented into what is described as the biosolids model, to assess the effects of a mixture of chemicals at real-life concentrations in an in vivo model.

2.7. The Biosolids Model

As described, a relatively large volume of work has been carried out pertaining to the individual effects of various environmental chemicals, some of which has been outlined above. However, typical daily exposure is not limited to one or two chemicals but to a complex mixture of varying chemicals. As many of these chemicals act through a variety of cellular mechanisms, they have the potential to have additive or synergistic effects on one another, or possibly cancel out one-another's effects, making it difficult to accurately describe the risk of individual chemicals present within the environment (Bellingham et al., 2012, Darbre, 2019).

To closely replicate the typical mixture of these chemicals at truer to life concentration, studies are utilising treated human sewage sludge, also known as biosolids as a source of chemicals that approximates human exposures (Paul et al., 2005, Bellingham et al., 2012, Elcombe et al., 2021, Elcombe et al., 2022, Elcombe et al., 2023). Biosolids are used routinely as an agricultural fertiliser and are the physically and chemically treated byproduct of domestic, agricultural and industrial wastewater treatment (Paul et al., 2005, Elcombe et al., 2023, Pozzebon and Seifert, 2023).

Approximately 87% of all processed sewage sludge is applied to agricultural land, with 1.3% of UK farmland being fertilised with biosolids (Elcombe et al., 2023). Processed sewage sludge is a relatively cheap fertiliser high in nitrogen, phosphorus and organic carbon (Marchuk et al., 2023, Pozzebon and Seifert, 2023). It is a good source of phosphorus, which is a non-renewable mineral (Marchuk et al., 2023). In the UK it is estimated that biosolids provide approximately 35,000 tonnes of phosphate per year, which is believed to be worth £25 million. There is also evidence to suggest that biosolids application improves soil structure, soil workability and water holding capacity (BAS, 2019).

Despite the benefits of spreading biosolids it is important to note that studies have found that sewage sludge contains various/low levels of potentially toxic contaminants, including heavy metals (e.g. chromium, zinc, copper, cadmium and lead), organic chemicals and pathogens. The mixture of organic contaminants can include PCBs, PAHs, dioxins, alkyl sulfonates and pharmaceuticals (Sharma et al., 2017, Beduk et al., 2023). Also identified within biosolids are several human pathogenic and zoonotic bacteria (Sharma et al., 2017, DEFRA, 2018, Beduk et al., 2023).

Research has been conducted into the risk factors of the presence of heavy metals and pathogens present in the sludge (Gale, 2005). Although in the UK, DEFRA provide a code of practice for biosolids use in agriculture, it describes the regulation of heavy metals but not organic contaminants (DEFRA, 2018). While focus has previously been on the risk factors of heavy metals and pathogens (Gale, 2005) there is rising concerns over the effects of organic chemicals also present in the biosolids. The biosolids model allows for the examination of the potential effects of the accumulation of organic chemicals and the possible synergistic or additive effects of these chemical mixtures not normally seen in studies examining one chemical at a time (Bellingham et al., 2012).

Humans are also exposed to these chemicals through various exposure pathways (e.g., diet, inhalation), making investigations into the effects of these chemicals difficult to realistically replicate exposure to similar conditions on animal models. The chemicals present within the biosolids are largely reflective of daily human exposures because the sewage sludge comes from domestic wastewater (Bellingham et al., 2012).

2.7.1. Limitations

The use of the biosolids mixture does leave a source of variability as different mixtures may contain different levels of environmental chemicals (Abad et al., 2005, Clarke and Smith, 2011). Alterations to the balance of chemicals may mean they interact in different ways, or the mixture may contain or be missing specific contaminants which have an additive, synergistic or inhibitory effect.

The DEHP level in Spanish sewage sludge across multiple wastewater treatment plants was highly variable with concentrations varying between plants (i.e. 1.5 mg kg^{-1} dw in one and 3513.8 mg kg^{-1} dw.at another), and across time (i.e. 3513.8 mg kg^{-1} dw and 76.8 mg kg^{-1} dw 14 days later) (Abad et al., 2005, Clarke and Smith, 2011).

Only a limited number of chemicals within the sewage sludge has been measured. It is thought that thousands of varying chemicals are present within the model making the logistics of defining specific mixes nigh impossible (Rhind et al., 2010). This unfortunately means that within the various studies examining the impacts of sewage sludge fertilised pasture on sheep fertility the exact mixture may differ between one another. However, studies utilising the biosolids model are highlighting the effects of exposure to a generalised mixture of EDCs (Paul et al., 2005, Rhind et al., 2010, Bellingham et al., 2012, Lea et al., 2016, Elcombe et al., 2021, Elcombe et al., 2022, Lea et al., 2022, Elcombe et al., 2023, Evans et al., 2023, Tanner, 2023).

The comparison of ewes grazed on biosolid treated pastures with ewes grazed on pastures treated with inorganic fertiliser which has comparatively minimal contaminants demonstrates the effects of generalised exposure against a comparative negative control (Rhind et al., 2010). The model can also allow for variation within the animals based on factors such as preferences for different grazing area, variance in uptake across the fertilised pasture, and variance within the biosolids itself. Due to the nature of exposure, there was no way of measuring oral dosage precisely (Elcombe et al., 2022).

There are currently several studies which utilise the biosolids model in examining the effects of in-utero exposure to chemical mixtures on male offspring at various ages, including the foetus, neonate, prepubertal lamb and adult ram. These studies were carried out by the same research groups and explored the effects of different exposure periods on various reproductive parameters at differing ages.

2.8. Current Research on Effects on Sheep Reproductive Development and Fertility

Studies have examined the effects of in-utero exposure to environmental chemicals on 110-day-old (Paul et al., 2005), 80-day-old and 140-day old male foetuses, as well as the effects of temporal exposure during early-, mid- and late-gestation to target different differentiation windows in 140-day-old foetuses (Lea et al., 2022).

Others have focused on the effects of in-utero exposure on neonates (Elcombe et al., 2021), and the persistence of effects on 8-week-old lambs (pre-pubertal) (Elcombe et al., 2022) and 11-month-old rams (Elcombe et al., 2023). Another study examined the effects of prolonged chemical exposure throughout gestation and the first 7 months of life on 19-month-old rams (Bellingham et al., 2012).

2.8.1. Body weight and Organ Weight

Of the studies examining the effects of biosolids exposure on body weight and organ weights there are conflicting results. No significant difference was observed in the body weight or testis weight in neonatal lambs (Elcombe et al., 2021), continuously exposed 80-day old and 140-day old foetuses, or 140-day-old foetuses exposed during early and mid-gestation. However, body weight and testis weight was significantly reduced in the 140-day old foetuses exposed during late-gestation (Lea et al., 2022) and in the 110-day old foetuses (Paul et al., 2005). Neither body weight nor testis weight were assessed in the 8-week-old lambs or 11-month-old rams. In the 19-month-old rams there was no significant difference in body weight between treatment groups (Bellingham et al., 2012).

It was noted that the body weight of 110-day-old male foetuses in the Biosolids group was comparable to that of the female foetuses in the Biosolids treatment group. As there is usually a difference in body weight between the sexes, a suggested cause was a reduction in testosterone levels. Though there was an evident decrease in mean plasma testosterone levels this difference was not significant in these animals (Paul et al., 2005). However, in the 60-140T transiently exposed foetuses, which were also significantly lighter than their Control group, they did have significantly lower testosterone levels.

Due to the natural variations in testosterone expression during different developmental periods and within individuals it is unclear if the effects are due to the treatment. Testosterone levels were significantly reduced in the neonates exposed to the biosolids, these animals also had significantly higher nuclear localisation of HIF1α, a transcription factor which has been shown to block promotor regions for STAR (Elcombe et al., 2022, Elcombe et al., 2023). Although

there was significant activation nuclear localisation of HIF1α in the prepubescent lambs, testosterone levels were not reported (Elcombe et al., 2022).

Although HIF1α may disrupt testosterone production by limiting the transportation of cholesterol, it is unlikely that this is the only cause of reduced testosterone levels. Reductions in the steroidogenic enzymes CYP11A1 and CYP17A1 have also been observed in the transiently expressed foetuses which would similarly decrease testosterone production (Lea et al., 2022).

Within one study of adults (Bellingham et al., 2012) and in neonates (Elcombe et al., 2021) the Biosolids group was sub-divided into animals within control ranges and those outside of the control range. This subdivision showed that results which were grossly insignificant, i.e. germ cell number in 19-month-old rams were significant within a specific population of rams (Bellingham et al., 2012). This highlighted the possibility of chemicals impacting individuals within a population more potently than others (Bellingham et al., 2012, Elcombe et al., 2021). It also raises an issue with comparing the effects of environmental chemicals on a group only through the utilisation of means which can diminish the perceived effects on individuals.

2.8.2. Testicular Cells

2.8.2.1. Germ cells

In the 110-day-old foetuses, initially a significant decrease in gonocyte number was observed but this was no longer significant when corrected for the weight of the testis and expressed as the number of cells per gram of testis (Paul et al., 2005). In both the neonates (Elcombe et al., 2021) and 8-week-old lambs (Elcombe et al., 2022) the Biosolids lambs had significant lower mean relative germ cell to Sertoli cell ratio compared to the Control group. No significant differences between the mean total number of germ cells per testis for the Biosolids and Control groups were seen in the 19-month-old rams. The research group did however note considerable variation within the Biosolids group so divided it into 2 subgroups. Rams in the S2 subgroup had significantly lower total germ cell numbers, lower germ cell to Sertoli cell ratios, lower germ cell absolute volume per testis and fewer spermatids and spermatocytes. Several of these rams had noticeably smaller testis (Bellingham et al., 2012). In the 11-month-old rams it was found that although there was a greater proportion of seminiferous tubules with degenerated and depleted spermatids in the Biosolids exposed rams, there was no significant difference in germ cell number between the Biosolids and Control groups (Elcombe et al., 2023).

Observation of the seminiferous tubules in all 3 ages examined revealed an increase in the percent of tubules without germ cells. Sertoli cell only (SCO) tubules were identified in the neonates (Elcombe et al., 2021), the pre-pubescent lambs (Elcombe et al., 2022) and in the 19-month old rams (Bellingham et al., 2012). However, interestingly no SCO tubules were observed in the 11-month old cohort which were half-siblings to the pre-pubescent lambs (Elcombe et al., 2023).

Reduced numbers of germ cells and higher percentage of SCO tubules seen in these studies are associated with TDS like phenotypes. Additionally, in the pre-pubescent lambs differentially expressed genes (DEGs) identified in the Biosolids exposed lambs correlated with DEGs identified in studies of humans with TDS (Elcombe et al., 2022).

Due to the lack of significance difference between Control and Biosolids germ cell numbers in the 11 month old rams it has been suggested that with age and a lack of continued exposure, the reproductive system may recover partially from its TDS-like state (Elcombe et al., 2023). However, despite the number of germ cells and percentage of SCO being similar between

Control groups in the 11-month-old rams there were a greater proportion of tubules with degenerated and depleted elongated spermatids which may be due to reduced testosterone levels in the ram, which has previously been identified in the foetal and neonatal lambs (Elcombe et al., 2021, Lea et al., 2022). Additionally, in the 19-month-old rams (Bellingham et al., 2012) the division of the Biosolids group showed that some rams had significantly less germ cells. This raises concerns as it is unclear if there may have been unseen differences in the 11-month-old rams in which only the mean was used or if the greater exposure period in the 19-month-old rams lead to the decrease in germ cells in the S2 Biosolids subgroup.

2.8.2.2. Sertoli Cells

Studies in the foetus found that Sertoli cell number was initially significantly reduced in the Biosolids lambs (Paul et al., 2005, Lea et al., 2022). However, when Sertoli cell numbers was expressed as number of cells per gram of testis the difference in the 110-day old foetuses was no longer significant (Paul et al., 2005). Only the transiently exposed foetuses, showed a significant decrease in Sertoli cell number per gram of testis, no difference was observed in either the continuously exposed 140-day-old or 80-day old foetuses (Lea et al., 2022). A reduction in Sertoli cell number might disrupt the Blood Testis Barrier (BTB), which could the cause a reduction in germ cell number.

In the neonatal and pre-pubertal lambs Sertoli cell number is only recorded in relation to germ cell number (Elcombe et al., 2021, Elcombe et al., 2022).

In the adult, Sertoli cell number was examined in the 19-month-old rams in which there were observable decreases in Sertoli cell numbers in the smaller testis belonging to the S2 group this difference was not significant (Bellingham et al., 2012).

2.8.2.3. Leydig cells

Within the transiently exposed foetuses, different populations of Leydig cells were identified using two cell specific cell markers, CYP11A1 and CYP17A1 (Lea et al., 2022). Whilst CYP17A1 percentage expression was significantly reduced in all the transiently exposed Biosolids groups, and those exposed for 140-days only foetuses exposed during early gestation and for all 140 days of gestation showed reduced levels of CYP11A1 staining. This indicated that the antibodies were staining different sub-populations of Leydig cells (Lea et al., 2022).

The only other investigation into Leydig cell numbers was in the 110-day-old foetuses in which only CYP11A1 was used. They initially found a difference in cell numbers, but this was no longer significant when expressed as number of cells per gram of testis (Paul et al., 2005). However, if subpopulations of cells are being missed by only utilising one Leydig cell marker, it suggests the observed difference in Leydig cell population may have been greater than reported and may in fact have been significant.

Interestingly, despite being exposed for the same time period, i.e. 80 days, the 0-80T transient Biosolids showed significant differences in all measured parameters at day 140, whereas those exposed for 80 days and then sacrificed saw no significant differences (Lea et al., 2022). This may imply that although the EDC may have an effect at this point during gestation it is not necessarily visible at this point (Paul et al., 2005).

It is suggested by Paul and colleagues that the main change in the Biosolids group is the reduction of Leydig cells which may then result in the reduction in Sertoli and gonocyte cell numbers (Paul et al., 2005).

2.8.3. Gene Studies

2.8.3.1. Differentially Expressed Genes (DEGs)

Differential gene expression analysis in the 140-day-old foetuses exposed continuously and during early gestation identified 202 differentially expressed genes (DEGs) between lambs exposed during early-gestation and the Control group, of which 174 were downregulated and 28 were upregulated. Between the continuously exposed group and the Control group, 47 genes were differentially expressed, with 27 being downregulated and 20 genes being upregulated. Only 22 of these genes were differentially expressed in both the continuously exposed 140-day old foetuses and those exposed during early gestation (Lea et al., 2022).

Analysis of these common DEGs identified associated enriched canonical testicular pathways, including VEGFA and several signalling pathways (SMAD2/3, IGF1, ERK1/ERK2, MAPK and ErbB1,2,3). Genes differentially expressed only in lambs exposed during early gestation were associated with androgen signalling, cell signalling and angiogenesis. The DEGs identified in the continuously exposed group were associated with metabolism (e.g. insulin receptor pathway), cell signalling (including the mTOR signalling pathway), and angiogenesis (Lea et al., 2022).

Elcombe and colleagues identified DEGs in three age groups. The neonates had the greatest number of DEGs, followed by the prepubertal lambs and then the adults (Table 2) (Elcombe et al., 2021, Elcombe et al., 2022, Elcombe et al., 2023). Interestingly, no single DEG was common between all three age groups. Seven DEGs were common between the neonates and prepubescent lambs, three between the neonates and the 11-month-old adults and only one DEG was common to the prepubescent lambs and the adults (Elcombe et al., 2023).

Table 2. Table showing total number of differentially expressed genes (DEGs) in neonatal lamb (1-day-old), prepubertal lamb (8-week-old) and adult ram (11-month-old) testis, and of this total, the number of DEGs upregulated and downregulated in the Biosolids group comprared to the same aged Control group.

Age	Total DEGs	DEGs Upregulated	DEGs Downregulated	Reference
1-day-old lambs	296	21	275	(Elcombe et al., 2021)
8-weeks-old lambs	99	60	39	(Elcombe et al., 2022)
11-month-old rams	33	13	20	(Elcombe et al., 2023)

In neonates, significant variation was identified within the Biosolids group, as such they subdivided it into the ‘resistant’ group and the ‘susceptible’ group. Analysis of differential gene expression between these two subgroups revealed 123 genes with higher expression and 36 genes with lower expression in the susceptible subgroup in comparison to the resistant subgroup. Seven pathways were identified to have substantial enrichment in the susceptible Biosolids group compared to the resistant subgroup, most significantly the cAMP signalling pathway (Elcombe et al., 2021).

Within the cAMP signalling pathway, three genes (NPY1R, IKBKG and PIK3CGs) had significantly higher expression in the susceptible lambs and significantly lower expression in the resistant lambs relative to the Control group. The gene FXYD1 had increased expression in the resistant lambs and lower expression in the susceptible lambs. The gene CREB3L3 was not expressed in either the Control group or the susceptible lambs but was expressed in the resistant subgroup (Elcombe et al., 2021).

2.8.3.2. Transcription Factors

One study (Elcombe et al., 2023) re-examined the neonatal and pre-pubertal lambs alongside the novel results from the adult (11-month-old) rams to identify transcription factors (TFs) with over-represented gene products within the DEG lists for each age group. In the pre-pubertal lamb, they identified no significant difference in the expression of the TFs FOSL1 and JUND, however, a significant decrease and increase respectively were seen in the expression of their gene products (Elcombe et al., 2023). Only three TFs (FOXA1, FOXP2 and BCL11A) had altered gene production expression in both the neonatal and pre-pubescent lambs. Whilst in the neonate there was a significant decrease in gene production expression there was a significant increase in the pre-pubertal lambs (Elcombe et al., 2023). When examining the expression levels of the TFs themselves no difference was seen in FOXA1 in either age group. Whilst there was also no significant difference in BCL11A or FOXP2 in the neonate, a significant increase in the expression of these two TFs was seen in the pre-pubertal lambs (Elcombe et al., 2023). A significant increase in the expression of gene products of GATA was seen in both the pre-pubertal and adult rams. However, there was no significant difference in GATA expression levels in either age group (Elcombe et al., 2023).

2.8.3.3. mTOR Signalling Pathway

The mTOR signalling pathway was highlighted as enriched by GO analysis of DEGs in the neonatal (Elcombe et al., 2021), foetal (Lea et al., 2022), prepubertal (Elcombe et al., 2022) and adult sheep (Elcombe et al., 2023). In the prepubescent lambs the DEGs which identified the mTOR pathway as enriched were common with DEGs from human TDS studies (Elcombe et al., 2022).

Spermatogenesis is controlled by a variety of signalling pathways, including the mTOR pathway (Oliveira et al., 2017, Ni et al., 2019, Correia et al., 2020). mTOR is a serine/threonine kinase which binds and interacts with other proteins to form either the mTOR complex 1 (mTORC1) or mTOR complex 2 (mTORC2). Humans treated with rapamycin, a specific inhibitor of mTORC1, had poor sperm quality with patients presenting with oligospermia (reduced sperm count), tetratospermia (low percentage of sperm with normal morphology), asthenospermia (poor sperm motility) and in some azoospermia (lack of sperm in ejaculate). They also presented with increased levels of LH and FSH and decreased levels of testosterone (Correia et al., 2020).

Rodent studies have supported these observations with inhibition of mTOR results in impaired spermatogenesis. Conditional knockouts of Rheb, which reduces the activity of mTORC1, resulted in oligozoospermia, asthenozoospermia, and teratozoospermia. It also resulted in abundant spermatogonia and low numbers of spermatocytes and spermatids indicating a role for mTOR in both the meiotic and post-meiotic stages of spermatogenesis (Jesus et al., 2017, Correia et al., 2020). Inhibition of mTOR in germ cells showed reduced expression of meiotic markers, suggesting a role for mTORs in the differentiation of germ cells (Correia et al., 2020). mTORC1 is thought to play an important role in spermatogonia proliferation and differentiation (Jesus et al., 2017, Cao et al., 2020, Correia et al., 2020).

Continuous activation of mTORC1 through the removal of normal inhibition, can cause increased differentiation of spermatogonia which can lead to premature depletion of undifferentiated spermatogonial stem cell pools (Hobbs et al., 2010, Xiong et al., 2015, Correia et al., 2020). Rapamycin mediated inhibition of the mTORC1 resulted in lower levels of downstream products including phosphorylated p70-S6K1, and rpS6 levels, which may implicate them as having a role in spermatogenesis (Jesus et al., 2017).

Adult humans treated with rapamycin presented with increased levels of LH and FSH and decreased levels of testosterone (Correia et al., 2020). Reduced testosterone levels were present in the neonates lambs (Elcombe et al., 2021), and foetuses exposed during late gestation. However, gene array analysis was only performed on foetuses continuously exposed for 140 days and during early gestation, in which no significant difference was observed in testosterone levels (Lea et al., 2022). The reduction in testosterone levels suggests mTOR may also have a role in Leydig cells, whilst most studies have focused on Sertoli cells.

In the neonatal lambs, 6 DEGs were identified in the mTOR signalling pathway with significantly lower gene expression in Biosolids lambs. These included RICTOR and mLST8 (Elcombe et al., 2021) genes which encode components of mTORC1 and mTORC2. mLST8 is present in both mTORC1 and mTORC2, whilst RICTOR is only present in mTORC2 (Jesus et al., 2017). Though the function of mLST8 (mammalian lethal with sec-13 protein 8, also known as GβL) in mTORC1 is unclear, its removal from mTORC2 is shown to significantly reduce the stability and function of mTORC2 (Jesus et al., 2017).

RICTOR (rapamycin-insensitive companion of mTOR) is thought to help form the structural basis of the complex. It is highly expressed in the early stages of the epithelial cycle in the seminiferous tubules and gradually reduces through the late stages of the cycle (Jesus et al., 2017). It is possible that the observed downregulation of RICTOR may be due to normal variations in the stages of the seminiferous epithelial cycle between animals in the Control and Biosolids groups. Conditional knockout of RICTOR in germ cells has shown evidence of spermatogenesis impairment with some seminiferous tubules lacking germ cells including elongated spermatids (Correia et al., 2020).

There is evidence to suggest that mTOR complexes may have an important role in the blood-testis-barrier (BTB) (Oliveira et al., 2017, Moreira et al., 2019, Correia et al., 2020). It is suggested that mTORC1 is needed to weaken the BTB during the later stages of the spermatogenic cycle to allow for the movement of spermatocytes to the adluminal region. mTORC2, however, is thought to be needed for the maintenance of the BTB during the early stages of the spermatogenic cycle. The conditional knockout of RICTOR in Sertoli cells did not affect the expression of tight junction proteins but instead resulted in their mislocalisation and disorganisation (Jesus et al., 2017, Oliveira et al., 2017, Moreira et al., 2019, Correia et al., 2020).

Both BPA and DEHP has been shown to inhibit the PI3K-Akt-mTOR signalling pathway leading to apoptosis and autophagy (Quan et al., 2017, Fu et al., 2020). Whilst exposure to another environmental chemicals, 4-nonylphenol (4NP), resulted in the upregulation of phosphorylated AMPK and inhibition of mTOR, leading to autophagy in Sertoli cells (Duan et al., 2017).

qPCR was performed in the pre-pubertal lambs to quantify the expression of downstream mTOR genes. Of these downstream genes, FASN, HK1, PDPK1 and VEGFA, all of which are transcribed by HIF1α, had significantly higher expression levels in the Biosolids group (Elcombe et al., 2022). HK1 and VEGFA mRNA expression was significantly increased in the neonate Biosolids group while no difference was observed in PDPK expression. No changes were seen in expression of these three genes in the adult sheep (Elcombe et al., 2023).

VEGFA is a growth factor with various isoforms, which can be angiogenic or antiangiogenic (McFee et al., 2012, Sargent et al., 2016). Within rodents, VEGFA and its receptors are expressed in Sertoli cells, germ cells and Leydig cells at various levels coinciding with the recruitment and development of germ cells (Sargent et al., 2016). Bovine testis treated with VEGFA164 (an angiogenic isoform) showed promoted germ cell survival and is believed to be

involved in the self-renewal of spermatogonia stem cells. However, antiangiogenic isoforms of VEGFA, such as VEGFA165b, are thought to promote differentiation (Sargent et al., 2016). Though an increase in VEGFA expression was seen in both the neonates (Elcombe et al., 2023) and the 8-week-old lambs (Elcombe et al., 2022), there was no indication of which isoforms of VEGFA was upregulated.

2.8.3.4. HIF1α

Hypoxia Inducible Factor 1 is a heterodimer transcription factor comprising of the subunits HIF1β (encoded by ARNT) and either HIF1α or HIF2α (encoded by EPAS1). The specificity of the HIF1 differs based on the presence of the HIFα subunit (Düvel et al., 2010, Wu et al., 2015, Graham and Presnell, 2017). Cells deficient in tuberous sclerosis complex 1 (TSC1 or hamartin) and 2 (TSC2 or tuberin), which removes inhibition of mTORC1, had increased levels of HIF1α protein and upregulation of HIF1α target genes. Inhibition of mTORC1 through rapamycin blocked the increase in HIF1α protein levels (Düvel et al., 2010).

mTOR has been demonstrated to regulate HIF1α translation and transcription (Laplante and Sabatini, 2013). mTORC1 is thought to selectively control HIF1 translation from the 5'UTR of the mRNA, through the 4E-BP1-eIF4E pathway. HIF1 protein expression is increased through promotion of cap-dependent translation (Düvel et al., 2010). A possible mechanism for mTORC1 influence on HIF1α transcription has been demonstrated in mouse embryonic fibroblasts. Glycogen synthase kinase 3 (Gsk3) promotes the phosphorylation of Foxk1, which in turn induces HIF1α transcription. mTORC1 inhibits Foxk1 phosphorylation through the inhibition of Gsk3 (He et al., 2018).

Nuclear localisation of HIF1α was significant in both the neonates and pre-pubertal lambs but to a greater degree in the neonates compared to the pre-pubescent lambs, suggesting a foetal origin (Elcombe et al., 2022, Elcombe et al., 2023). No significant localisation was observed in the adults (Elcombe et al., 2023).

Western blotting in the 8-week-old lambs showed significantly less HIF1α present in the Biosolids testes compared to the Controls. The effects of biosolids exposure on the nuclear localisation of HIF1 to Leydig cell nuclei is thought to overweigh the reduction in its relative protein levels within the testis, causing an overall increase in HIF1 activation. It was noted that the reduced HIF1 protein level may be due to changes within other cells in the testis which were not examined as closely as Leydig cells (Elcombe et al., 2022). The relative abundance of HIF1 was not examined in the neonates or adults (Elcombe et al., 2023).

2.8.3.4.1. STAR

The upregulation of HIF1 in hypoxic conditions in rodents significantly reduced testosterone synthesis both in vitro and in vivo (Zhang et al., 2013, Wang et al., 2019a, Karimi et al., 2023). Studies have found that hypoxia regulates steroidogenesis through abnormal STAR synthesis in the testis (Wang et al., 2019a) and ovaries (Kowalewski et al., 2015).

One study in TM3 Leydig cells found that under cobalt chloride induced hypoxia, testosterone levels were significantly decreased, the expression of STAR was significantly reduced and HIF1α expression was significantly increased (Karimi et al., 2023). Another study in mice testis identified significant increases in HIF1a and VEGFA expression and a significant decrease in STAR expression under hypoxic conditions. They also reported that silencing HIF1 in mice resulted in the upregulation of STAR and an increase in testosterone levels. HIF1 was shown to have three binding sites on the STAR promotor region through which HIF1 could regulate STAR transcription (Wang et al., 2019a).

The transcription factor NRF1 (nuclear respiratory factor 1) has also been implicated in the regulation of STAR translation under hypoxic conditions (Wang et al., 2017b, Zhang et al., 2017, Wang et al., 2019b). Reduced NRF1 expression in the testis following exposure to hypoxic conditions was correlated with reduced testosterone levels and STAR transcription levels. It is suggested that NRF1 binds to the promotor region of STAR to promote its expression (Wang et al., 2017b). NRF1 is thought to interact with the peroxisome proliferator-activated receptor gamma coactivator-related protein 1 (PPRC1) (Andersson and Scarpulla, 2001), a gene which was downregulated in the prepubescent Biosolid exposed lambs (Elcombe et al., 2022).

Further information can be attained from samples of these sheep to have a wider perspective on more potentially altered parameters. The study conducted on the pre-pubescent lambs only examined germ cells and DEGs common between those identified in human TDS patient studies and prepubescent Biosolids exposed lambs (Elcombe et al., 2022), rather than examining differences between the Control and Biosolids group. As of yet no work has been done to examine the populations of other vital testicular cells including Sertoli and Leydig cells. Alterations to these cell types could impact the germ cells previously identified as significantly reduced in the Biosolids group.

Work in rodents suggests the potential for persistent effects of in-utero exposure of individual EDCs in future generations (Doyle et al., 2013). This raises implications for continued effects of biosolids exposure in future generations and warrants further investigations into the F2 generation to identify potential continuous effects.

2.9. Hypothesis and Aims

We hypothesised that exposing pregnant ewes to a mixture of environmental chemicals at environmentally relevant concentrations would perturb somatic cell populations and alter the expression of key steroidogenic enzymes in the testis of 8-week-old male lambs in both the F1 and F2 generations.

The overall aim of this study is to examine histological and molecular effects of biosolids exposure on pre-pubertal lambs at 8-weeks of age in both the F1 and F2 generations. The previous study examining prepubescent lambs didn't examine Sertoli cell number or Leydig cell population. This study aims to quantify these histological parameters in the F1 generation to fill the gap of information as well as examine the same parameters in the F2 generation which have yet to be investigated. This study will also utilise qPCR to further examine genes highlighted by previous RNAseq analysis in the F1 generation, as well as analyse the expression of steroidogenic enzymes in both generations.

3. General Materials and Methods

3.1. Animals and Treatments

All animal procedures were approved by the Animal Welfare and Ethical Review Board (AWERB) of the University of Glasgow, where Biosolids exposure took place. In addition, procedures undertaken on living donors were performed under the auspices of the Animal Scientific Procedures Act (1986). Associated protocols complied with the ARRIVE guidelines.

Ewes were mated through artificial insemination using semen from four rams maintained on control pastures. To limit the impact of paternal genetics on the results, a record was kept of which rams sired which lambs to allow for the inclusion of 'sire' into the statistical analysis.

The EasyCare F0 ewes were either maintained on pasture treated with biosolids or inorganic fertiliser. Biosolids was applied to pasture using conventional rates (i.e. 4 tonnes per hectare) once in April and again once in September. The control pasture was fertilised using inorganic fertiliser (225kg Nitrogen per hectare per year) to supply equivalent nitrogen levels. Exposure occurred for 1 month prior to mating and throughout gestation. Though the ewes were brought indoors for the last two weeks of gestation they were maintained on concentrates and forage harvested from their respective pastures. Following parturition all ewes and lambs were kept on control pastures limiting lamb exposure to that taking place in-utero.

Once 8-weeks old, a cohort of 11 Control ram lambs and 11 Biosolids lambs were weighed and then euthanised via an intravenous barbiturate overdose (140 mg/kg Dolethal, Vetroquinol, UK). These lambs were from separate ewes and were balanced across sires. The testes were removed at necropsy.

Remaining lambs were reared to sexual maturity and bred within exposure groups (i.e., Control or Biosolids) at around 19 months of age to produce the F2 generation. Mating took place between half-sib family groups thus avoiding mating of close relatives.

A group of 12 F2 Control and Biosolids ram lambs were weighed and euthanised via an overdose of intravenous barbiturates.

3.2. Immunohistochemistry

3.2.1. Tissue Processing and Fixation

Histological analysis utilised the left testis, with samples consisting of two transverse sections from the centre of the left testis. Samples were fixed in 10% buffered formalin (Thermo Scientific) overnight, dehydrated in 70% ethanol and then embedded in paraffin wax.

3.2.2. Sectioning of Tissue

The paraffin wax blocks were chilled on ice to support the cutting of 5µm sections for immunohistochemistry. The blocks were placed in the microtome (Leica rm2235) and representative section were cut at a thickness of 5µm and placed on Superfrost microscope slides before baking.

3.2.3. Dewaxing and Rehydration

Wax was removed through exposure to two successive xylene bath for 5 minutes each. The slides were rehydrated by soaking in successively weaker solutions of absolute ethanol (100% to 75% ethanol solutions). They were then placed in two baths of distilled water for 5 minutes each.

3.2.4. Antigen Retrieval

The fixation process results in the formation of cross-linkages between proteins which can restrict the binding of primary antibodies to their antigen targets. The antigen retrieval process removes these linkages, exposing the antigens for binding to primary antibodies.

A heat-induced epitope retrieval method was utilised. In brief, slides were submerged in 10mM sodium citrate buffer (pH 6.0) and microwaved on medium for 4 x 5 minutes with 1 minute rest between each. The slides were then left in the hot buffer and allowed to rest for a further 20 minutes. Following this, slides were washed in distilled water.

3.2.5. Peroxidase blocking

To block endogenous peroxidase activity and prevent non-specific background staining, the slides were incubated in 3% hydrogen peroxide (H_2O_2) (Sigma, H1009-500ML) solution for 15 minutes. The slides were then submerged in two washes in phosphate buffer saline containing 0.2% Tween-20 (PBST) (Gibco, 18912-014) (Sigma, P-1754, 073k006) for 5 minutes each.

3.2.6. Serum blocking/non-immune blocking

The tissue was drawn around with a wax pen to keep the treatment solutions over the tissue and then placed in a humidity chamber. To block non-specific binding sites slides were individually incubated for 30 minutes at room temperature in 200 µl of blocking serum at a dilution of 10%. As horse anti-mouse/rabbit secondary antibodies were used to bind to the primary antibodies, normal horse serum (Vectastain Elite ABC universal kit, PK-6200, Vector laboratory USA) was used.

3.2.7. Non-specific background staining Avidin and biotin blocking

Endogenous biotin was blocked through incubation with avidin and then with biotin for 15 minutes each to block any additional biotin binding sites present on the avidin molecules. The blocks were both washed off with PBST.

3.2.8. Primary Antibodies

The primary antibodies were diluted in 10% horse serum buffer, previously used as the serum block. The concentrated primary antibodies were aspirated to ensure good mixing and added to the serum buffer in 1.5 ml microtubes. The diluted solution was again aspirated before 200 µl was added to each tissue section. The tissue was left to incubate in the primary antibody overnight in a humidity chamber in the fridge at 4°C.

Non-specific rabbit IgG at the same concentration as the primary antibody was added to 2 slides as a negative control.

Table 3. Primary antibodies used to stain Sertoli cells and Leydig cells

Cell Marker	Antibody	Type	Dilution Used	Source
Sertoli Cell	AMH	Mouse monoclonal IgG	1/100	Santa Cruz Biotechnology
	Vimentin	Rabbit Polyclonal	1/500	Proteintech 10366-1-AP
Leydig Cell	CYP17A1	Rabbit polyclonal	1/100	Biorbyt Ltd
Negative Controls	Rabbit IgG	Normal Rabbit IgG	Matched to primary antibody	ABCam
	Mouse IgG	Normal Mouse IgG		

3.2.8.1. AMH

The F1 generation was stained with AMH antibody by a previous student using a 1/100 dilution (Tanner, 2023). This AMH antibody was no longer available for purchase at the time of this experiment. An alternative AMH antibody (ab229212) was purchased for use on F2 testis as it was predicted to work in sheep. Initial attempts at optimising and verifying this AMH antibody utilised a range of dilutions between 1/50 and 1/800. Staining with a 1/50 dilution resulted in minimal staining or localised staining of Sertoli cells surrounding germs cells so it was agreed that this AMH antibody would not be used going forward.

As the AMH antibody was only being used to help indicate the location of Sertoli cells for the manual counting of cells, rather than to measure the expression of AMH, it was deemed that an alternative antibody target (vimentin) could be used to demonstrate Sertoli cell locations.

3.2.8.2. Vimentin

Vimentin (Proteintech 10366-1-AP) (initial concentration 500 µg/ml) was used as an alternative to the AMH antibody. Adult sheep testis stained with 1/1000 dilution of vimentin resulted in clear staining of Sertoli cells so 8-week-old samples were similarly stained, which yielded faint staining of Sertoli cells. Additional runs using a dilution of 1/500 resulted in much clearer staining which highlighted the Sertoli cells within the tubules.

3.2.8.3. CYP17A1

F1 slides were staining with CYP17A1 (biorbyt ltd. orb228533) by a previous student (Tanner, 2023), whilst the F2 slides were stained by a laboratory technical specialist.

3.2.9. Secondary Antibody Application

The primary antibody was removed and sections washed with phosphate buffer saline (PBS) twice for 5 minutes each. As the primary antibodies used were either rabbit or mouse derived antibodies, the secondary antibody utilised was horse anti-mouse/rabbit IgG (H+L). Biotinylated antibodies supplied in a kit (Vectastain Elite ABC universal kit, PK-6200, Vector laboratory USA) and diluted in 10% horse serum buffer. Following aspiration to ensure the antibodies were adequately mixed 200 µl was pipetted onto the slide within the wax ring and left to incubate for 30 minutes at room temperature in a humidity chamber.

3.2.10. ABC

The secondary antibody was removed and washed in PBS twice for 5 minutes each. Following this avidin-biotin-horseradish peroxidase enzyme complex (ABC-HRP) solution was added (Figure 3) to amplify signal intensity and increase the sensitivity of the method.

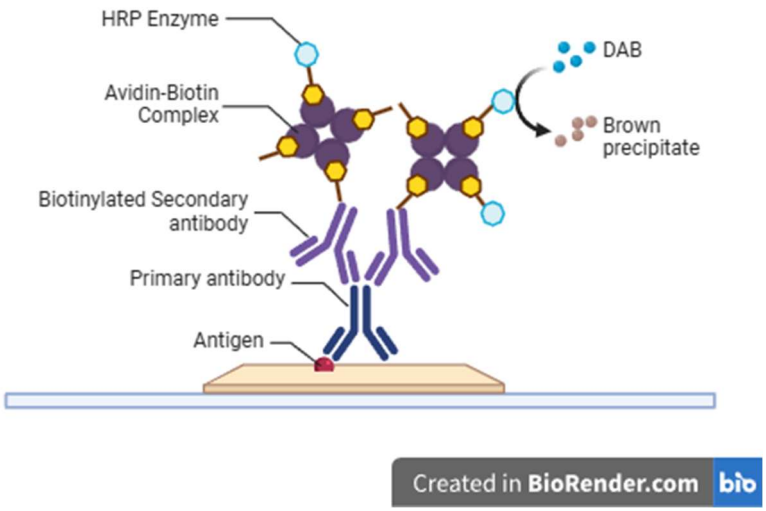

Figure 3. Diagram of Avidin-Biotin Complex binding. The Primary antibody binds to the antigen. The biotinylated secondary antibodies bind to the primary antibody. Avidin-biotin complexes bind to the biotinylated secondary antibodies. The Avidin-biotin complexes are conjugated to the detection enzyme horseradish peroxidase (HRP). In the presence of HRP DAB (diaminobenzidine) will form a brown precipitate. Created in BioRender.

A kit was used to make the ABC solution (Vectastain Elite ABC universal kit, PK-6200, Vector laboratory USA). Two drops of reagent A (avidin) and two drops of reagent B (biotinylated HRP) was added to 5ml of normal horse serum diluted in PBST. The ABC solution was made up and allowed to rest for 30 minutes before 200µl of ABC solution was pipetted onto each slide and left to incubate for 30 minutes.

3.2.11. DAB

After incubation the ABC solution was removed and sections washed in PBST twice for 5 minutes each. To visualise and locate the presence of the primary antibodies the chromogenic substrate 3,3'-diaminobenzidine (DAB) was added to the samples. The Avidin biotin complex was HRP conjugated, in the presence of HRP, DAB produced a brown precipitate, indicating positive staining for the specific antigen bound to the primary antibody.

Following mixing of DAB following the kit protocol, 200µl of DAB (Dab Substrate Kit, SK-4100, Vector laboratory USA) was added to each slide and once clear brown staining was visible, or 20 minutes had passed the excess DAB was removed and the slide was dipped in water for 5 minutes to stop the reaction.

When staining a full batch of slides 20 seconds were left between each application of DAB to allow for the knocking off of excess DAB, placement into a slide rack and dipping in distilled water. They were then rinsed again and kept in distilled water until counterstaining. Leftover DAB was mixed with bleach and once deactivated, disposed of.

3.2.12. Counterstaining

The slides were counterstained in haematoxylin for 5 minutes to stain the nuclei of the surrounding tissue blue, following incubation the slides were rinsed with running water. They were then dunked 5 times into acidified industrial methylated spirits (IMS) to remove non-specific background staining before being rinsed in running water. Following this they were dunked 5 times in ammoniated water, which turned the nuclear stain blue in the alkaline solution and then rinsed in running water.

3.2.13. Dehydrating and Cover-Slipping

The slides were then dehydrated by dunking the slides in 75%, and then 90% absolute ethanol. They were then submerged in 2 separate baths of 100% absolute ethanol for 2 minutes each. Following this they were added to 2 separate xylene baths for 5 minutes each.

A small droplet of the mounting medium DPX was added to glass coverslips five at a time. Slides were then removed from the slide rack submerged in xylene and pressed into the coverslip and DPX so that the coverslip covered the tissue. Any air bubbles were pushed out. Slides were left to dry for 24 hours before being viewed under a microscope.

3.3. Image Analysis

3.3.1. Image Capturing

A total of 20 images were captured of each slide. Five images were taken at each pole of the tissue moving along the horizontal and vertical axis with the first image taken at each pole (as demonstrated in Figure 4) to avoid the inclusion of the rete testis in the images taken. Images were captured using a Leica microscope with a X20 objective lens at a magnification of X200.

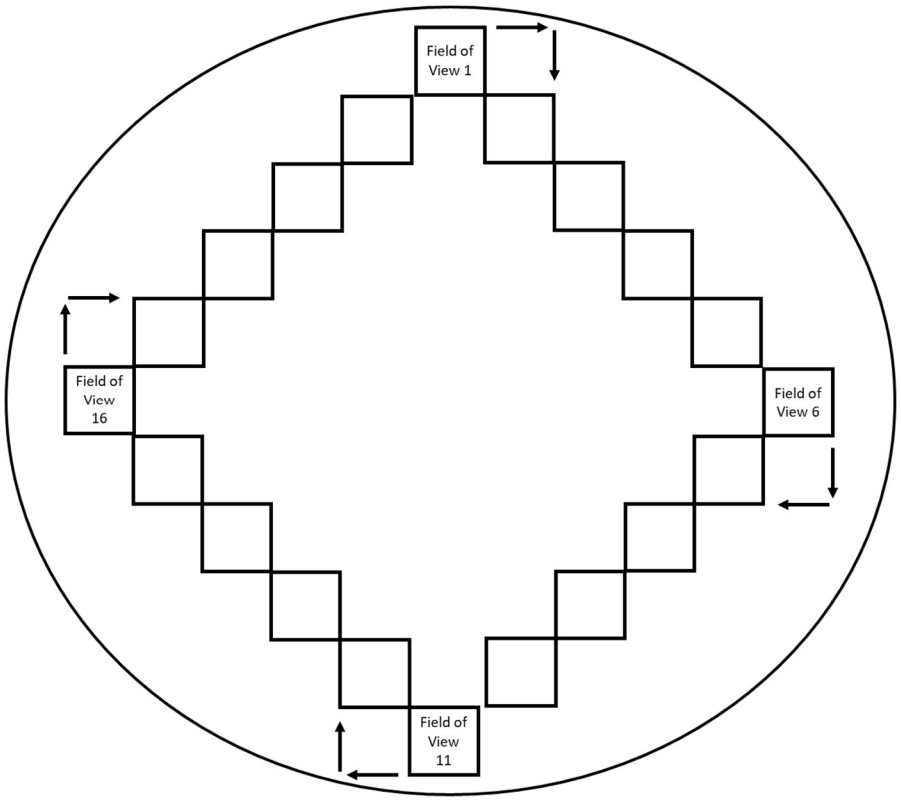

Figure 4. Diagram of fields of view captured on all tissue samples.

3.3.1.1. Image Pro vs ImageJ

To conduct the different analyses of the stained images a mixture of two software packages were utilised. ImageJ was used when counting Sertoli cells as it was more accessible and could be used away from office computers, without the need for organising a timeshare between members of the research group for the use of a licencing dongle. The multipoint tool and the grid tool already existed in the core ImageJ software and were simple and easy to use.

Image Pro Premier was used to determine the average diameter of Sertoli cells by drawing around individual nuclei and exporting the results to an excel file. This method was more accurate and faster than the alternative in ImageJ in which individual diameters could be drawn onto a cell by hand and an average calculated. As such, Image Pro was used for this part of the method.

To determine the percentage area stained of CYP17A1 both pieces of software were considered as both allowed for the stained areas to be limited to the interstitial area. In ImageJ, different shades of staining could be selected with the point tool, with the colour segmentation window displayed the red, green, and blue values as well as σ. The primary disadvantage of this method was that it did not highlight the stained area, so it was difficult to ensure that all the stained tissue was being counted.

Whereas, within the Image Pro software there is a dropper tool which when hovered over the selected colour will magnify the selected area and show all the coloured pixels being measured which allows for more accurate selection of staining, it also highlights the selected areas giving a clear indication of which staining is being measured.

As the initial analysis of the F1 CYP17A1 staining was carried out using Image Pro as well as the advantages previously described, this software was used to analyse the CYP17A1 F2 generation.

3.3.2. Determining Sertoli cell Numbers

3.3.2.1. *Counting Sertoli Cells*

Using Image J software, a grid of 432 intersecting points with 24 lines across and 18 lines down was superimposed onto each image (Figure 5).

The number of intersections within the grid can only be altered through the input of area per point in pixels^2.

$$(No.of\ pixels\ across/24) \times (No.of\ pixels\ down/18) = Area\ per\ point\ (pixels^2)$$

The F1 images were 2560 pixels by 1920 pixels, whilst the F2 images were 2592 pixels by 1944 pixels.

$\left(\frac{2560}{24}\right) \times \left(\frac{1920}{18}\right) = 11378\ pixels^2$ and $\left(\frac{2592}{24}\right) \times \left(\frac{1944}{18}\right) = 11664\ pixels^2$ for the F1 and F2 images respectively.

If a Sertoli cell nuclei was present in all 4 quadrants around an intersection of the grid it was manually tagged using the ImageJ Multipoint tool (Figure 5). As each image was counted the number of Sertoli cell nuclei in each image was recorded to an Excel file where the total number of Sertoli cell nuclei across all 20 images was calculated.

The total number of Sertoli cells was then divided by the total number of intersections of the grid to calculate the total Sertoli cell number as a percentage of possible points

$$Total\ Sertoli\ Cell\ Count\ Per\ cent = \frac{Total\ no.\ of\ Sertoli\ cells\ counted}{Total\ no.of\ intersections = (20\ x\ 432)}$$

The total Sertoli cell count percent was then multiplied by the weight of the testis minus the epididymis to calculate the testis weight occupied by Sertoli cells (i.e. if testis weight was 1g and the total Sertoli cell count percent was 10%, Sertoli cells would occupy 0.1g of the testis). Absolute volume of Sertoli cells (cm^3) was then calculated based on the assumption that 1g of testis weight is equal to 1cm^3 volume. This was then converted from cm^3 to μm^3.

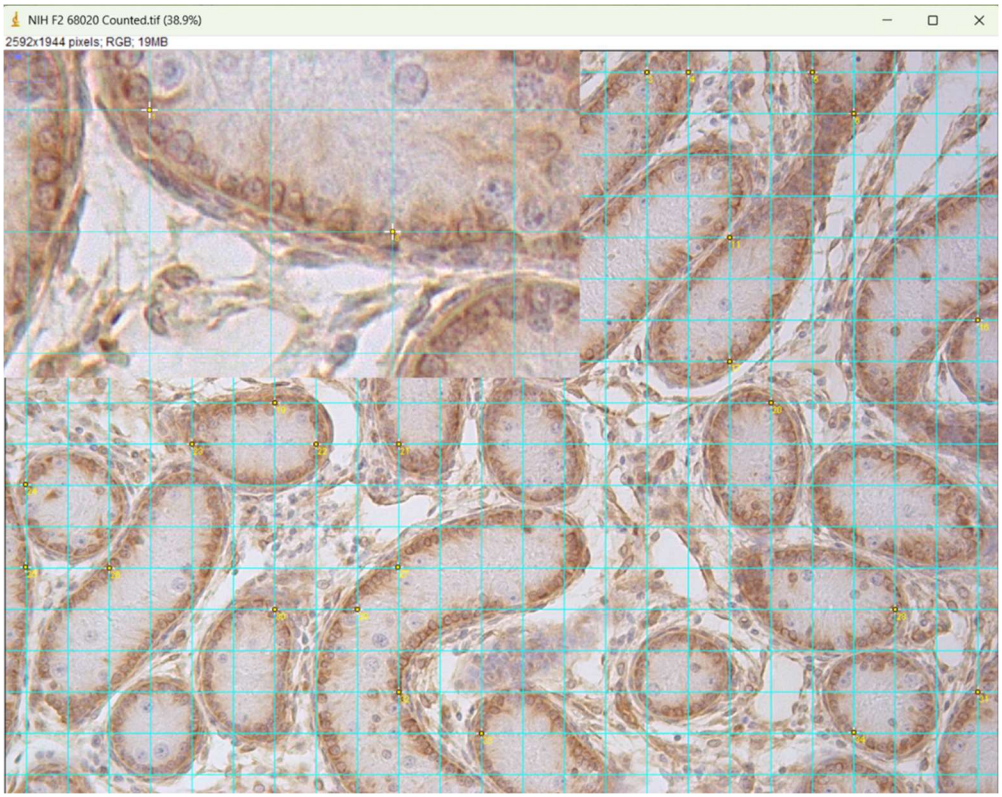

Figure 5. Section of testis stained with vimentin with a grid superimposed using ImageJ software. The yellow crosses with numbers beside them indicate the Sertoli cells which have nuclei which intersects with the overlayed grid and are present in all four quadrants around a grid intersection. The total number of Sertoli cells which coincided with the grid intersections was recorded. Insert of magnified image with yellow crosses indicating Sertoli cell nuclei which are present in all four quadrants around a grid intersection.

The mean nuclear diameter of 4 randomised Sertoli cells at each pole (or as close to the pole as possible) of an image was measured before calculating an average Sertoli cell diameter in pixels per image and then per sample. A total of 80 Sertoli cell nuclei were measured for each testis sample. The average diameter was then converted into microns using a calibrated scale bar taken at the same magnification.

The mean nucleic volume of the Sertoli cells was then calculated using the mean nuclear radius of Sertoli cells in the following equation:

$$\left(\frac{4}{3}\pi\right)r^3$$

The absolute volume of Sertoli cells previous calculated was then divided by the mean nuclear volume to estimate the total number of Sertoli cells in the testis.

$$Total\ Number\ of\ Sertoli\ Cells\ = \frac{Absolute\ volume\ of\ Sertoli\ cells\ (\mu m^3)}{Mean\ Nuclear\ Volume\ (\mu m^3)}$$

This was then adjusted for the weight of the testis and expressed as the number of Sertoli cells per gram of testis.

3.3.3. Determining Leydig cell Percentage Area Stained

Image Pro Premier was used to calculate the percentage of interstitial cells stained with CYP17A1.

The seminiferous tubules were excluded from the analysis to ensure that only the cellular staining in the interstitial area was measured. Regions of interest were drawn around the interstitial areas, excluding the seminiferous tubules from future analysis.

Images of the F2 CYP17A1 stained slides were analysed by the same technician. The F1 testis had been previously stained and analysed by a previous student, however, upon re-analysis by the technician different results were calculated of the F1 testis, which may be due to differences between the individual's perception of positive staining or when removing the area of the seminiferous tubules. Because of these discrepancies 8 Control and 8 Biosolids lambs were completely reanalysed for more consistent results.

The seminiferous tubules were individually drawn around to exclude them from further measurements (Figure 6). The area of positive staining both for CYP17A1 (Figure 6) and haematoxylin was selected for and measured as pixels^2. The proportion of CYP17A1 positive cells was calculated as a percentage of total cellular staining in the interstitial area.

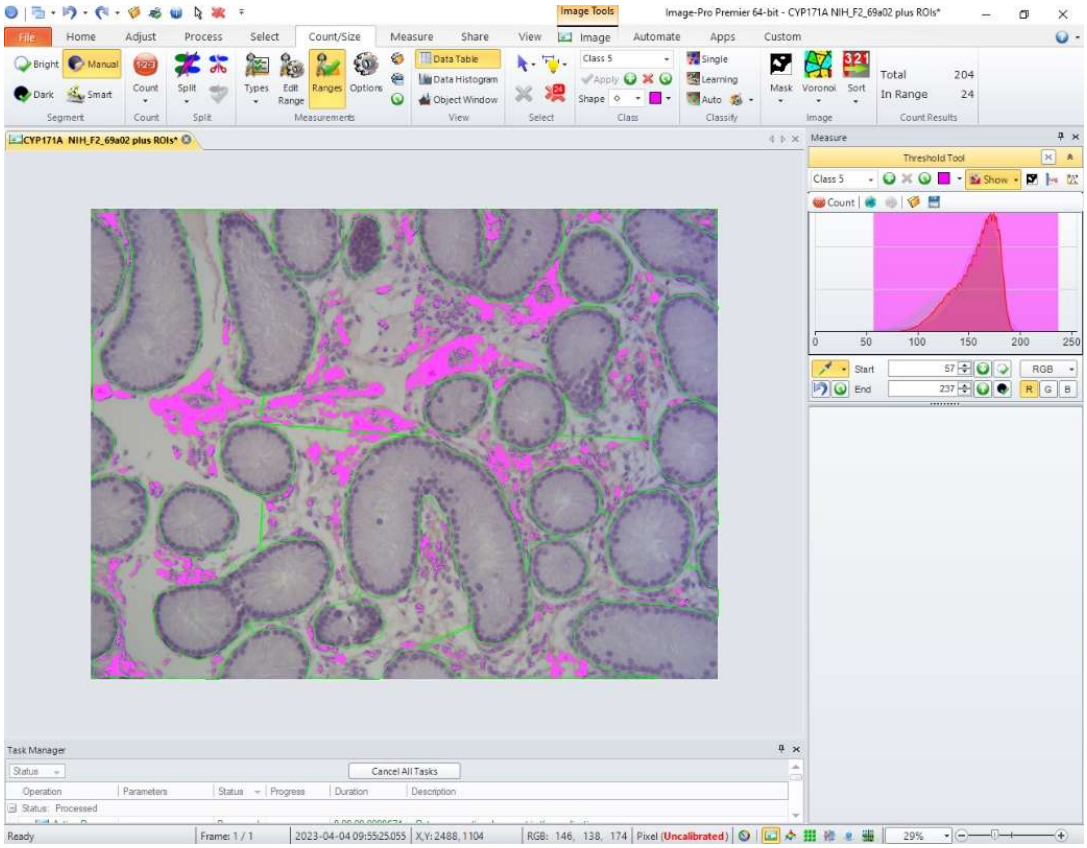

Figure 6. Selection of CYP17A1 positive staining in the interstitial area.

3.4. Molecular Biology

3.4.1. Extraction and Reverse Transcription

A section of the right testis was frozen on dry ice and stored at -80°C until RNA and DNA extraction. RNA and DNA were extracted using a QIAwave DNA/RNA Mini Kit (Qiagen 80504) according to the protocol provided by the manufactures. Testis samples were disrupted and homogenised in lysis buffer (RLT Plus provided in kit) and β-mercaptoethanol (β-ME) and milled with a 5mm stainless steel bead. DNA was separated using the AllPrep DNA spin columns provided in the kit and purified and eluted using EB buffer. RNA was separated using RNeasy spin columns provided in the kit. The additional optional step of DNase digestion was included utilising a RNase-Free DNase Set (Qiagen 79254). The RNA was purified and eluted using RNase-free water. All samples were aliquoted and frozen at -20°C.

The extracted RNA was quantified (Quibit) to calculate the concentration of each sample to calculate the exact volume of RNA needed to produce uniform cDNA samples. 1µg of RNA was converted into cDNA using Qiagen's QuantiNova Reverse Transcription kit (205411).

Any gDNA was removed through addition of 2µl of gDNA removal mix to 1µg of RNA and RNase free water to make up to a total volume of 15µl. This was then incubated for 2 minutes at 45℃ before being placed on ice. Reverse transcription master mix containing reverse transcription enzyme and reverse transcription mix (including dNTPs and Mg^{2+}) was then added to the template RNA. This was then heated in a PCR thermocycler at 25℃ for 3 minutes for annealing, at 45℃ for 10 minutes for reverse transcription and a further 5 minutes at 85℃ to inactivate the reaction. They were then kept at 4℃ until being removed from the machine and placed on ice. The newly transcribed cDNA was aliquoted into PCR tubes and stored at -20℃ until being used for qPCR.

3.4.2. Selection of Genes

3.4.2.1. Reference Genes

Details of the housekeeping genes, *GAPDH* and *B2M* used previously on the F1 8-week-old animals by Elcombe and colleagues (Elcombe et al., 2022) were requested. Neither the *GAPDH* primers previously used by Elcombe or alternative primers for *GAPDH* were stable enough across the Control and Biosolids treatment groups to move forward with *GAPDH* as a housekeeping gene. Primers for *Cyclophilin* (sourced from (Lea et al., 2008)) and primers designed for *B-ACTIN (ACTB)* were also trialled as possible reference genes. A standard curve was generated for *B2M*, *B-ACTIN* and *Cyclophilin* primers (Table 4). These primers were trialled using 3 Control and 3 Biosolids samples with triplicates to assess their stability (Table 4).

3.4.2.2. Target Genes

Due to the suggested changed in Percentage staining of CYP17A1 positive Leydig cells some key Leydig cell markers were investigated with qPCR. These included several proteins involved in steroidogenesis, namely STAR, CYP11A1 and CYP17A1.

Elcombe identified several GO terms following RNAseq analysis of the same F1 testis as examined here. Of particular interest was the GO term spermatid development. Within this category the genes CIB1 and CELF1 were downregulated and upregulated respectively (Elcombe et al., 2022).

Elcombe (Elcombe et al., 2022) also previously analysed the expression of 14 genes using qPCR and identified a significant difference in the expression of 4 genes between the Control

and Biosolids groups. Analysis of primers previously used identified large product sizes, so primers were redesigned, and these 4 genes were reanalysed to confirm previous findings.

3.4.3. Primers

Several of the primers were designed on IDT using their PrimerQuest Tool. These were then blasted to ensure they would sequence the correct gene and further analysed using IDT's OligoAnalyser to assess for probability of primer interactions. Other primers were designed using NCBI's BLAST tool to ensure the primers spanned exon junctions. Two of the housekeeping genes used came from published literature, these included B2M previously used on the same animals (Elcombe et al., 2022) and cyclophilin (Lea et al., 2008). Primers least likely to have hairpins, homodimers and heterodimers were trialled using temperature gradients in the annealing step to identify optimum conditions to avoid production of non-specific double stranded DNA. Standard curves were plotted using 6 concentrations with five four-fold serial dilutions for all target and housekeeping genes to calculate primer efficiencies. When calculating primer efficiencies for *CYP11A1, CYP17A1* and *HK1* the neat cDNA was discounted as it overlapped with the next highest concentration, likely due to the presence of inhibitors (Table 5). As *PDPK1*'s primers well exceeded 100% it was not examined in the full generations.

3.4.4. Quantitative Polymerase Chain Reaction

As previously quantitative real-time polymerase chain reaction (qPCR) was performed on the 8-week-old lambs using qPCR Brilliant II SYBR Master Mix a similar methodology was applied here.

The 100µM primers were aliquoted and diluted to 10µM to avoid repeated thawing and freezing. Forward and reverse primers, RNase free water and qPCRBIO SyGreen Mix mix Lo-ROX (PCR Biosystems Ltd, London, UK) were combined to make up the master mix and 15µl were pipetted into PCR plate wells. Diluted cDNA (1:10) was then added to PCR plate wells with a multi-headed pipette. All samples were done in triplicate with no template controls as the negative control.

Samples were run on a BioRad machine using CFX-Connect software. The polymerase enzyme in the mastermix was activated by heating the sample to 95℃ for 2 minutes. The cDNA was then denatured at 95℃ for 5 seconds. The Primers annealed to the template cDNA at 60℃ for 15 seconds, followed by an extension period of 30 seconds at 72℃. The steps for denaturing, annealing and extension were then repeated for a total of 40 cycles. Melt curves were run after each analysis to confirm the presence of only one product.

Gel electrophoresis was run alongside 100bp DNA ladder molecular weight marker (Promega G2101) to further confirm the presence of only one product in the positive samples and confirm the lack of any product in the negative control.

3.4.5. Relative Gene Expression Calculation

Relative gene expression was calculated using a method described by Vandesompele et al (Vandesompele et al., 2002).

An average of Ct value was calculated for each sample for all of the housekeeping genes (HKG) and the genes of interest (GOI). Following this the calibrator Ct value was calculated for each gene (HKG and GOI) by averaging the Ct values of the Control group samples. This meant that results were presented as relative to the Control group average Ct values for each gene.

To determine ΔCt values the sample Ct values were subtracted from the calibrator Ct value individually.

The primer efficiency values for each gene were converted from a percentage. A converted primer efficiency value of 2 would equal a 100% efficiency value.

$$Converted\ Primer\ Efficiency = \frac{Efficiency\ (\%)}{100} + 1$$

The relative quantification (RQ) of each gene (HKG and GOI) was calculated using both the converted primer efficiency which functioned as the base of exponential amplification (E) and the ΔCt value for each sample.

$$Relative\ Quantification\ (RQ) = E^{\Delta Ct}$$

The Geometric mean of the housekeeping genes RQ values was calculated to allow for the combination of multiple HKGs to strengthen data normalisation.

Following this the relative gene expression was calculated using the following calculation:

$$Relative\ Gene\ Expression = \frac{RQ_{GOI}}{GeoMean\ (RQ_{HKG})}$$

3.5. Statistical Analysis

Neonatal weight, lamb weight, organ weights (testis, pituitary, combined thyroid, and combined adrenal), histological data and relative gene expression data were analysed using General Linear Regression (GLR: Genstat statistical package version 20; https://www.vsni.co.uk/). Terms fitted to the model were 'Sire Family' and Treatment (Biosolids vs Control). Residuals were plotted to ensure that data were normally distributed and of equal variance. Statistical significance was declared at $p<0.05$. Histological and relative gene expression data are presented as scatter plots with means and standard error of means (SEM) (GraphPad Prism Prism, version 10.2.0).

Table 4. Forward and Reverse Primers of Housekeeping genes used for RT-qPCR

Gene	Accession no.	Forward Primer	Reverse Primer	Product Size	Efficiency (%)	Stability	Primer Volume
B2M	XM_012180604.3	CCTGCTGTCGCTGTCTGGACTG	TCTCCCCGTTCTTCAGCAAATCG	161	96.5%	0.649	0.4
ACTB	NM_001009784.3	GCAAGTACTCCGTGTGGATT	CAGTCCGCCTAGAAGCATTT	133	89.0%	1.673	0.4
Cyclophilin/PPIA	NM_001308578.1	CATTCTGAAGCATACAGGTCCTG	TCCATGGCTTCCACAATATT	165	88.6%	1.673	0.4

Table 5. Forward and Reverse Primers of Target genes measured in RT-qPCR

Gene	Accession no.	Forward Primer	Reverse Primer	Product Size	Efficiency (%)	Primer Volume	Final Primer Conc
CYP17A1	NM_001009483.1	GGCTATCATTGACTCCAGCATT	TGCTGCCACTCCTTCTCATT	102	90	0.4	200nM
CYP11A1	NM_001093789.1	GGATCCTACCCACAGAGATATGA	CCAGGCTCCTGACTTCTTAAAC	99	84	0.4	200nM
STAR	NM_001009243.1	TGTCAAGGAGATCAAGGTCCTG	TCACGAAGTCTCGGGGACC	107	102.4	0.4	200nM
CIB1	NM_001114765.1	GCCTTCCGCATCTTCGACTTT	TCATCTCTGAAGCACTGAGCC	118	88	0.8	400nM
CELF1	XM_060400106.1	GTCAGCTCTTTGGCAGGGATG	GTCAGCAGGCTCTGGTTGTA	164	103.7	0.8	400nM
RBM23	XM_060418963.1	CTGGGATCGTCGACATAGTAATG	CTGGGCTCTTCTCTCTGAAATG	140	90	0.4	200nM
HIF1a	XM_027971913.2	CAGTCGTCACAGTCTGGATATG	GTCAGAGTCCAAGGCATGATAA	127	96	0.4	200nM
FASN	XM_027974304.3	CTTAACAGCACGTCCCCCAT	CAGTCTGGGTCCTCACCATTGA	149	89	0.4	200nM
VEGFA	NM_001025110.1	ACCAAAGCCAGCACATAGGA	CCTCGGCTTGTCACATTTTTC	104	100.2	0.8	400nM
HK1	XM_060406861.1	GACGAGCTTAGATGCGGGTA	CCACCACGTCCAGGTCAAAT	134	90.2	0.4	200nM
PDPK1	XM_027961765.2	CCACCAGCCAGCTGTATGAC	CAGTCTGGGTCCTCACCATTGA	90	148 or 138	-	-

4. Results

4.1. Lamb and Organ Weights of F1 Generation

No significant difference was observed between the birth weight of the Control and Biosolids exposed lambs. However, by eight weeks of age there was a significant decrease in body weight in the F1 Biosolids group ($p = 0.003$) when compared to their matched Controls (Table 6).

The testis plus epididymis weight was reduced but not significantly so in the Biosolid lambs, however upon removal of the epididymis weight, this difference approached statistical significance ($p = 0.051$: Table 6). Pituitary weight was significantly reduced ($p=0.04$) in the Biosolids lambs compared to Controls. No difference was found between the Biosolids and Control group for either combined adrenal weight or combined thyroid weight (Table 6).

4.2. Testis Histology of F1 Generation

A visual difference in the size of the seminiferous tubules within different F1 testis was noted when initially viewed. Multiple samples in both the Control and Biosolids groups in the F1 generation had smaller seminiferous tubules (Figure 7A and 7C) compared with seminiferous tubules from the testis of other animals (Figure 7B and 7D). This is visible in both the samples stained with AMH and CYP17A1. Seminiferous tubules were visually categorised into three groups, normal, smaller and smallest. Those designated as normal (n=11) had seminiferous tubules consistent with most other samples, those described as smaller (n= 5) had visually slightly smaller tubules than the normal group and those in the smallest subgroup (n=3) had visually smaller seminiferous tubules compared to both the normal and smaller group. These groups contained a mixture of Control and Biosolids samples. Trends were identified between the three groups with the smallest seminiferous tubules having the smallest testis weights (Figure 8A), smallest Sertoli cell nuclear diameters (Figure 8B), and greatest Sertoli cell number per gram of tissue (Figure 8C).

Quantification of F1 testis were stained with AMH (Figure 9A) showed no significant difference in the total number of Sertoli cells (million) (Table 6) or the number of Sertoli cells (million) per gram of testis (Figure 10A) was observed between the Biosolids (5202 ± 500.2) and Control (4332 ± 527.6) groups.

The percentage of the interstitial area positive for CYP17A1 staining (Figure 9C) was not significantly different between the Control (28.36 ± 4.022) and Biosolids treatment (24.19 ± 4.022) group. Within the Control group, results were highly variable whilst in those exposed to biosolids, percentage area of interstitial tissue stained with CYP17A1 were much less variable (Figure 10B).

Table 6. Lamb weight and organ weights of 8-week-old F1 lambs

F1	Control	SE	Biosolids	SE	P Value
Number of animals	11		11		
A. Lamb organ weight					
Lamb (birth) (kg)	4.364	0.1677	4.136	0.1677	-
Lamb (8 weeks) (kg)	25.23	0.8441	21.0	0.8441	**0.003**
Testis (g)	21.54	1.497	18.04	1.497	-
Testis minus epididymis (g)[a]	13.60	1.0147	10.65	0.9673	**0.051**
Pituitary (g)[a]	0.4868	0.01591	0.4376	0.01517	**0.040**
Combined Thyroid (g)	2.038	0.1463	1.748	0.1463	-
Combined Adrenal (g)	1.914	0.1276	2.148	0.1276	-
B. Testis Histology					
Total Sertoli Cell (million)[a]	55610	2890	50354	2740	-

[a] number of animals equals 10 for Control and 11 for Biosolids. Standard error (SE) values vary in parameters with uneven sample groups.

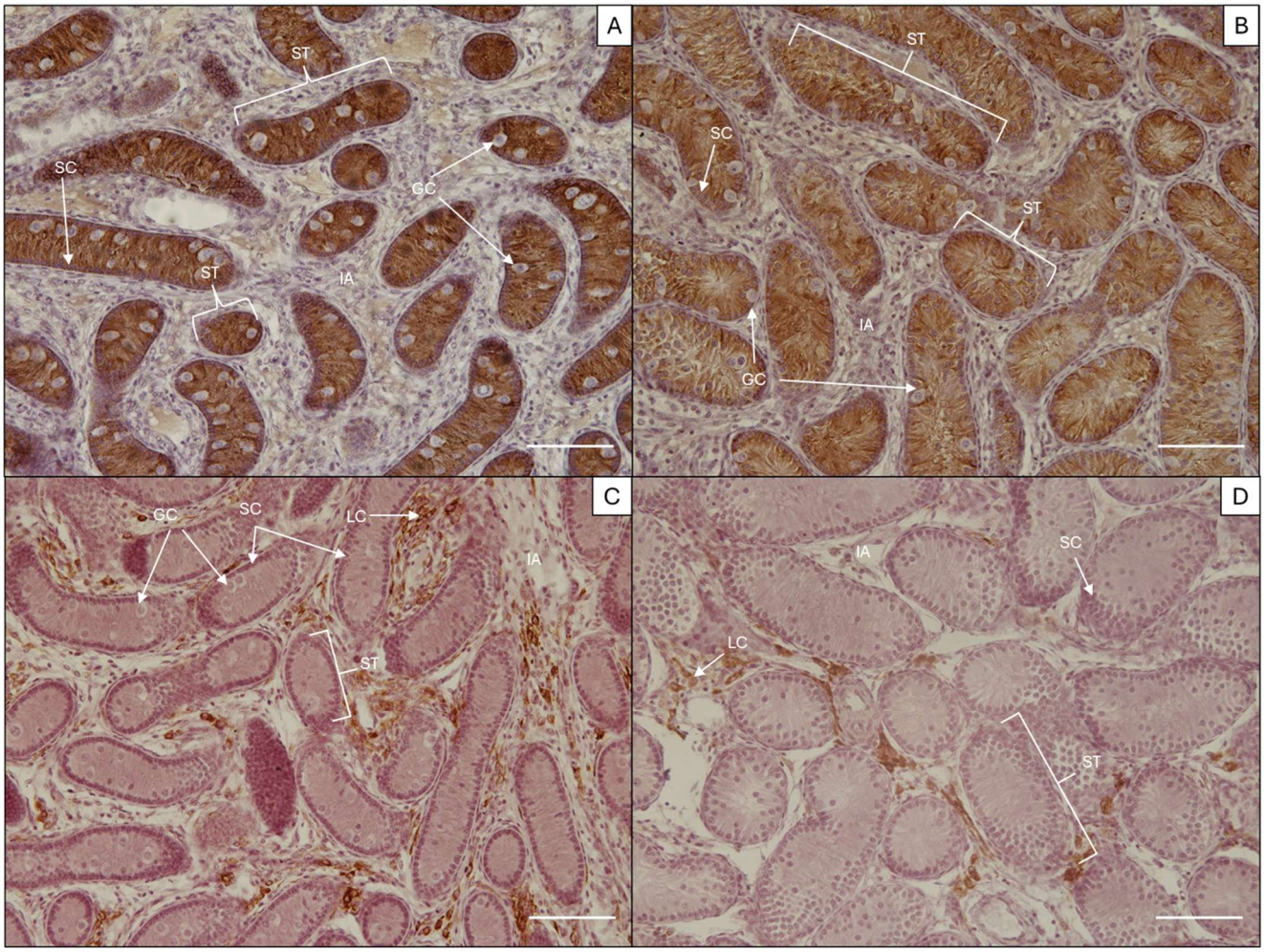

Figure 7. Images demonstrating variation in seminiferous tubules size within select individuals when stained with either AMH (A and B) or CYP17A1 (C and D). Images A and C are examples of an animal with the visually smallest seminiferous tubules. Images B and D are examples of an animal with visually normal seminiferous tubules. ST: Seminiferous Tubules, GC: Germ Cells, IA: Interstitial testicular area SC: Sertoli cells, LC: Leydig cells. Magnification x200. Scale bar = 50µm

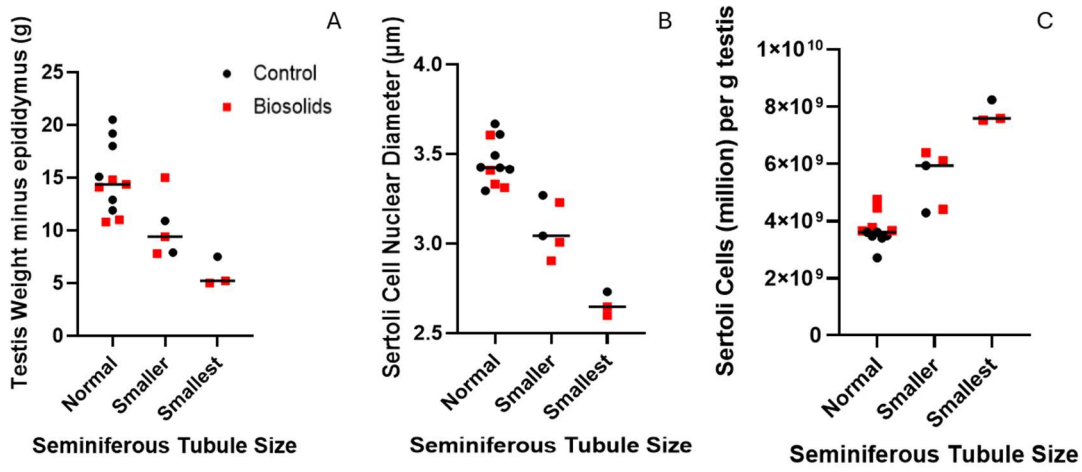

Figure 8. Variations in Testis weight, Sertoli cell nuclear diameter and Sertoli cell density across seminiferous tubules size categories. Black circles indicate Control animals and red squares are representative of Biosolids exposed animals. Data is presented as a scatter plot with simple means.

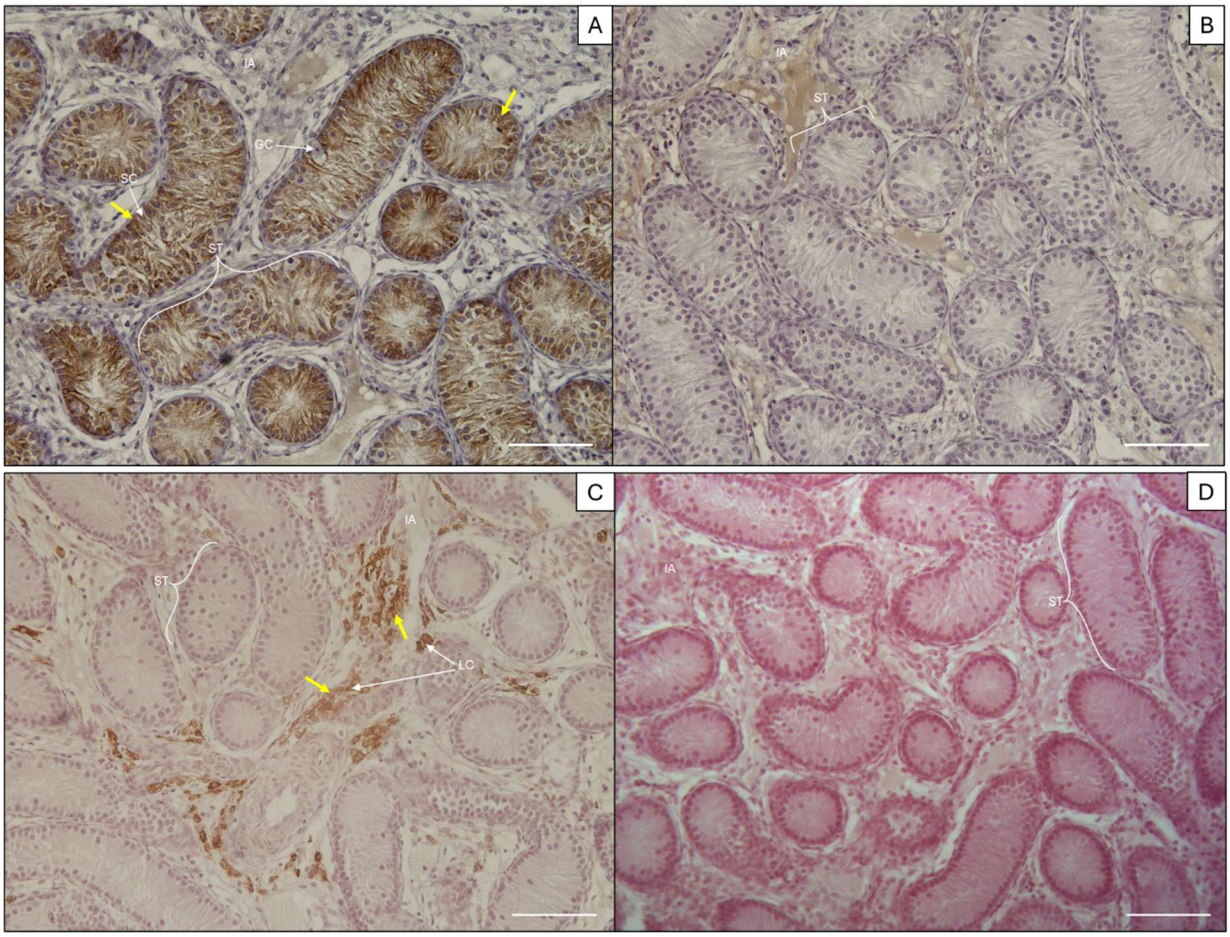

Figure 9. Staining of somatic testicular cells in F1 lambs (A) Seminiferous tubules stained with AMH to identify Sertoli cells and (B) matched IgG negative control. (C) Interstitial testicular area stained with CYP17A1 to highlight presence of Leydig cells and (D) matched IgG negative control. Yellow arrows indicate examples of positive staining. ST: Seminiferous Tubules, GC: Germ Cells, IA: Interstitial testicular area SC: Sertoli cells, LC: Leydig cells. Magnification x200. Scale bar = 50µm.

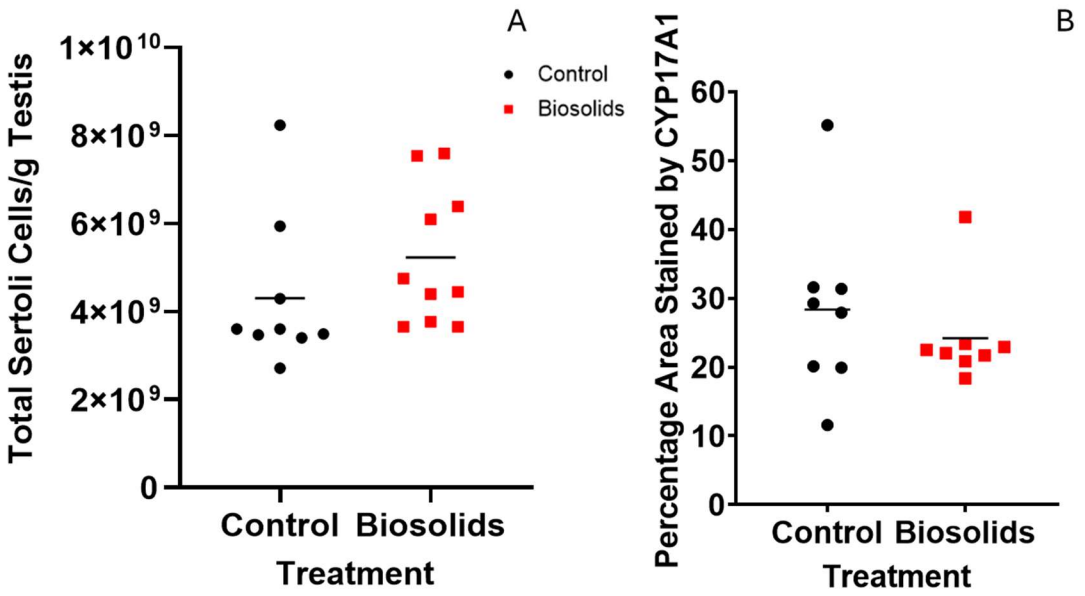

Figure 10. Quantification of testicular cell types in the F1 testis (A) Total number of Sertoli cells per gram of testis (minus epididymus) (Control n=9 Biosolids n=10). (B) Percentage area of Interstitial tissue stained positive for the Leydig cell marker CYP17A1 (n=8 for Control and Biosolids groups). Black circles indicate Control animals and red squares are representative of the Biosolids group. Data is presented as a scatter plot with simple means.

4.3. Molecular Biology of F1 Generation

This study utilised qPCR to quantify the relative expression of a total of 10 target genes. Gel electrophoresis was run for all 10 target genes and three housekeeping genes. A positive sample and negative control were selected from a single PCR plate for each gene. A single band was present in each positive well and no band was present in the negative well confirming the presence of only one product (Figure 11).

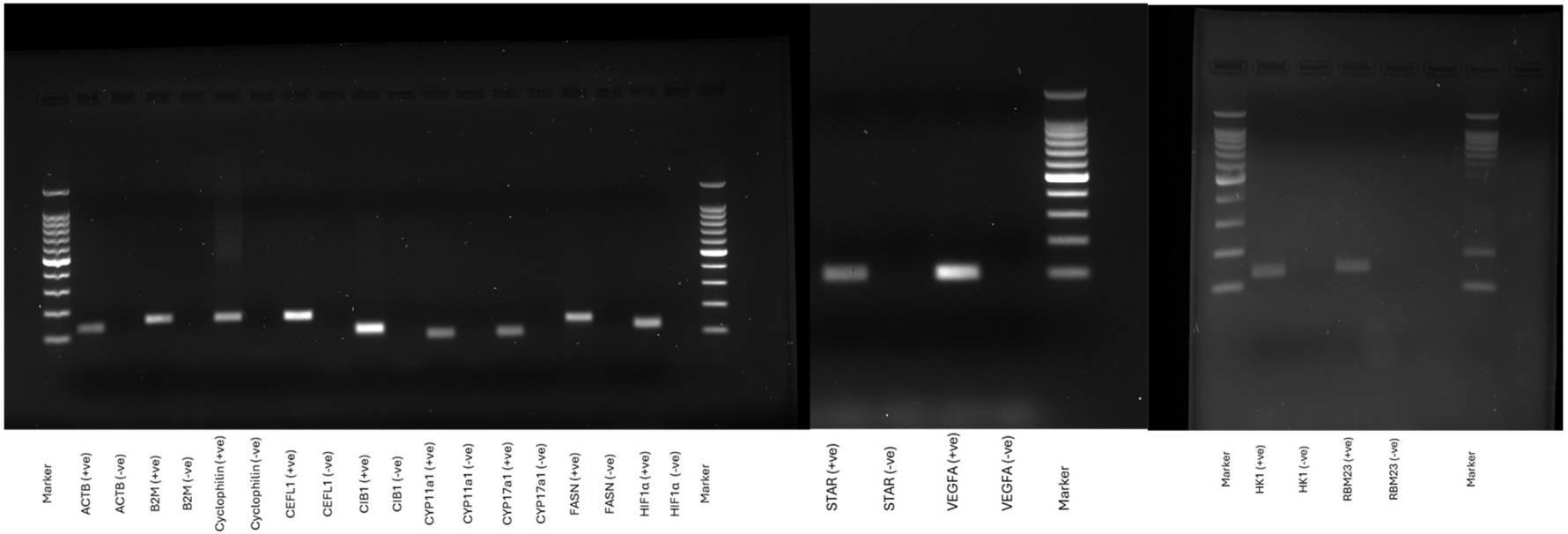

Figure 11. Gel Electrophoresis of housekeeping genes, ACTB, B2M, and *Cyclophilin, and the target genes CELF1, CIB1, CYP11A1, CYP17A1, FASN, HIF1α, STAR, VEGFA, HK1 and RBM23. Each gene has a positive well (+ve) followed by a sample from the negative control (-ve).*

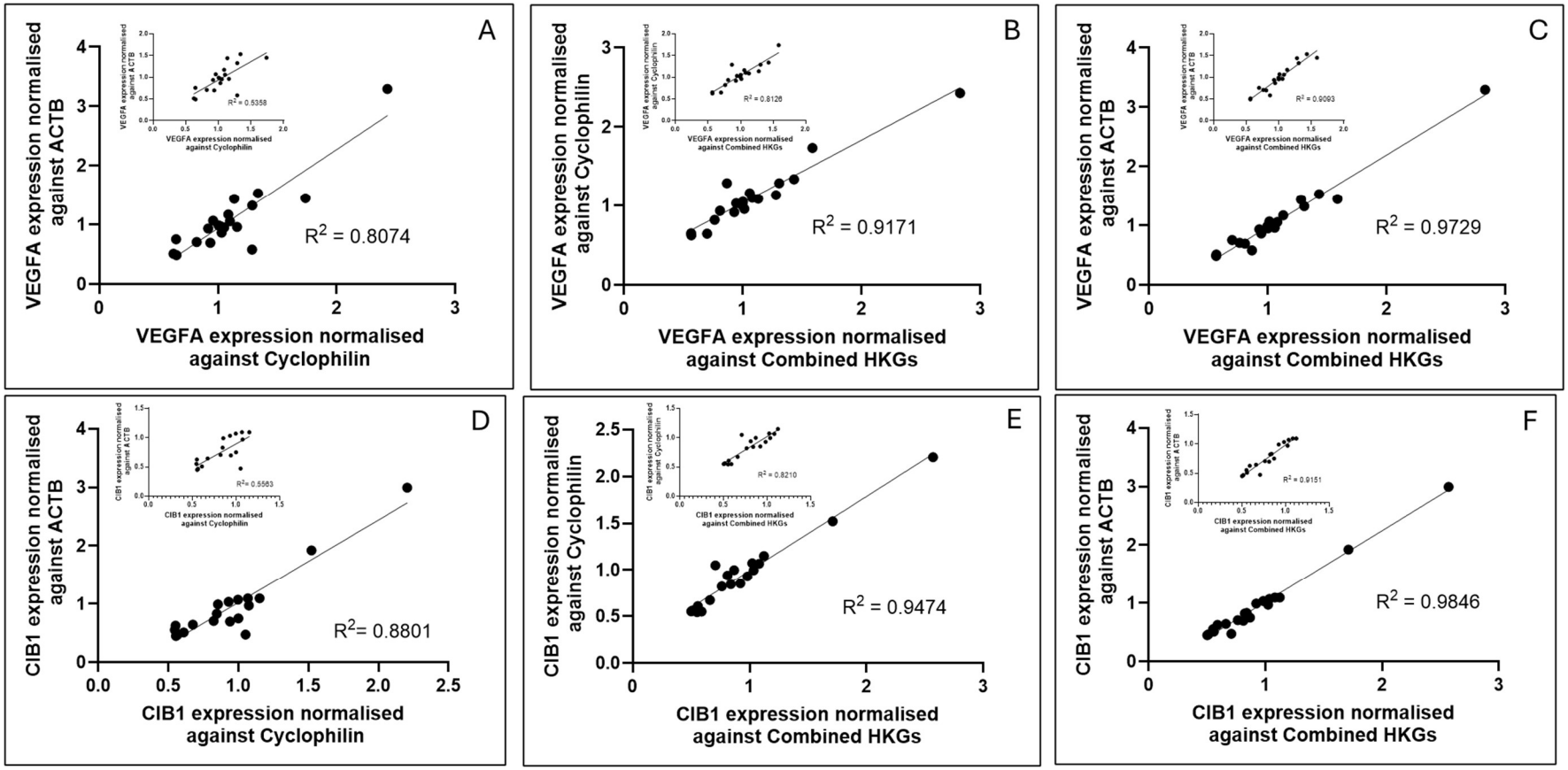

Figure 12. Relative Expression of VEGFA (ABC) and CIB1 (DEF) normalised against individual or combined housekeeping genes (HKG) RQ (relative quantities) values. (A and D) Expression of VEGFA and CIB1 respectively, normalised against individual RQ values for ACTB and Cyclophilin. (B and E) Expression of VEGFA and CIB1 respectively, normalised against the combined geomean of ACTB and Cyclophilin's RQ values and the individual RQ value for Cyclophilin. (C and F) Expression of VEGFA and CIB1 normalised against the combined geomean of ACTB and Cyclophilin's RQ values and the individual RQ value for ACTB. Each figure contains an insert showing continued correlation even with the removal of the largest values.

To improve confidence in the correlation of housekeeping genes the relative expression of VEGFA and CIB1 when normalised against ACTB and Cyclophilin and a combination of both housekeeping genes were plotted. All Control and Biosolids samples from the F1 were plotted. All graphs were significantly correlated, even with the removal of the largest values (Figure 12). Given these results a combination of the housekeeping genes ACTB and Cyclophilin were used to normalize target genes.

Quantification of target genes using qPCR found no significant difference in the relative gene expression of the steroidogenic markers *CYP17A1, CYP11A1*, or *STAR*. Only *CIB1* was reduced (P=0.054) out of the genes highlighted by previous RNAseq data (Table 7). Of the genes previously quantified in the 8-week-old lambs no difference was seen in the relative expression of *FASN* or *VEGFA* (Table 7) but a significant increase (p= 0.037) in expression of *HK1* was observed in the Biosolids (1.0737 ± 0.5714) lambs compared to the Control group (0.8875 ± 0.5714) (Figure 13). No difference was seen in the expression of *HIF1α* (Table 7).

Table 7. Relative Gene Expression of target genes in F1 8-week-old lamb testis normalised against ACTB and Cyclophilin.

F1	Control	SE	Biosolids	SE	P Value
Number of animals	10		10		
CYP17A1[a]	1.108	0.1954	0.1556	0.1954	-
CYP11A1[b]	1.1346	0.2696	1.250	0.2829	-
STAR[b]	1.389	0.586	1.781	0.5546	-
CIB1	0.9412	0.07600	0.7153	0.07600	**0.054**
CELF1	0.9046	0.07818	0.8644	0.07818	-
RBM23[c]	0.9236	0.07913	1.0238	0.08345	-
VEGFA	0.9230	0.08503	1.0573	0.08503	-
FASN	0.9134	0.06024	0.9720	0.06024	-
HIF1a[d]	0.8902	0.07967	0.8429	0.08425	-

[a]= number of animals: Control (n=11) and Biosolids (n=11); [b]=number of animals: Control (n=11) and Biosolids (n=10); [c]=number of animals: Control (n=10) and Biosolids (n=9); [d]=number of animals: Control (n=11) and Biosolids (n=9). Standard error (SE) values vary in parameters with uneven sample groups.

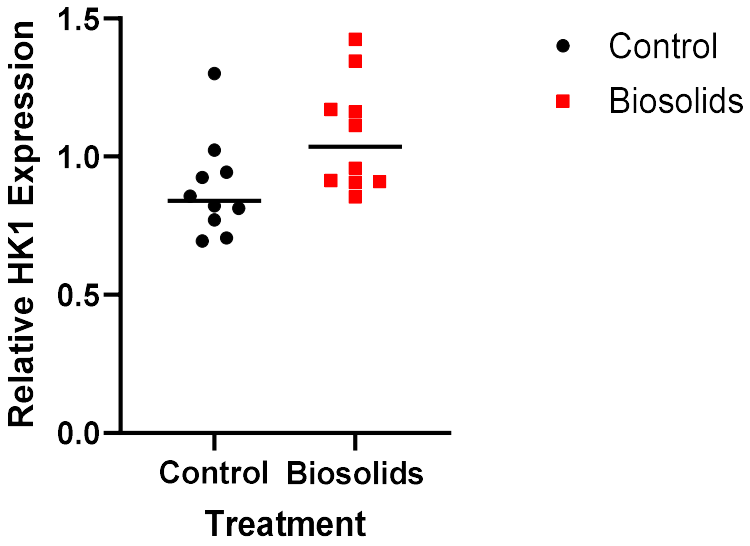

Figure 13. Relative gene expression of HK1 in 8-week-old F1 testis normalised against the combined housekeeping genes ACTB and Cyclophilin (n=10 for Control and Biosolids groups). Black circles indicate Control animals and red squares are representative of the Biosolids group. Data is presented as a scatter plot with simple means.

4.4. Lamb and Organ Weights of F2 Generation

When comparing the F2 Biosolids group with the F2 Control group, no significant difference is observed in body weight at birth and, at 8-weeks of age, the difference in body weight is only significant at p=0.07 (Table 8). Although not statistically significant, the body weight of Biosolids treatment lambs is noticeably greater than that of the Control lambs (Table 8). This pattern is also seen in the weight of the testis. Following the removal of the weight of the epididymis, the testes of the Biosolids animals are significantly heavier than that of the Control group (p = 0.05: Table 8). However, no significant change is observed in either the combined adrenal, combined thyroid or pituitary weights.

4.5. Testis Histology of F2 Generation

F2 testes were stained with vimentin (Figure 14A). No significant difference was observed between the Biosolids (4491 ± 290.8) and Control (4000 ± 304.2) group for the number of Sertoli cells (million) per gram of testis (Figure 15A).

Percentage area of the interstitial area stained with CYP17A1 was highly variable in both the F2 Biosolids group and Control group. There was a noticeable less CYP17A1 positive staining (Figure 15B) in the Biosolids group (18.37 ± 2.614) than in the Control lambs (25.07 ± 2.614), although not significantly so (p = 0.095). Removal of the lowest percentage values, both below 10%, from both the Control and Biosolids group indicates a significant decrease (p = 0.035) in CYP17A1 staining in the F2 Biosolids lambs (19.21 ± 2.370) compared with the Control group (27.12 ± 2.370). These samples were removed from statistical analysis after examining residual plots and identifying these samples as abnormally distributed.

Table 8. Lamb weight and organ weight for 8-week-old F2 lambs

F2	Control	SE	Biosolids	SE	P value
Number	12		12		
A. Lamb organ weight					
Lamb (birth) (kg)	3.654	0.2067	3.850	0.2067	-
Lamb (8 weeks) (kg)	18.57	0.8380	20.93	0.8380	0.070
Testis (g)	11.92	1.381	15.99	1.381	**0.058**
Testis minus epididymis [a] (g)	8.83	1.049	12.10	1.097	**0.050**
Pituitary (g)	0.3526	0.02218	0.3838	0.02218	-
Combined Thyroid (g)	1.112	0.1092	1.172	0.1092	-
Combined Adrenal [b] (g)	1.587	0.08394	1.745	0.08017	-
B. Testis Histology					
Total Sertoli Cell (million)[a]	39636	5983	49485	6259	-
CYP17A1 % nuclear interstitial area with outliers removed[c]	27.12	2.370	19.21	2.370	**0.035**

[a] number of animals equals 12 Control and 11 Biosolids. [b] number of animals equals 11 Control and 12 Biosolids. [c] number of animals equals 11 Control and 11 Biosolids. Standard error (SE) values vary in parameters with uneven sample groups.

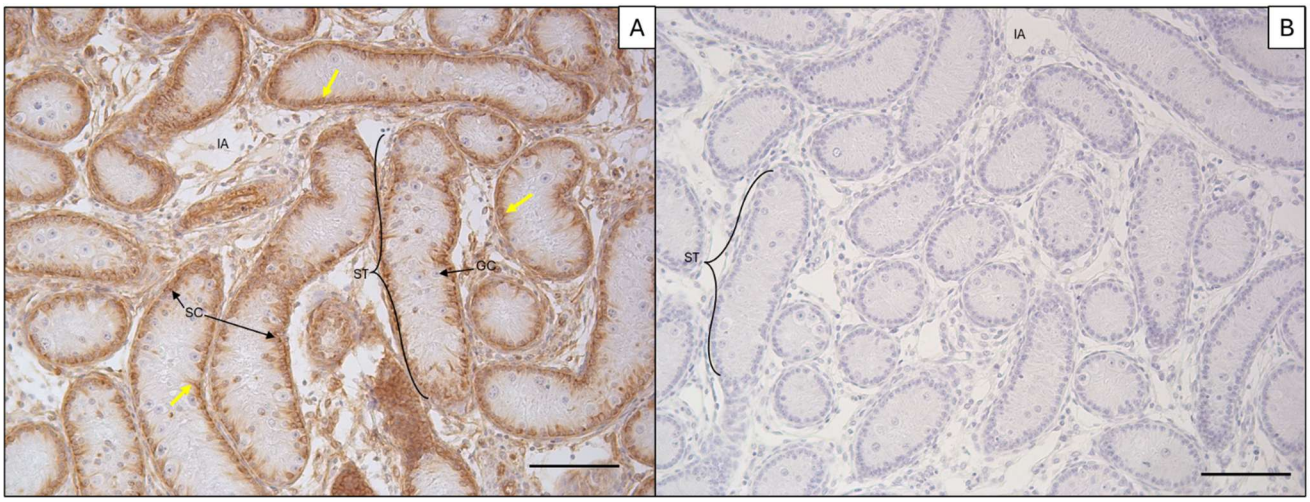

Figure 14. Staining of somatic testicular cells in the F2 generation (A) Sertoli cells stained with vimentin and (B) matched IgG negative control. Yellow arrows indicate examples of positive vimentin staining within the seminiferous tubules. ST: Seminiferous Tubules, GC: Germ Cells, IA: Interstitial testicular area SC: Sertoli cells. Magnification x200. Scale bar = 50µm.

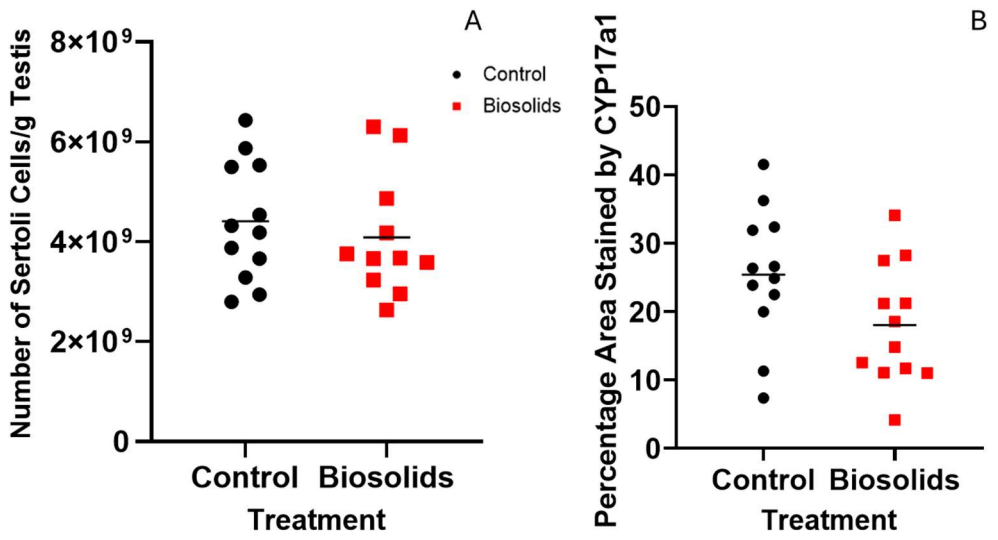

Figure 15. Quantification of testicular somatic cell types in the F2 testis (A) Total number of Sertoli cells per gram of testis (minus epididymus) (Control n=12 Biosolids n=11). (B) Percentage area of Interstitial tissue stained positive for they Leydig cell marker CYP17A1 (n=12 for Biosolids and Control groups). Black circles indicate Control animals and red squares are representative of the Biosolids group. Data is presented as a scatter plot with simple means.

4.6. Molecular Biology of F2 Generation

No significant difference was observed in the relative gene expression of any of the steroidogenic enzymes including *CYP17A1, CYP11A1* and *STAR* (Table 9). There was no change in the expression of HIF1α, CIB1, RBM23, VEGFA, FASN or HK1 between the Control and Biosolids groups (Table 9). Only *CELF1* expression was significantly different with relative expression being increased in the Biosolids exposed lambs (Table 9). Technical artifacts associated with qPCR were removed from statistical analysis after they were identified as being abnormally distributed following examination of residual plots.

Table 9. Relative Gene Expression of Target genes normalised against ACTB and Cyclophilin

F2 Number of animals	Control 12	SE	Biosolids 11	SE	P Value
CYP17A1[a]	0.9631	0.09475	0.8603	0.090354	-
CYP11A1[b]	1.527	0.7308	2.044	0.8174	-
STAR[c]	2.053	1.229	3.599	1.439	-
CIB1	1.244	0.4957	1.809	0.5195	-
CELF1[d]	1.254	0.5687	3.275	0.6764	**0.049**
RBM23[e]	0.9631	0.1475	1.0259	0.1556	-
VEGFA[b]	1.1414	0.2323	0.6797	0.2763	-
FASN[f]	1.208	0.1776	1.583	0.1965	-
HK1	1.147	0.1218	1.081	0.1276	-
HIF1a	1.322	0.2257	1.375	0.2366	-

[a] number of animals equals 11 Control and 12 Biosolids. [b] number of animals equals 11 Control and 9 Biosolids. [c] number of animals equals 12 Control and 8 Biosolids, [d] number of animals equals 11 Control and 8 Biosolids, [e] number of animals equals 11 Control and 10 Biosolids, [f] number of animals equals 12 Control and 10 Biosolids. Standard error (SE) values vary in parameters with uneven sample groups.

5. Discussion

Exposure to environmental chemical mixtures has been associated with perturbations in human and animal male reproductive development and function including declining fertility (Paul et al., 2005, Bellingham et al., 2012, Knez, 2013, Buñay et al., 2018, Elcombe et al., 2021, Elcombe et al., 2022, Lea et al., 2022, Elcombe et al., 2023, Evans et al., 2023). Since chemical mixtures are complex in their nature, determining real-life exposure effects is challenging and requires an animal model which approximates human exposures. This project used such a model in which pregnant ewes were exposed to human sewage sludge derived biosolids one month prior to and throughout gestation.

This study has shown for the first time that exposure limited to the period of gestation only, affects testes of 8-week offspring in both the F1 and F2 generations despite no further periods of exposure. Notably, staining of the steroidogenic enzyme CYP17A1 was reduced in the F2 Biosolids group and the relative gene expression of *CIB1* and *HK1 in the F1 generation and CELF1 in the F2 generation was altered.*

Previous studies with foetal endpoints have found effects on somatic testicular cell numbers: reduced Sertoli cell and Leydig cell numbers (Paul et al., 2005, Lea et al., 2022) in mid and late gestation. This contrasts with the current study in that, in 8-week offspring, the data presented at the histological level identified no significant difference in mean Sertoli cell number in both the F1 and F2 generation. Consequently, this raises the suggestion that there may be a degree of compensation during the first 8-weeks of postnatal development. To an extent this observation is supported by the study on 19-month-old adult rams, in which only reductions in germ cell populations are statistically significant (Bellingham et al., 2012).

Of note however, is that there were two subsets of animals observed in the 19-month-old Biosolid exposed group one of which had smaller testis and fewer Sertoli cells, though this was not significant (Bellingham et al., 2012). Additionally, these 19-month-old rams were exposed during gestation and the first 7-months of post-natal life, the lack of direct effects on Sertoli cell population suggests that if Sertoli cells are going through a degree of compensation during the prepubertal period, continued exposure to biosolids up until puberty has no observable effect. This could imply that Sertoli cells populations may potentially be less susceptible to the effects of biosolids exposure during post-natal life.

The current study found a noticeable difference in the size of the F1 seminiferous tubules when viewing the AMH stained slides done previously. As this was unrelated to processing, an alternative explanation was sought.

Interestingly, the samples with smaller seminiferous tubule diameters had the greatest total number of Sertoli cells and number of Sertoli cells per gram of testis. This may be connected to these seminiferous tubules having Sertoli cells with the smallest average nuclear diameters and occurring in the lightest weighting testis. Both testis weight and average Sertoli cell diameter were included in calculations to determine total Sertoli cell numbers. Whilst testis weight would influence the total number of Sertoli cell, by correcting for the weight of the testis the reduced weight in these samples should not affect the total number of Sertoli cells per gram of testis. The smaller Sertoli cell mean nuclear diameter and radius in these samples resulted in reduced mean nuclear volume of Sertoli cells. Dividing the absolute volume of the testis occupied by Sertoli cells by this reduced mean nuclear volume resulted in greater total number of cells and cells per gram of testis. However, as there were examples of all three seminiferous tubule size categories in both the Biosolids and Control group this did not appear to be linked to biosolids exposure and time constraints meant this could not be investigated further at the time.

Interestingly, Elcombe and colleagues have previously identified testes with significantly smaller seminiferous tubule diameters in the 8-week-old Biosolids lambs to have compared to their Control group (Elcombe et al., 2022). Further analysis comparing the diameters calculated by Elcombe and colleagues with the testis diameters, Sertoli cell nuclear diameters, Sertoli cell total counts and Sertoli cell per gram of testis may identify subgroups present in the Biosolids group. This supports Bellingham and colleagues interpretation of biosolids exposure on 19-month-old rams, in which they suggest that by comparing whole groups of animals without taking into account variation within the group, potentially adverse testicular alterations may go unnoticed in the larger population (Bellingham et al., 2012).

Uniquely in this study and one other (Elcombe et al., 2023) the sire was incorporated into the experimental study and statistical analysis thus allowing analysis of the sires genetic impact. Of interest, the smaller sized tubules appear to be present in lambs sired by two specific rams which may suggests a potential genetic cause for these smaller seminiferous tubules. However, as there is no additional evidence to support this suggestion, further study would be required.

No significant difference in Sertoli cells were seen in the F2 generation nor were any tubules observed as markedly smaller. Sertoli cell number per gram of testis was variable in both the Control and Biosolids groups with no suggestion of subgrouping.

The percentage area of CYP17A1 positive staining in the interstitial area was used to quantify Leydig cell populations. Though there were markedly reduced levels of CYP17A1 producing Leydig cells in the F1 generation this difference was not statistically significant. This contrasts with an earlier study (Lea et al., 2022) in which reduced staining of CYP17A1 was seen in 140-day-old foetuses exposed transiently during early, mid and late gestation, or continuously for 140 days. This could reflect a degree of compensation in the post-natal development of the lambs; however, a previous study (Lea et al., 2022) highlighted that using a single Leydig cell marker may not be adequate to accurately identify all Leydig cells present in the interstitial area. Where previously lower levels of CYP17A1 were identified in 140-day-old foetuses exposed during mid and late gestation, no significant difference was observed in CYP11A1 expression (Lea et al., 2022). Thus, staining with an additional steroidogenic Leydig cell marker, such as CYP11A1 could be beneficial in gaining a better assessment of Leydig cell populations.

Within the F2 generation, there is also a noticeable difference in Leydig cell populations. However, this difference is only statistically significant with the removal of the lowest percentage value from the Control and Biosolids groups.

Given previous reports (Paul et al., 2005, Lea et al., 2022) in changes in steroidogenic enzymes and the current study exploring CPY17A1 and the trend of CYP17A1 decreased across both generations, *CYP17A1* and two additional steroidogenic enzymes (*CYP11A1 and STAR*) were selected for qPCR analysis. Surprisingly no significant difference was observed in the relative expression of any of the three steroidogenic targets. Though the lack of difference in the F1 generation is consistent with the histological analysis the same cannot be said for the F2 generation. The lack of significance in the F2 generation may be due to the homogenised nature of the RNA extracted from the testis. The population of CYP17A1 expressing Leydig cells was calculated as a percentage of the interstitial area whilst the relative expression of the CYP17A1 gene was calculated from a homogenised sample of testis which would have included multiple cell types from the interstitial area and the seminiferous tubules. It is therefore possible that any change in *CYP17A1* mRNA expression may be too small to be identified as statistically significant when assessed as a part of the whole testis.

Further molecular data presented in this study supports some of the qPCR data previously published by Elcombe and colleagues (Elcombe et al., 2022) that highlighted four genes downstream of mTOR to have significantly altered expression in the Biosolids group (Elcombe et al., 2022). Three of these genes, *VEGFA, FASN* and *HK1* were reanalysed in this study. No significant difference was seen in the expression of *VEGFA* or *FASN*. However *HK1* relative expression was significantly increased in the Biosolids group in the F1 generation in this study which is consistent with previous findings (Elcombe et al., 2022). No significant difference was observed in *HK1* expression in the 11-month-old rams (Elcombe et al., 2023) suggesting that effects observed in the foetus and prepubescent animal may not persist into adulthood.

One possible explanation for the lack of significance in *VEGFA* and *FASN* expression is at the technical level with previously published primers showing large product lengths, greater than the recommended range. For this reason, this study used redesigned primers with product lengths between 104-150 base pairs.

Calcium and Integrin Binding protein 1 (*CIB1*) was previously identified as being significantly downregulated by Elcombe and colleagues (Elcombe et al., 2022). This study confirms this through the quantification of relative CIB1 expression in the F1 testis which found a significantly lower expression of CIB1 in the Biosolids group. This may be significant as CIB1 is known to be highly expressed in the testis and thought to be involved in the proliferation of and survival of germ cells through the ERK and AKT-dependent signalling pathways (Leisner et al., 2016). A study has found oligoasthenozoospermia patients had lower mRNA and protein expression of CIB1 compared to fertile men and knockout studies in mice showed disruption to spermatogenesis, irregular histology and smaller testis resulting in sterile mice (Yuan et al., 2006, Sun et al., 2014).

CUG-BP, Elav-like family 1 (*CELF1*) was previously shown to be significantly upregulated in the F1 Biosolids lambs (Elcombe et al., 2022), however this study found no difference in the relative expression of *CELF1* in the F1 testis. In contrast, analysis of the F2 testis revealed a significant increase in *CELF1* relative expression.

CELF1 is an RNA binding protein involved in the post-transcriptional regulation of mRNA. It is of particular interest as it is expressed in germ cells, Sertoli cells and Leydig cells. Knockout mice for CEFL1 have exhibited impaired fertility in both males and females as well as reduced growth (Kress et al., 2007). Further analysis identified subgroups of knockout mice with delayed spermatogenesis and steroidogenesis during the pre-pubertal period and others showing spermiogenesis arrest. Additionally, at 25-days-postpartum, significant lower expression of the Leydig cell markers *CYP11A1, Hsd3b1* and *CYP17A1* were also observed (Cibois et al., 2012). This contradicts the results of this study in which increased expression of *CELF1* and reduced levels of CYP17A1 staining was both observed in the F2 males. Though this may be due to a difference in animal model.

However, the F2 relative gene expression was variable with several samples missing or reaching threshold after 30 cycles. This is likely due to a technical issue during reverse transcription as it was typically the same samples which reached threshold after 30 cycles or did not reach threshold at all. No issues were seen with these samples in the previous batch of cDNA, so it is unlikely that there was an issue with the RNA. However, the reverse transcription could not be rerun at the time due to time and resource constraints.

Moving back and looking at the more gross level, testis weight was reported as significantly reduced in the foetus (Paul et al., 2005), this study also found testis weight in the F1s was significantly lower in the Biosolids group. Reduced testis weight is consistent with rodent studies in phthalate exposure during the MPW (Moore et al., 2001, Carruthers and Foster,

2005). This is further supported by a study examining testicular volume in lambs between 21 and 49 weeks of age, following in-utero exposure to biosolids. They found that between 21-weeks and 29-weeks of age the Biosolids group had significantly smaller testes. However, post-29 weeks the difference in testis volume between groups became minimal and after 45-weeks testis volume of the Biosolids lambs was greater than the Control group, but not significantly so (Evans et al., 2023). Similarly, no significant difference was observed in the 19-month-old rams (Bellingham et al., 2012). This likely suggests that any compensation in testis weight occurs post-week 29. Interestingly, in the F2 generation, testis weight is significantly greater in the Biosolids group compared to their Control group. This may imply a potentially compensatory effect on testis weight in the F2 generation.

A similar pattern was also observed in lamb body weight. At 8-weeks of age a statistically significant decrease in the body weight of F1 Biosolid lambs. This pattern has also been observed in lambs from 9-weeks-of-age to 45-weeks-of-age, with the difference in body weight of Biosolids groups being statistically significant between 9-weeks and 29-weeks of age. Following 45-weeks, Biosolid lamb weight was greater than Control body weight, however, not significantly (Evans et al., 2023). This supports the findings in the 19-month-old rams in which no difference was observed in body weight (Bellingham et al., 2012).

Interestingly in the F2 generation, though not significantly so, Biosolids lamb weight is noticeably increased when compared with the F2 Control group. This along with the changes seen in testis weight may suggest a potential compensatory response to environmental chemical exposure during F1 gestation.

It is possible that the differences seen in lamb body weight could have been due to alternative factors. Ewe body weight and ewe body condition score was not recorded at the time of lambing so it is unclear whether there could have been a difference in nutritional quality of forage of the two pastures. As both groups were separated into pastures fertilised by different treatments, it is possible the effects observed in lamb weight may be due to differences in pasture rather than caused directly by the chemicals present in the biosolids. For example, it is possible that the biosolids pasture had a higher parasitic burden than the control pasture (Fthenakis et al., 2015).

6. Conclusion

In conclusion, this study has shown for the first time that exposure limited to one gestation (F0) has an impact on 8-week-old testes in both the F1 and 2 generations. The histological differences identified in the foetus during mid and late gestation, compared to the lack of significant difference observed in this study of 8-week-old lambs and other studies on adult rams may indicate a degree of compensation. The decrease in CYP17A1 expression at the protein level is particularly interesting as it suggests a multigeneration effect following gestational exposure. This raises concerns around exposure to environmental contaminants for the immediate generations and for generations to come. It illustrates the need for further studies on the epigenetic effects of exposure to real-life concentrations of environmental chemicals in a model closely related to human exposure.

7. References

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