The Effects of in Utero Exposure to Biosolids on Ovine Testicular Histology and Semen Quality in F1 offspring

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Contents

Abstract

Human male fertility has been put under a microscope in recent decades, due to a rise in reproductive disorders and decline in semen quality. Exposure to individual anthropogenic environmental chemicals (ECs), has been associated with reproductive dysregulation during gestation and into adulthood. 'Real-life' exposure in humans encompasses a complex cocktail of ubiquitous ECs. This study examined ovine prepubertal testes and pubertal ram semen from sheep that were exposed in utero to a mixture of ECs. Pregnant ewes were grazed on pastures treated with biosolids fertiliser (processed human sewage sludge) eight weeks prior to mating until parturition. Prepubertal testes (*n*= 22) were subjected to immunohistochemical staining of Sertoli cells and Leydig cells for specific biomarkers (AMH and CYP17A1 respectively) and index of proliferation (PCNA). No significant changes in testicular stained area were reported for AMH, CYP17A1 or PCNA. Examination of pubertal ram semen (*n*=16) using a new portable CASA platform (iSperm), revealed a significant increase in sperm motility within fresh samples from biosolids exposed males. No significant changes in sperm motility or kinematic parameters were reported in cryopreserved semen from the same rams. An evaluation of the iSperm was conducted to review the three methodologies (i.e., Dropper, Dipper and Pipette methods) for semen analysis and compared the iSperm to a validated CASA system (IVOS II). Upon review of the three methodologies, the Pipette method produced the most consistent readings across the measured semen analysis parameters. The Dropper method had unreproducible results and continually gave higher readings for percentage sperm motility and progressive motility. The IVOS II CASA comparison showed no agreement in sperm motility or kinematic parameters between the two systems. This study indicated that exposure to ECs throughout gestation had little to no significant effect on prepubertal testicular histology or adult semen quality. This proves controversial to previous studies and could inspire future work to explore compensatory effects during fetal development to restore the testicular phenotype, which might mitigate reproductive effects in adulthood.

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Introduction

Despite technological advancements in artificial reproduction, fertility in men and women has witnessed a global decline in recent decades (Carlsen et al.,1992; Mascarenhas et al., 2013). The broad documentation of semen quality has indicated adverse effects in the male genital tract, particularly in western men (Dissanayake et al., 2019). There is evidence of increasing testicular cancer, cryptorchidism, hypospadias and poor sperm quality (Oliva et al., 2001). Female fertility also appears to have deteriorated; the birth rate in 2015 per woman had halved when compared to 1975 (United Nations et al., 2017). In addition, advancing parental age within developing economies, obesity and malnutrition have been widely linked with infertility (Sun et al., 2019). Collectively however, the rapid decline in male and female reproductive health cannot be due to genetic factors alone, suggesting environmental and/ or social factors could be influencing this trend (Younglai et al., 2005).

In recent decades, the awareness of environmental chemical (EC) exposure, the potential links to infertility and altered reproductive health, have sparked ecological studies in numerous species (Table 1). Environmental anthropogenic chemicals arise from a variety of sources and there is a considerable body of evidence to suggest that they inflict harmful consequences on health and wellbeing including reproductive health (Annamalai and Namasivayam, 2015; Harrison et al., 1997).

Certain ECs act as endocrine disruptors, impeding the biosynthesis, metabolism and action of hormones with adverse effects (Annamalai and Namasivayam, 2015). Aquatic wildlife populations have exhibited impaired reproductive functions when exposed to ECs. Juvenile male alligators inhabiting Lake Apopka experienced feminisation and testicular abnormalities after a spillage of dichlorodiphenyltrichloroethane (DDT) was biomagnified up the food chain thus impacting on reproductive function (Matsushima, 2018). Harbour porpoises naturally exposed to polychlorinated biphenyls (PCBs) had reduced testicular weights, an indicator of lower reproductive fitness and subfertility (Williams et al., 2021). Evidence from rodent studies exposed in utero to ECs, supports the observational findings that have occurred in wildlife populations (Pocar et al., 2011; Zhou et al., 2017; Kuriyama and Chahoud, 2004; Axelstad et al., 2018). The most documented effects in male offspring were poor sperm viability and altered seminiferous tubule structure; in female offspring, increased follicular atresia, decreased ovarian weight and decreased anogenital distance were often reported (Pocar et al., 2011; Zhou et al., 2017; Kuriyama and Chahoud, 2004; Axelstad et al., 2018). In the real world, humans and animals are exposed to mixtures of ECs, many of which interact (Lee, 2018). Replicating this in a laboratory setting is difficult and therefore there is a need for a model to assess the effects of mixtures of ECs. One such model is the exposure of sheep to

biosolids-treated pastures which, unlike previous in vivo studies, addresses the effects of exposure to mixtures of ECs at various stages of gestation, providing a basis for real world interpretation (Evans et al.,2014).

In the developed world, application of biosolids as fertilisers for pastoral and arable land is common agricultural practice (Tezel et al., 2011). Biosolids consist of nutrient rich organic matter that is recycled from treated sewage sludge. Under legislation guidelines and controlled management application, biosolids are considered safe to use (Lu et al., 2012; Laturnus et al., 2007). However, biosolids contain a complex cocktail of ECs, many of which are resistant to biological and chemical degradation, and remain at low concentrations below the safety threshold reported to pose an immediate risk to health (Bellingham et al., 2009; Smith, 2008). Animal tissues present an environment for lipophilic ECs to be stored and to bioaccumulate through the food chain. At each trophic level the concentration of ECs increases, peaking in the tertiary consumer (Smith, 2009). Continuous consumption of food produced from biosolids treated land may expose humans to ECs and further, link to incidences of reproductive and metabolic disorders (Rorat et al., 2019).

Previous studies by our group have indicated that grazing pregnant sheep on biosolids treated pastures, can lead to offspring with reproductive and metabolic disorders. It has been shown that:

(i) The duration and timing of exposure can influence the development of the fetal neuroendocrine system in a sexually-dimorphic manner, predisposing to reproductive disorders once sexually mature (Bellingham et al., 2016).

(ii) Differential temporal sensitivity of fetal ovaries to ECs can perturb the development of fetal ovarian follicles and is dependent upon the period of gestation at which exposure occurs (Lea et al., 2016a).

(iii) Spermatogenic abnormalities in rams exposed during gestation and post-natal life to ECs, exhibit reduced germ cell (GC) numbers per testis, per Sertoli cell and abnormalities in the testis including Sertoli cell only tubules (Bellingham et al., 2012).

(iv) Disruption can occur to hormonal function and the cellular development of the mid-gestation fetal testis (Paul et al., 2005).

(v) Physiological changes to the liver affecting xenobiotic and detoxification responses, predisposes to metabolic disorders (Filis et al., 2019).

(vi) Disruption of fetal thyroid morphology as a possible presage of future thyroid function complications (Hombach-Klonisch et al., 2013).

Evidence from some of the studies had indicated greater reproductive and metabolic effects were reported in males than females, however the reason behind this was not fully understood (Bellingham et al., 2016; Hombach-Klonisch et al., 2013).

Recent studies indicate that some ECs can induce epigenetic effects thus have the potential to affect multiple generations (Xin et al., 2015; DeCourten et al., 2020). Previous studies have focussed on short lived rodent models whereas the current research forms part of a larger programme, looking at multi and trans-generational effects when exposed to chemical mixtures in biosolids. Specifically, this study utilises sheep exposed pre-conceptionally and in utero, to a cocktail of chemicals in order to assess the consequences for male reproductive health.

The study reported in this thesis will focus initially on the histological development in testes. Sertoli and Leydig cell populations will be evaluated through immunohistochemical staining in order to discover the effects environmentally relevant doses of ECs have on these cell types. Abnormal testicular histological development may arise as a consequence of EC in utero exposure and may have sequential effects on semen quality. Studies that have explored the relationship between ECs and semen quality have produced conflicting data (Bloom et al., 2015; Den Hond et al., 2015; Specht et al., 2014; Mendiola et al., 2010). This relationship will be the subject of the secondary study which will assess sperm quality in terms of fertilisation potential in F1 progeny exposed in utero to a cocktail of ECs. The aim of the study will analyse whether sperm parameters (sperm motility (%), progressive motility (%), curvilinear velocity (VCL) (µm/S), average path velocity (VAP) (µm/S), straight line velocity (VSL) (µm/S), straightness (STR) (%) and linearity (LIN) (%)) are compromised as a result of this exposure.

Table 1. Species specific reproductive effects when exposed to different environmentally relevant concentrations of ECs.

Chemical	Species	Effects	Reference
Polychlorinated biphenyls	Harbour porpoise (Phocoena phocoena) Bald eagle (Haliaeetus leucocephalus)	Reduced testicular weights Reduced hatching rates	(Williams et al., 2021) (Annamalai and Namasivayam, 2015)
Polybrominated diphenyl ethers	American Kestrel (Falco sparverius)	Delayed egg laying, smaller egg volume, mass, width and length, lighter eggshell weight	(Fernie et al., 2009)
Polycyclic aromatic hydrocarbons	Zebrafish (Danio rerio)	Testicular hyperplasia and reduction in spawning success	(Vignet et al., 2016)
Bisphenol A	Tilapia (Oreochromis niloticus) Common carp (*Cyprinus carpio*)	Feminisation of gonads and skewed sex ratios Oocyte atresia, altered testes morphology and alterations in the pattern of sex steroids for both males and females.	(Chen et al., 2014) (Mandich et al., 2007)
Phthalates	CD-1 Mice	Smaller adult gonads, prostate and seminal vesicles, lower adult testosterone concentration and decreased sperm count and motility	(Barakat et al., 2019)
Dichlorodiphenyltrichloroethane	Alligator (Alligator mississippiensis) American sparrow hawk (Falco sparverius)	Feminisation of male embryos and testicular abnormalities. Reduced eggshell thickness, egg disappearance and egg breakage	(Matsushima,2018) (Porter and Wiemever,1969)

Chapter 1. Review of Literature

1.1 Sheep as a Model of Human Reproductive Function

Sheep are ideal to use as a comparative animal model for human pregnancy. The ability to monitor vital signs by inserting sampling catheters into the maternal and fetal vasculature is feasible without pregnancy loss in sheep; this is not the case in other species (Barry and Anthony, 2008). An extended gestation length with large physiological and anatomical size allows for physical monitoring; rodent species possess short gestation periods and difficulties arise collecting sufficient blood samples for endocrine and steroid hormone analysis (Abedal-Majed and Cupp, 2019). In addition, activation of sheep fetal liver occurs earlier than in fetal rodents, again showing greater similarities to humans (Evans et al., 2014). Furthermore, the sheep model shares similarities to humans by finalising gonadal development during gestation, unlike rodents where much development occurs postnatally (Evans et al., 2014)

1.1.1 Comparative Differences Between Human and Sheep Fetal Testes and Endocrine Development

At a glance, human and sheep reproductive development share similarities in endocrinology and physiological formations, however there are noticeable differences in the timing of events that set the species apart (Figure 1.1).

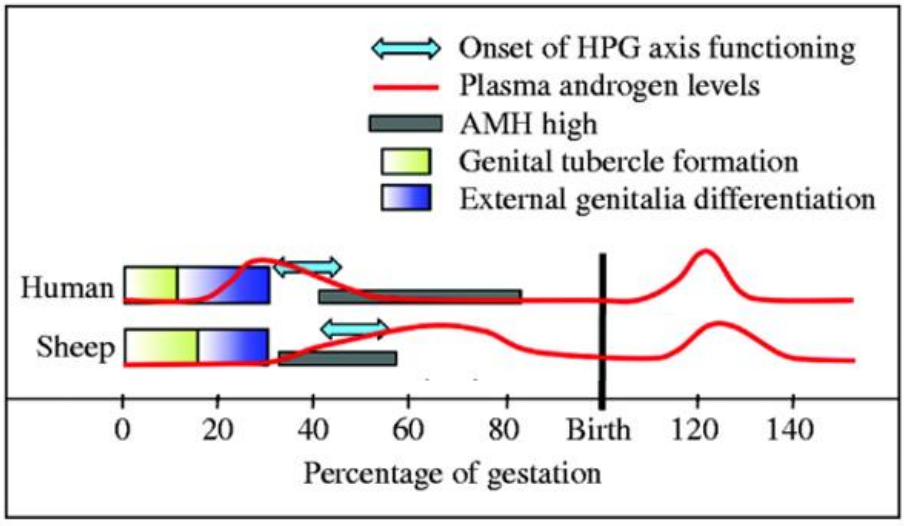

Figure 1.1: A schematic diagram representing key developmental differences as a percentage across gestation between sheep and humans. The figure is adapted from O'Shaughnessy and Fowler, (2011) highlighting the transitions of endocrine events and physiological development of the internal and external genitalia.

The formation of the genital ridge and the testis occurs relatively at the same time for both species, but differences arise along the transition phases of testicular descent (Klonisch et al., 2004). In humans, the beginning of the transabdominal phase occurs earlier in gestation

(~26% of gestation) whereas in sheep this is significantly later (~40-44% of gestation) (Klonisch et al., 2004). Interestingly, the initiation of the inguinoscrotal phase is relatively rapid in sheep (~48-50% of gestation) but is delayed until much later in humans (~68% of gestation) (Klonisch et al., 2004).

During the transabdominal phase, Anti-Müllerian hormone (AMH) is secreted by the developing gonad to regress the Müllerian ducts, inhibiting the female reproductive ducts (paramesonephric ducts) from developing. AMH can be detected in sheep around sexual differentiation at Day 30 and increasing rapidly in production as represented by intense staining in Sertoli cells at Day 40 (Brooks et al., 1996). AMH is present in human testicular tissues from 8 weeks and high serum concentrations of AMH have been identified from 19-30 weeks of gestation followed by a decrease after 30 weeks until birth (Josso et al., 1993). The pattern of AMH is reflective of the species-specific duration of the transabdominal phase, short and rapid in sheep and prolonged in humans. Furthermore, differences in AMH concentration can be linked to the extent of rough endoplasmic reticulum development within immature Sertoli cells (Tran et al., 1987). The organelle involved in the protein synthesis pathway, is significantly more abundant in the developing sheep Sertoli cells in comparison to humans (Tran et al., 1987). The shorter sheep transabdominal phase requires greater numbers of rough endoplasmic reticulum to fuel the demand for AMH synthesis.

During the first trimester of human pregnancy, testosterone production is significantly amplified, before resuming to a stable level for the rest of gestation (Collu et al., 1983). The rise of testosterone production is not seen until later in sheep gestation, representing an extended incline initiated from Day 35 onwards (Quirke et al., 2001). A study following the ontogeny of steroidogenesis in fetal sheep showed testosterone production at 121.9, 10488 and 33928 pg/gonad for Days 35, 55 and 75 respectively, demonstrating the prolonged period of rising androgen levels (Quirke et al., 2001). Inhibin is another hormone that exhibits different secretory patterns across gestation. Sheep fetal testes present increasing concentrations of inhibin mRNA with gestational age. However, humans display a gradual decrease in inhibin mRNA expression across gestation (Hochereau-de Riviers et al., 1995; Thomas et al., 1995).

The development of the hypothalamic-pituitary gonadal (HPG) axis begins with the migration of the gonadotropin-releasing hormone (GnRH) neurones from the olfactory placode to the fetal hypothalamus (Bizzarri and Cappa, 2020). This occurs relatively early in human gestation at Day 40/ 280 compared to Day 35/146 in the sheep (Bizzarri and Cappa, 2020; Brooks et al., 1996). Human gonadotropes begin synthesising luteinising hormone (LH) and follicle stimulating hormone (FSH) relatively earlier in gestation at Day 84/280 compared to Day 60/146 in sheep (Guimeiot et al., 2012; Brooks et al., 1996) In both species, towards the end

of gestation the gonadotropic axis is suppressed and it not activated again till the onset of puberty (Guimiot et al., 2012; Brooks et al., 1996).

1.1.2 Comparison of Sheep and Human Male Reproductive Anatomy and Physiology

1.1.2.1 Copulatory Organ

The male reproductive system of both species is comparatively similar but significant differences are found in the copulatory organ. The penis of the sheep can be described as 'fibroelastic' due to the presence of the sigmoid flexure and retractor muscle which work as an antagonist pair to extend and retract the penis (Schimming and Moraes, 2018). In contrast, humans do not have a sigmoid flexor or retractor muscle and instead rely upon a vasculature system for erection to occur (Karacan et al., 1983).

1.1.2.2 Testes

The ram is a seasonal breeder and undertakes reproductive changes in response to photoperiodic signals (Milczewski et al., 2015). Under shortening daylength from autumn to winter, gonadal involution occurs followed by the recovery in spring as daylength increases (Milczewski et al., 2015). The testicular mass for rams in relation to their body mass is high, on average each testicle measures 300g with variations between breeds (Johnson, 1991). Comparatively, humans tend to not follow seasonal reproductive patterns and can reproduce year-round unlike most breeds of sheep (Mortimer et al., 1983). Furthermore, men in comparison to rams have proportionately smaller testicles relative to body mass, weighing approximately 20g per testis (Condorelli et al., 2013).

1.1.2.3 Spermatozoa

Spermatogenesis is the development of mature spermatozoa from spermatogonia through a series of mitotic and meiotic divisions and cytologic changes (Zeng et al., 2013). The total duration of spermatogenesis is 47-48 days in sheep (Zeng et al., 2013) and 74 days in humans (Griswold, 2015). Spermatogenic cycles last 10 days in sheep and 16 days in humans (Johnson, 1991). The efficiency of spermatogenesis is measured by the daily production of spermatozoa per gram of testicular parenchyma (Johnson et al., 1999). Humans have lower spermatogenesis efficiency compared to sheep, (4-6 $x10^6$/g and $21x10^6$/g respectively) (Johnson et al., 2000) which has been attributed to the longer duration of spermatogenesis and lower density of germ cells (Johnson, 1986).

Table 1.1 Standard semen parameters for humans and sheep showing normal ranges (Tibary et al., 2017; Larsen, 2021; Synnott et al., 1981; Campbell et al., 2021; WHO, 2021)

Semen parameter	Human	Ram
Ejaculate volume (ml)	1.3-1.5	0.5-1.8
Sperm concentration (10^6/ml)	15-18	2500
Total sperm number (10^6 per ejaculate)	35-40	50-300
Progressive motility	≥30%	≥30%
Normal morphology	≥4%	≥70%

Sheep and human spermatozoa show similarities in morphological appearance, and both have sperm head morphometric subpopulations (García-Vázquez et al., 2016). Ram spermatozoa heads are larger in terms of surface area as well as length and width in comparison to human spermatozoa heads (Yániz et al., 2016; Pérez-Sánchez et al., 1994).

1.2 Sheep Biosolids Model

Although laboratory rodent studies can underpin specific effects and develop an understanding of the mechanisms of an individual EC, the Sheep Biosolids Model provides a real-world insight into simultaneous exposure to a medley of ECs, many of which are still unknown.

The abundance of knowledge surrounding ECs has grown dramatically over the past few decades. The 1962 book *Silent Spring* (Carson,1962) awakened the scientific community and started the conversation that the discharge of manufactured chemicals entering the ecosystem was harmful to wildlife and human populations (Schug et al., 2016). However, the modern industrial and agricultural world continues to eject a plethora of chemicals into the land, water and air. Agricultural land is routinely exposed to a concoction of chemicals and in particular, fertilisers such as biosolids. Temporal changes to soil EC concentrations provided preliminary evidence that after continuous yearly application of biosolids the abundance of soil ECs increases (Rhind et al., 2013; Mangas et at., 1998). Increased EC exposure was reflected in wildlife and domestic animals who presented adverse effects which triggered numerous studies and the development of the Sheep Biosolids Model (Evans et al.,2014; Rhind et al., 2010; Rhind et al., 2013; Rigby and Smith, 2020; Viguié et al., 2020).

Real world exposure was investigated by developing a sheep paradigm; pregnant ewes were either maintained on pastures treated with biosolids generated from human sewage sludge or grazed on organic pastureland free of ECs (Lea et al., 2022). The establishment of the

paradigm allowed different periods of embryonic development to be investigated in the presence of EC mixtures, providing a representation of human exposure to ECs (Evans et al., 2014). Animal products raised from biosolids treated pasture enter the human food chain; recycling of municipal waste generates new biosolids and re-enters in a bioaccumulating cycle of ECs. Synthetic and biological contaminants are incorporated daily into the human population; consumption, inhalation and topical absorption are all routes of exposure (Rhind, 2002). The model offers an insight into the consequences that continuous exposure to low concentrations of ECs have on the metabolic and reproductive health of an individual and this closely approximates human exposure to chemical mixtures.

The model challenges the traditional toxicology concept of the dose dependant nature of poison because ECs have a non-linear relationship between dosage and effect (Vandenberg et al., 2012). The lowest observed adverse effect level (LOAEL) proposes that exposure beneath this threshold will not incur an adverse effect; ECs undermine this principal triggering adverse effects below the LOAEL (Zoeller and Vandenberg, 2015). Furthermore, studies have highlighted the non-monotonic dose response of ECs, noting effects recorded at lower doses were not reciprocated at higher doses (Pelch et al., 2010; Saal et al 1997). Moreover, ECs exist in varying concentrations of agonists and antagonists throughout gestation, resulting in unpredictability and differing physiological responses (Bellingham et al., 2016; Duarte-Guterman et al., 2014).

1.3 Environmental Chemicals

Environmental chemicals are ubiquitous, including industrial effluent, by-products of pharmaceuticals, solvents, plastics, pesticides and herbicides (Frye et al.,2011). Some ECs are more persistent within the environment, designed to have longer half-lives and resist degradation or metabolise into more toxic compounds (Diamanti-Kandarakis et al.,2009). In addition, environmental xenobiotics have the power to perturb endocrine and steroidogenic pathways, disturb DNA methylation status and manipulate lipid metabolism and adipogenesis (Tabb and Blumberg, 2006). Governments recognised the damaging capabilities of PCBs and DDT resulting in a ban during the 1970's across America and industrialised countries (Frye et al.,2011). However, PCBs residing in electrical appliances and the continued use of DDT for malaria control maintains exposure for decades to come (Korrick and Sagiv, 2008). Biosolids are composed of a synergy of chemicals acting as persistent organic pollutants: polycyclic aromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDEs), bisphenol A (BPA), PCBs, phthalates, and heavy metals (Evans et al., 2014).

1.3.1 Phthalates

Phthalates are environmental toxicants, derived from alkyl diesters of phthalic acid and commonly used in construction materials, pharmaceuticals, medical devices and toys (Hannon and Flaws, 2015; Schettler, 2006). Although phthalates are not persistent in the environment, the anti-androgenic properties can result in profuse perturbations of the reproductive tract (Dziewirska and Hanke, 2018; Magnusson and Ljungvall, 2014; Latini et al., 2008).

In recent rodent toxicity studies, phthalates have been identified to induce defects in folliculogenesis and are associated with increased malformations and resorptions (Hannon and Flaws, 2015; Kaul et al., 1982). Phthalate metabolites di(2-thylhexyl) phthalate (DEHP) and mono-ethylhexyl phthalate (MEHP) were shown to be significantly higher in endometriotic women, presenting the potential for the EC to be a contributing factor (Cobellis, 2003). In humans the metabolites have further been associated to decrease the expression of trophoblast differentiation markers which can perturb placental development and function (Fowler et al., 2012). The anti-androgenic action of phthalates impedes normal androgen dependent pathways and has been shown extensively in male offspring exposed prenatally (Swan, 2008; Foster et al.,2001). Testosterone biosynthesis is governed by a cascade of enzymatic dependent reactions. Phthalates can modify the expression of these enzymes and down regulate testosterone production (Latini et al., 2006). Fetal androgen suppression leads to a variety of malformations and steroidogenic imbalances which have been characterised by the phthalate syndrome model (Schug et al., 2011). This encompasses poor semen quality, abnormalities in the epididymis, vas deferens, seminal vesicles, prostate and phallus with hypospadias and cryptorchidism (Blessinger et al., 2020). Demasculainsation and development of feminine characteristics including the retention of nipples and a shortened anal-genital distance have also been attributed to phthalate syndrome (Gray et al., 2000). The activation of peroxisome proliferator activated receptors by phthalates has been proposed as the mechanism to down regulate other nuclear receptor activities, altering normal testicular function (Latini et al., 2006).

1.3.2 Brominated Flame Retardants

Flame retardants encompass a diverse group of chemicals comprising 209 congeners that are used to reduce flammability in consumer products such as clothes, toys, electrical equipment, and appliances (Segev et al., 2009; Kim et al., 2014). The anthropogenic chemicals are persistent within the environment and are lipid soluble with bio accumulative tendencies due to the halogen moiety (Segev et al., 2009). Laboratory studies identified flame retardants to

cause chronic lung inflammation, reproductive toxicity, and disruption to thyroid hormones (Annamalia and Namasivayam, 2015).

1.3.2.1 Polybrominated Diphenyl Ethers

Recent work has uncovered reproductive effects in males and females exposed to polybrominated diphenyl ethers, most notably during the fetal and prepubertal stages (Kuriyama et al., 2005; Talsness et al., 2008). Subjecting fetal male rats to low doses of PBDE-99 on gestation day (GD) 9 hindered offspring once matured by impairing spermatogenesis and reducing epididymis weight (Kuriyama et al., 2005). High dosage exposure (60mg/kg) to postnatal male rodents resulted in reduced prostate and seminal vesicle weights and postponed the onset of puberty (Birnbaum and Staskal, 2004). Female rats under the same conditions in an independent study also experienced a delay in vaginal opening (Kodavanti et al., 2010). PBDEs have been proposed as androgen receptor antagonists, therefore delays to the onset of puberty could be indicative of interference with androgen production and/or function (Stoker et al., 2004, Stoker et al., 2005). It is important to note that PBDEs have different mixtures of several congeners, particular variants have more pronounced effects on the androgen receptor than others (Magnusson and Ljungvall, 2014). In humans, a preliminary study identified multiple PBDE congeners at high concentrations within the blood are associated with reduced fecundability in women (Harley et al., 2010). Adult human males exposed to BDE-47 presented lower inhibin-B levels than controls (Makey et al.,2016). Inhibin-B is an endocrine marker for spermatogenesis and irregularities, specifically diminished serum volumes, are associated with testicular dysfunction (Alhalabi, 2016). However, other studies have reported inconsistent results and identify a positive correlation between BDE-47 exposure and increase in inhibin-B (Johnson et al., 2013; Meeker et al., 2009).

1.3.2.2 Bisphenol A

Bisphenol A (BPA) possesses both oestrogenic and antiandrogenic characteristics which can perturb the HPG axis of subsequent generations (Cariati et al., 2019). Pregnant rat dams exposed perinatally to an environmental dose of BPA had deleterious effects on the male germ line (Salian et al., 2011). F1 offspring showed significant impairments in spermatogenesis, and this was replicated in F2 and F3 generations indicative of a transgenerational effect (Salian et al.,2011). Intratesticular testosterone levels have been shown to be reduced during adulthood after perinatal exposure (2.4µg/kg body weight per /day) suggesting a disruptive effect within the testosterone biosynthesis pathway (Akingbemi et al., 2004; Salian et al., 2009). Perinatal exposure in female rats also perturbed steroid synthesis of LH resulting in disruption to the oestrous cycle (Rubin et al., 2001). An epidemiological study of 218 men exposed to BPA while at work presented a detectable increase in urine BPA irrespective of dose and increased

the risk of compromised semen quality (Li et al., 2011). The oestrogenic activity of BPA can induce modifications to the genetic makeup of the prostatic cell and stimulate carcinogenic activity with age (Prins et al.,2008). Epigenetic modifications within the prostatic epigenome after gestational and neonatal exposure are shown to predispose males to prostate cancer (Salian et al., 2011; Prins et al.,2008).

1.3.3 Persistent Organic Pollutants

Persistent organic pollutants (POPs) are ubiquitous around the world (Alharbi et al., 2018). Agricultural and industrial processes are key sources to distributing POPs and infiltrating the food chain (Guo et al., 2019). These ECs withstand environmental degradation and persist for extended periods of time with lipophilic properties promoting bioaccumulation (Alharbi et al., 2018). Exposure has been linked to a variety of conditions including obesity, cancer, reproductive and neurological disorders, and hormonal disruption (Carpenter, 2011).

1.3.3.1 Polychlorinated Biphenyls

Temporal trends indicate semen quality is decreasing and growing evidence suggests ECs are responsible (Swan et al., 2000). Despite the ban on PCBs decades ago, the EC is still inflicting harmful effects because of the extensive half-lives (Ritter et al., 2011). Like PBDEs, PCB congeners have oestrogenic, antiestrogenic and antiandrogenic actions with the primary source of exposure determining the prominence of the congener effect (Meeker and Hauser, 2009). Studies involving PCB exposure have associated the chemical with decreased sperm motility (Toft et al., 2006; Bonde et al., 2008) and diminished sperm DNA chromatin integrity to an extent (Rignell-Hydborn et al., 2005; Rozati et al., 2002). In Taiwan 1979, rice oil contaminated with PCB resulted in mass poisoning and adverse effects in the next generation (Guo et al., 2000). Teenage boys who were exposed in utero exhibited increased percentage of abnormal sperm morphology and decreased daily sperm production (Guo et al., 2000). Generational consequences after prenatal exposure have also been seen in female rodents. Low exposure during late pregnancy perturbed the proestrus hormones LH and progesterone resulting in dysregulation of the rat reproductive tract (Steinberg et al., 2008). An in vitro study using the PCB mixture Aroclor-1254 on rat prostate cells identified: disruption in the cell morphology, double stranded DNA breaks and the expression of gap junction proteins was hindered (Cillo et al., 2007). Several studies have explored links between PCBs and testicular cancer, but the relation remains uncertain (Sweeney et al., 2015).

1.4 EC Mechanism of Action on Endocrine Receptors

The perturbation of hormone synthesis and function is the focal point of attack by many ECs (Diamanti-Kandarakis et al., 2009). Several pathways are targeted including nuclear and non-nuclear steroid receptors, the aryl hydrocarbon receptor and enzymatic mechanisms that support endocrine biosynthesis and/or action (Maire et al., 2010). ECs can interact and cause disruption to receptors through a variety of mechanisms: activation, antagonism, altering receptor expression, altering signal transduction, induction of epigenetic modifications, and alterations in endocrine synthesis, transport, distribution, and metabolism (La Merrill et al., 2020). Hormone receptors normally interact with cognate ligands and inhibit interactions with non-specific endogenous molecules. Synthetic molecules can compete with endocrine ligands due to the similarities in shape and size to hormones and the high quantities can swamp a receptor, disabling access from genuine ligands (Combarnous and Nguyen, 2019). It is important to emphasise that steroid hormone receptors will have greater affinity for their endogenous ligands, and a lower affinity for ECs when present at environmental concentrations (Lee et al., 2013). Furthermore, many studies that have investigated ECs and steroid hormone receptor interaction have been at a toxicological level and/or single chemical exposure (Birnbaum and Staskal, 2004; Hannon and Flaws, 2015; Kaul et al., 1982). Hence, a mechanism of action is very challenging to ascribe for individual chemical interactions with receptors let alone a complex mixture of ECs.

1.4.1 Oestrogen Receptor

Oestrogen receptors (ERs) are part of the nuclear receptor family and mediate oestrogenic activity by two receptor subtypes: ERα and ERβ (Shanle and Xu, 2011). Oestrogen is a steroid hormone synthesised from cholesterol through a series of enzymatic reactions and serves a biological role in differentiation and homeostasis of numerous target tissues (Li et al., 2013). ERα and ERβ are expressed within the male reproductive tract and are important for Leydig cell function and testes development respectively (Lee et al., 2013).

Synthetic and natural agents that mimic oestrogen are known as xenoestrogens (Lee et al., 2013). In spite of the wide structural diversity (Fig 1.2), the common phenolic rings enable these ECs to perform as endogenous hormones acting on the promiscuous ERs (Watson et al., 2006; Lee et al., 2013). Xenoestrogens derived from anthropogenic agents interact directly with the ligand binding domains of ERs and indirectly by activating transcriptional protein pathways (Shanle and Xu, 2010). ECs can induce dysregulation of these signalling pathways through agonistic and antagonistic mechanisms at the ER resulting in adverse impacts to the reproductive function and sexual behaviour (Craig et al., 2011; Lee et al., 2013).

BPA is a known chemical that exerts weak oestrogenic effects, disturbing the hypothalamic-pituitary-gonadal axis resulting in downstream impacts on sperm production (Cariati et al., 2019). Xenoestrogens impeding LH production will advertently reduce testosterone production from Leydig cells; testosterone is a key steroid for spermatogenesis and after converting to oestradiol, maintains Sertoli cell function (Cariati et al., 2019). ECs not only inappropriately activate ERs but also impede on oestrogen metabolism and synthesis (Frye et al., 2011). Furthermore, the inappropriate activation can disrupt ER-mediated transcriptional activity of target genes and alter gene expression (Lee et al., 2013). In vivo studies have linked fetal BPA exposure to greater susceptibility to carcinogenesis stemming from oestrogen mediated transcriptional signalling pathways (Seachrist et al., 2016).

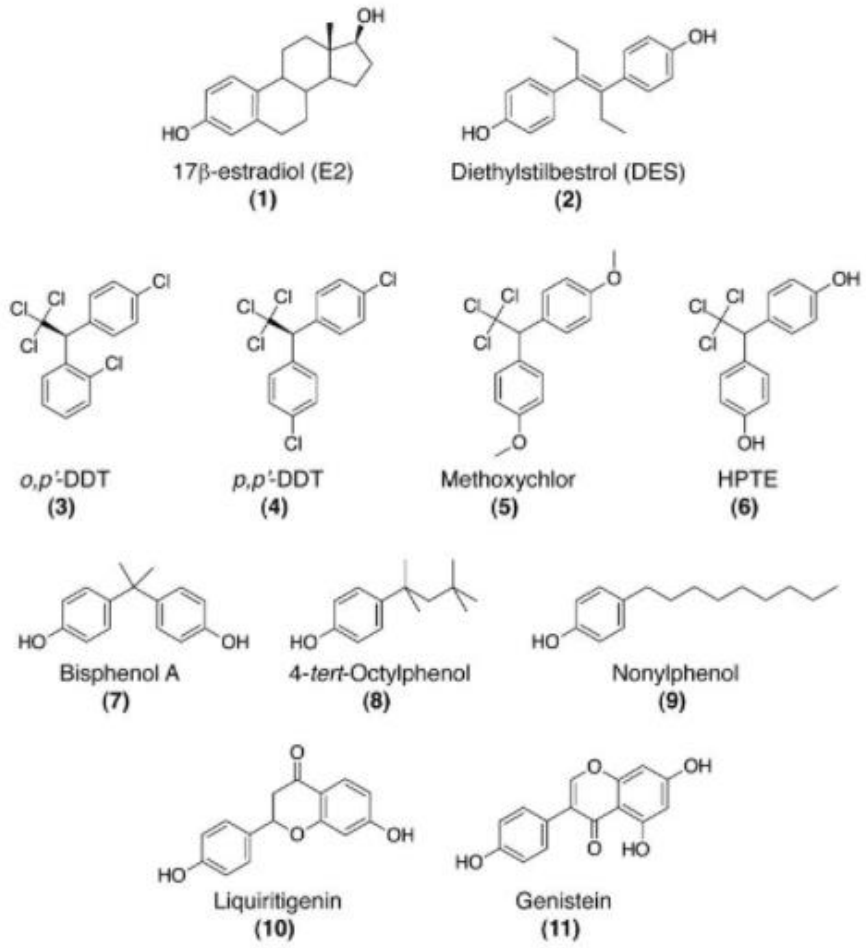

Figure 1.2 Examples of chemical structures of oestrogenic ligands and ECs compared with the endogenous hormone 17 β-oestradiol that affect ER signalling (Shanle and Xu, 2011)

1.4.2 Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AhR) is an important regulator for steroidogenesis and similar to the ER, is promiscuous (Bonefeld-Jørgensen et al., 2007; Shanle and Xu, 2011). The receptors are ubiquitously distributed throughout tissues and are expressed by human sperm (Faure et al., 2014). Usually the AhR remains inactive, however when xenoestrogens such as PCBs bind, the receptor is activated and is translocated to the nucleus (Rehman et al., 2018). Interactions with the xenobiotic response element on the DNA can interfere with steroid synthesis and potentially influence steroidogenesis (Rehman et al., 2018). The ramifications could impact sperm capacitation, the acrosome reaction and fertilisation (Faure et al., 2014).

1.4.3 Epigenetics and the Transgenerational Consequences

Environmental chemicals can induce modifications to the epigenome during fetal development and introduce multi and transgenerational changes to phenotypes (Perera and Herbstman, 2011). Epigenetic modifications refer to alterations in molecular processes which change gene expression but are independent of the DNA sequence which remains unaltered (Jirtle and Skinner, 2007).

During development, the fetal epigenome undergoes epigenetic reprogramming, removing all epigenetic markers excluding those on imprinted genes (Uzumcu et al., 2012). The period of reprogramming coincides within a critical development window and poses greater susceptibility to epigenetic errors (Cescon et al., 2020). If EC exposure occurs during this timeframe, reprogramming processes such as DNA methylation can be influenced and could potentially affect fertility (Morgan et al., 2005). There are two stages during development when critical epigenetic modifications occur: in the pre-implantation embryo and in the primordial GC (Uzumcu et al., 2012). In utero exposure to ECs at the time of pre-implantation can affect all three germ layers and therefore all the tissues of that individual (Dolinoy et al., 2007). This presents an opportunity for postnatal diseases to arise in the future and potential for multigenerational effects to persist (Skinner et al., 2011). Pregnant mothers (F0) exposed to environmental stimuli will not only affect their offspring (F1), but also the sequential offspring (F2) could be affected as these GCs are developing during the gestation of F1 (Xin et al., 2015). An in utero study of pregnant rats demonstrated perinatal exposure to the PCB mixture Aroclor 1221, skewed the sex ratio towards female in both F1 and F2 generations (Steinberg et al., 2008). Furthermore, LH concentrations were significantly different in F1 females and F2 females experienced dysregulation of reproductive physiology (Steinberg et al., 2008). In utero exposure during the reprogramming of the primordial GCs will permanently modify the epigenome of the germline and affect subsequent generations (Stouder and Paoloni-

Giacobino 2011). Transgenerational effects are noted when the unexposed generation (F3) acquire the modified phenotype (Van Cauwenbergh et al., 2020).

1.5 ECs and Male Reproductive Dysfunction

1.5.1 Testicular Development

In precocial mammals like the sheep, the differentiation and development of the reproductive physiology and neuroendocrine function occurs before birth (Jackson et al., 2008). Prior to sexual differentiation, the gonads are indifferent and develop identically. The SRY gene encoding the testis determining factor, induces the indifferent gonad to differentiate to testes (Makivan, 2006). Sertoli cells form in the coelomic epithelium and, in part, orchestrate testicular development by inducing the formation of the seminiferous tubules and the subsequent function of fetal Leydig cells (FLCs) (Rebourcet et al., 2017). Several studies have emphasised the importance of Sertoli cell proliferation throughout fetal and early neonatal life and use Sertoli cell populations as a marker for spermatogenetic capacity (Meroni et al., 2019). Often described as “nurse cells”, Sertoli cells nourish the developing GCs and provide immunological protection from the remainder of the testis (Petersen and Söder,2006; Gonzalez-Mariscal et al., 2010). FLCs reside in the testicular interstitial fluid and are responsible for androgen production (Griswold and Behringer, 2009). Masculinisation is dependent upon sufficient secretion of testosterone and insulin-like peptide 3 (INSL3) to trigger downstream events and testicular descent (Bellingham et al., 2012).

The gestation period has several stages of critical reproductive development, opening up windows of susceptibility (Fig 1.1). Fetuses lack adequate defence mechanisms to protect against insults from ECs and are therefore vulnerable to exposure (Tang et al., 2020). In the fetal sheep, sexual differentiation extends from Day 30 – Day 100 with steroidogenic activity proving crucial for successful development (Roselli and Stormshank, 2010). Studies have shown that the masculinisation programming window is highly sensitive to testosterone secretion and abnormal levels can produce reproductive consequences (Scott et al., 2009). Welsh et al., (2010) reported pregnant rats exposed to flutamide, an androgen antagonist, produced male offspring with abnormal penis growth or formation (Welsh et al., 2010). Furthermore, an earlier study showed administration of an androgen antagonist to pregnant rats in late gestation affected penile growth of offspring but not morphology (Welsh et al., 2008). In contrast, excessive exposure to testosterone during embryonic Days 16-19 in rats, induced reproductive disruption in male offspring: reductions in testes weight, Sertoli cell, spermatocyte and spermatid numbers and lower motility (Ramezani Tehrani et al., 2013). Therefore, the window of exposure in conjunction with androgen availability heavily influences male reproductive morphology and development (Welsh et al., 2009). A peptide hormone that

is important during critical development is FSH. Xenoestrogens have been shown to inhibit fetal gonadotropin secretion in sheep, resulting in a 40% reduction in testes size at birth (Sweeney et al., 2000). Early neonatal suppression of FSH during Sertoli cell multiplication in rats threatens the reproductive output in adulthood (Orth et al., 1988). A diminished Sertoli cell population produces males with smaller testes and lower spermatogenic output (Orth, 1984). Early development as well as infancy and puberty show increased sensitivity to ECs that disrupt the hormonal milieu: androgens in particular are a crucial factor in development and perturbations can result in testicular dysgenesis syndrome (Sweeney et al., 2015).

1.5.2 Testicular Dysgenesis Syndrome

In recent decades, there has been a rising trend in incidences of testicular cancer, poor semen quality, undescended testis and hypospadias (Skakkebaek, 2003). Classified under the singular entity testicular dysgenesis syndrome (TDS), experimental and epidemiological studies have attributed TDS to disruption during embryonic programming and fetal gonadal development (Skakkebaek et al., 2001).

1.5.2.1 Cryptorchidism

Cryptorchidism refers to the failure of testicular descent either unilaterally or bilaterally and can result in infertility (Niedzielsk et al., 2015). Testicular descent is governed by hormones across two separate phases: gubernaculum enlargement stimulated by INSL3 and the migration of the gubernaculum and testis to the scrotum guided by androgens (Hutson and Hasthrorpe, 2005). Several ECs have already been associated with cryptorchidism including phthalates, PBDEs, BPA and PCBs (Kumar et al., 2020).

The expression of INSL3 by FLCs is crucial for regulating and differentiating the gubernaculum in preparation for testicular descent (Skakkebæk et al., 2001; Le Moal et al., 2021). A Danish study of 227 human cryptorchids reported phthalates inflicted a negative effect on amniotic fluid INSL3 levels during the critical period (Wang et al., 2019). Administrating 750mg/kg/day of DEHP to pregnant rats during sexual differentiation produced demasculinised male offspring with undescended testes (Gray et al., 2000). Phthalates have been shown to inhibit gene expression of steroidogenic proteins and INSL3 resulting in fewer FLCs (Hu et al., 2009). INSL3 knockout mice studies produced male offspring with bilateral cryptorchidism and presumed infertile (Zimmermann et al., 1999; Skakkebæk et al., 2001). However, a study by McKinnell et al, (2005) using rats, contradicts these findings reporting a suppression of INSL3 expression did not correlate with abnormal testes positions after exposure to di (n-butyl)

phthalate (DBP) on embryonic Days 17.5 -19.5 (McKinnell et al., 2005). The conflicting results may stem from differences in exposure periods or concentrations of phthalates used. A separate study did show phthalate exposure reduced INSL3 mRNA expression and testosterone resulting in inhibition of testicular descent (Toppari et al., 2006).

FLC biosynthesis of testosterone is a regulating factor for the inguinoscrotal phase of testicular descent (Hu et al., 2009). Anti-androgens like DBP alter steroidogenic gene expression within the testes, suppressing testosterone production (Barakat et al., 2019). Exposing rats to DBP at 100-500mg/kg/day from GD 12-21 downregulated expressions of StAR (steroidogenic acute regulatory protein) and SR-B1 (scavenger receptor class B1) (Shuktz et al., 2001). These proteins are involved in the mediation of cholesterol uptake, a precursor molecule to testosterone, and disruption could impact cholesterol transfer and therefore the sequential steroidogenic pathway (Shultz et al., 2001). However, it is important to note the exposure dose was not environmentally relevant, therefore lower doses may not incur the same results. A study investigated environmental exposure of BPA (2.4 µg/kg/day) in pregnant rats from GD12 to postnatal Day 21 and measured testosterone production at Day 90 (Akingbemi et al., 2004). Testosterone production and testicular interstitial fluid levels were reduced and the decline was associated with the suppression of the CYP17 gene (Akingbemi et al., 2004). Although these studies are not directly focused upon ECs relationship to cryptorchidism, they have demonstrated the deleterious impacts ECs have on testosterone production. Disruption to steroidogenic pathways by EC exposure could attribute to cryptorchidism and sequentially affect fertility. Undescended testes experience heat stress which hinders the development of GCs and therefore could impair spermatogenesis (Nakai et al., 2000).

1.5.2.2 Declining Sperm Quality

A systematic review and meta-analysis indicated that semen parameters have declined and environmental pollutants are likely to influence this trend (Pizzol et al., 2020). Spermatogenesis requires a complex communication system between cells and molecular signallers. Disruptions within these pathways can affect multiple aspects of sperm quality and sequentially fertility (Rehman et al., 2018).

Poor motility can arise from several EC induced factors stemming from abnormal morphology and physiology. An in vivo mice study involving gestational BPA exposure showed inhibition in sperm motility parameters and decreased intracellular ATP, protein kinase A activity and protein tyrosine phosphorylation (Rahman et al., 2017). ATP production is dependent upon phosphorylation proteins stimulating mitochondria therefore downregulation of such activity may reduce ATP synthesis and sequentially the energy required for sperm motility. An in vitro mice study also associated BPA (100µM) with reduced sperm motility and motion kinetic

parameters. After a six-hour incubation, evidence showed lower ATP levels which the authors hypothesised stemmed from EC induced mitochondrial dysfunction (Rahman et al., 2015). Endocrine mimicry by ECs can inappropriately activate CatSper, a calcium ion channel on spermatozoa, desensitising the channel from the physiological ligands (Brenker et al., 2018). Desensitisation could impair sperm hyperactivation, a vital process relying on rapid flagellum movements to propel the spermatozoa through the zona pellucida (Lishko and Mannowetz, 2018). Morphological abnormalities can also arise following EC exposure. In a cohort of 161 men, higher urinary BPA concentrations were associated with abnormal tail morphology (D'Angelo and Meccariello, 2021). A similar study comparing 50 infertile men with 50 fertile men, highlighted greater BPA levels in the infertile group and significantly decreased sperm morphology (Omran et al., 2018). Normal morphology in terms of the spermatozoa head, mid-piece and tail all serve crucial roles in the lead up to and including fertilisation. Abnormalities in the tail piece can impact upon the linearity of forward progression and the curvilinear velocity of the sperm (Vasan, 2011).

Apoptosis serves a role to balance the ratio of GCs to Sertoli cells to sustain proliferation and differentiation (Urriola-Muñoz et al., 2014). However, ECs can induce the up-regulation of apoptotic factors, offsetting the cell balance and impairing spermatogenesis. An in vitro study using human fetal testes (7 -12 weeks) cultured in MEHP (10^{-4}M) for 3 Days found a 40% reduction in GC numbers and was attributed to an increase in apoptosis (Lambrot et al., 2007). No effect was recorded on Sertoli cell proliferation nor testosterone production from Leydig cells, leading the authors to conclude MEHP directly impeded the development of the germ cell lineage (Lambrot et al., 2007). Numerous studies have previously recognised the relationship between GC numbers and spermatogenesis (Doyle et al., 2013; Trefil et al., 2017; Romero et al., 2011), hence depletion of these building blocks could drastically affect sperm concentration. Compensatory mechanisms can recover GC populations as shown by (Ferrara et al., 2006). They noted a significant increase in the GC proliferation index after DBP exposure was halted and had initially increased gonocyte apoptosis. However, in terms of real world exposure, the plethora of chemical subjection is incessant. Therefore, there is limited recovery period for GCs which could impact male fertility moving into adulthood in terms of sperm concentration and sperm count.

Oxidative damage can be detrimental to sperm quality and can manifest as DNA fragmentation and lipid peroxidation (Dada,2017). The incubation of Aroclor 1254 (10^{-9},10^{-8},10^{-7} M), a PCB congener, with adult rat sperm significantly decreased sperm viability after three hours (Aly,2013). Lipid peroxidation increased pointing towards an intensification in oxidative stress generated by reactive oxygen species (ROS). The composition of the sperm membrane is largely polyunsaturated fatty acids which are easily oxidised by ROS (Wyck et al., 2018). Lipid

peroxide induced effects can compromise membrane integrity and fluidity and furthermore sperm motility and viability (Kao et al., 2008). In addition, poor membrane integrity could also present issues for capacitation, ova binding and the acrosome reaction (Eskandari and Momeni, 2016). Several other studies have also associated links between various ECs and lipid peroxidation in different sperm cellular components and demonstrated negative impacts upon the viability (Huffman et al.,2018; Khasin et al.,2020; Hauser et al.,2007;Kaur et al.,2018; Jiang et al., 2017). As male GCs develop into mature sperm cells, the ability to repair DNA damage is progressively lost, increasing sperm cells' sensitivity to ROS (Wyck et al., 2018). The phthalate metabolite DEHP (10µM and 100µM) was shown to increase ROS production in mouse sperm and altered the capacitation profile (Khasin et al., 2020). The sperm genome can experience single and double DNA fragmentation as a consequence of excessive ROS production (Moustafa et al., 2004). The mutagenic effects of the superoxide and hydroxy radicals can cause chromosomal deletions and clear associations have been shown between nuclear DNA damage and poor semen quality (Moustafa et al., 2004). Furthermore, poor DNA integrity has been correlated with sperm aneuploidy and a greater tendency to develop triploid zygotes (Jurewitz et al., 2013; Dada,2017).

1.5.2.3Testicular Cancer

An increasing body of evidence has shown a rise in testicular cancer (TC) cases within recent decades and there are marked differences between countries (Giannandrea and Fargnoli, 2017; Liu et al., 2000). However, less information is available surrounding its aetiology (Nava-Castro et al., 2019). Research has recognised that 95% of TCs are germ cell cancers and are believed to have been derived before birth from germ cell neoplasia in situ (GCNIS), a precursor lesion (Fénichel and Chevaler, 2019).

It has been postulated that ECs may be responsible for GCNIS by disturbing the hormonal stability of the testicular microenvironment in early development (Giannandrea and Fargnoli, 2017). A study investigating various POPs in 58 human TC cases found no significant difference compared to controls (Hardell et al., 2006). Interestingly, when reviewing the concentration of POPs in the mothers of TC males, there was a significant increase in certain POPs indicating in utero exposure could be a driving factor in the aetiology of TC (Hardell et al., 2006). Several studies have tried to assess the influence PCBs have on TC; however, the results are conflicting (McGlynn and Trabert, 2012). A Norwegian study found higher concentrations of some PCB congers were associated in males with TC, but others were apparently lower (Purdue et al., 2009). Paoli et al., (2015) showed a significant increase in the risk of developing TC in patients with detectable PCBs in seminal fluid (Paoli et al., 2015). In contrast, a significantly larger scale study did not support these findings and demonstrated an

inverse relationship between TC and PCBs (McGlynn et al., 2009). As previously mentioned, ECs have the power to modulate epigenetic mechanisms, and this could be a factor for the rise in TC. Rodent studies have suggested in utero exposure to ECs may modify epigenetic modification processes such as hypomethylation of oncogenes promoting expression or hypermethylation of tumour suppressive genes (Fénichel and Chevaler, 2019). However, it remains unknown whether ECs have a direct role in the aetiology of TC and if so, which chemicals are responsible.

1.7 Hypothesis

1. Exposing male sheep to environmentally relevant doses of environmental chemicals throughout fetal life, perturbs the histological development of the testes.

2. Fetal exposure to environmentally relevant doses of environmental chemicals will depreciate semen parameter values of F1 progeny.

3. Using a portable computer assisted sperm analysis (CASA) system will produce statistically similar results to a comparative validated CASA system for sperm motility, progressive motility and kinematic parameters.

1.8 Aims

The first research chapter of this thesis utilises the Sheep Biosolids Model to determine the following:

1. The effect of in utero biosolids exposure on ovine prepubertal testis' Leydig cell populations using the antibody, CYP17A1, as a steroidogenic marker within an immunohistochemistry study.

2. The effect of in utero biosolids exposure on ovine prepubertal testis' Sertoli cell populations, specifically the expression of Anti-Müllerian hormone (AMH) staining cells by immunohistochemistry.

3. The effect of biosolids exposure on ovine prepubertal testis cell proliferation capacity using proliferating cell nuclear antigen (PCNA) as a marker of cell proliferation by immunohistochemistry.

The second research chapter evaluates the iSperm, a new CASA platform, to determine the following

1. The repeatability and accuracy of three iSperm semen analysis methods using ram semen
2. The validity of iSperm ram semen analysis by comparison to a validated CASA system (IVOS 11)

The final research chapter uses the iSperm to analyse fresh and cryopreserved semen from F1 pubescent rams that had been exposed in utero to biosolids to determine:

1. Whether in utero exposure to environmental chemicals dosed at the LOAEL compromise semen parameters (sperm motility (%), progressive motility (%), VCL (µm/S), VAP (µm/S), VSL (µm/S), STR (%) and LIN (%)).
2. Whether sperm motility and kinematic parameter differences arise between fresh and cryopreserved F1 ram semen from the same rams

Chapter 2: Histological Analysis of F1 Ram Testes Exposed in Utero to Biosolids

2.1 Introduction

Fetal testicular development is orchestrated, in part, by Sertoli cells which induce the formation of the seminiferous tubules, support germ cell development and facilitate function of fetal Leydig cells (Rebourcet et al., 2017). The period during which the fetal testes develop is highly sensitive to environmental chemical influences and since such exposures have been linked with perturbed reproductive development this may pose significant risk to adult male fertility (Cescon et al., 2020). The Sheep Biosolids Model (described in 1.2), in which pregnant ewes (and sometimes the offspring) are exposed to environmental chemical mixtures, is widely recognised as reflecting human exposures and has been utilised to examine down-stream effects on reproduction (Evans et al., 2014).

Sertoli cells regulate germ cell development by controlling the hormonal milieu within the seminiferous tubules (Griswold, 1998). The Sertoli cell number dictates the number of germ cells that can be supported and in turn the spermatogenic output in adulthood (Sharpe et al., 2003). Anti-Müllerian hormone is secreted by immature Sertoli cells in fetal and early postnatal life and is used as a biomarker to analyse Sertoli cell numbers and staining by immunohistochemistry within the seminiferous tubules (Grinspon and Rey, 2010).

A key functional marker of the testes is the steroidogenic Leydig cells. These cells are a major site for synthesising testosterone and INSL3 which initiate downstream events such as testicular descent and masculinisation (Bellingham et al., 2012). Testosterone synthesis incurs a rate-limiting step during the metabolism of cholesterol to pregnenolone, mediated by cytochrome P450 family 11 subfamily A member 1 (CYP11A1), and is dependent upon cytochrome P450 enzymes, of which 17α-hydroxylase/ 17,20-lyase (CYP17) is central to the synthetic pathway (Payne, 1990; Creasey and Chapin, 2013).

Sufficient proliferation of Sertoli cells and fetal Leydig cells during perinatal life is critical for determining the extent spermatogenesis can occur in the adult testes (Bellingham et al., 2012).

A commonly used biomarker for cells in early G1 phase and S phase of the cell cycle, and hence a useful index of proliferation, is PCNA: a participant in DNA cellular pathways (Strzalka and Ziemienowicz, 2011).

This study aimed to assess the effects of continuous in utero exposure to biosolids on F1 8-week male sheep. Immunohistochemical analysis of Sertoli and Leydig cell markers (AMH and CYP17) and proliferation (PCNA) of these cell types provided a means of investigating biosolid exposure related effects on the developing testis.

2.2 Materials and Methods

2.2.1 Animals, Treatment Groups and Tissue Collection

The animal tissues used in the experiments were collected from an ongoing study at the University of Glasgow (Cochno farm & Research Centre), Clydebank, Glasgow. All procedures were under licence by the UK Government Animals Scientific Procedures Act 1986 and ethical approval was obtained by the University of Glasgow ethical review committee and by the Committee for Animals and Research ethics at the University of Nottingham.

Selected unused pastures were treated in September with one application of thermally dried sewage sludge (2.25 metric tons of dry matter/ha). Control pastures were treated with inorganic fertiliser (225kg/ha/year) and balanced with nitrogen to obtain similar nitrogen levels to the treated pasture. Pregnant ewes were grazed on either control or treated pastures from eight weeks prior to mating to parturition after which all animals were maintained on non-treated pastures. A cohort of male offspring were euthanised at eight weeks postpartum. When animals from treated pastures were moved, they were maintained on a separate pasture for a few days to avoid cross contamination from faeces and urine to the control pasture. Afterwards, all animals were maintained on the control pasture until euthanasia. All animals were monthly body condition scored and were not provided with additional supplements as these could expose the sheep to non-experimental EDCs.

All ewes were euthanised in accordance with Schedule 1 protocols as stated by the UK Animals (Scientific Procedures) Act (1986). At slaughter, samples and data were collected and processed as described by Bellingham et al., 2012 and Lea et al., 2016a. One testis per lamb was bouins fixed for histological analysis. A total of 22 testes were analysed in this study, 11 Control and 11 Biosolids.

2.2.2 Testis Immunohistochemistry

Immunohistochemical staining was completed for markers in Sertoli cells (anti-Mullerian hormone, AMH (Santa Cruz Biotechnology, CA, USA)), Leydig cells (CYP17A1 (Biorbyt, Cambridgeshire, UK)) and proliferation (proliferating cell nuclear antigen, PCNA (AbCam, Cambridge, UK)) (Table 2.1).Prepubertal testes fixed in paraffin blocks were sectioned at 5µm thickness using a Leica Microtome and placed on Polysine slides to dry on a 45 °C hot plate. For each marker, one section per testis was randomly selected for staining and sectioned approximately from the same area of each testis.

A manual bench method was followed for the immunohistochemistry protocol. The sections (one per testis) were deparaffinised in xylene (Fisher Scientific, Loughborough, UK) for two times five minutes. The sections were then submerged in histoclear (National Diagnostics, USA) for five minutes then rehydrated through a series of decreasing ethanol (Fisher Scientific, Loughborough, UK) steps (100%, 95%, 70%), two minutes each. Sections were washed in distilled water for two five minute periods then followed with two five minute washes in Phosphate Buffer Saline (PBS) buffer solution (Life Technologies Limited, UK).

The antigen retrieval step comprised of microwave heating treatment for twenty minutes at medium power submerged in a pH 6 sodium citrate buffer (Sigma-Aldrich, USA). The treatment was divided into four times five minute time periods and allocated one minute rest between each period of heating. The sections were rested for twenty minutes in the citrate buffer.

The slides were washed in an endogenous blocking solution (3% H_2O_2: Sigma-Aldrich, USA) for five minutes followed by two five minute washes in PBS. The slides were transferred to a humidified box for incubation. A Vectastain Elite ABC Universal Kit (Vector Laboratories, Burlingame, USA) was used for the immunohistochemistry staining. The sections were incubated in 5% normal horse blocking solution for twenty minutes and then washed in PBS for five minutes. To reduce background staining, the sections were subjected to an avidin-

biotin blocking treatment (Vector Laboratories, Burlingame, USA). The primary antibody was added and left to incubate overnight at 4 °C (Table 2.1): (a) mouse monoclonal PCNA at 0.6mg/ml (AbCam); (b) monoclonal mouse AMH at 0.2mg/ml (Santa Cruz Biotechnology); (c) polyclonal rabbit CYP17A1 at 1mg/ml (Biorbyt Ltd). Negative controls were incubated as nonspecific rabbit IgG at 5mg/ml (Vector Laboratories), mouse IgG at 1mg/ml (Vector Laboratories) and RabMAb immunizing peptide 1mg/ml (AbCam).

After incubation, excess primary antibody was removed and slides were submerged for five minutes in PBS containing Tween20 (0.01%) (Merck, Germany). Slides were incubated with a secondary antibody (300 µl horse serum, 15ml normal horse blocking solution and 300 µl biotinylated secondary antibody (anti-mouse horse/rabbit IgG)(Vector Laboratories, Burlingame, USA) for thirty minutes then washed in PBS/T for five minutes. Sections were then incubated with Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, USA) for thirty minutes followed by a five minute PBS/T wash. Sections were incubated with DAB substrate (Vector Laboratories, Burlingame, USA) with twenty second intervals between slides to ensure consistency across applications. The DAB substrate was left for two -ten minutes before stopping the reaction by washing in distilled water for two five minute periods. The sections were counter stained in Harris Haematoxylin stain (Cell Path, Newton, UK) for two minutes then dehydrated through an increasing concentration series of ethanol (70%,95% and 100%) each for two minutes. The sections were cleared in two five minute periods of xylene before mounting slides with a cover slip using DPX mounting media (Sigma-Aldrich, USA).

For the CYP17A1 negative IgG control, a RabMAb immunizing peptide blocking method was used. A solution of 5% Non-fat dairy milk (NFDM) (Sainsbury's supermarkets Ltd, London,UK)/PBS was made. CYP17A1 was diluted to 1:100 using 4µl of antibody in 236µl of NFDM/PBS and 160 µl of immunizing peptide (AbCam, Cambridge, UK) then incubated with agitation overnight at 4°C.

Table 2.1: Antibodies used for immunohistochemistry protocol.

Antibody	Type	Dilution	Source
PCNA	Mouse monoclonal	1:3000	AbCam
AMH	Mouse monoclonal	1:100	Santa Cruz Biotechnology
CYP17A1	Rabbit polyclonal	1:100	Biorbyt Ltd
Mouse IgG	Mouse monoclonal	1:100	Vector Laboratories
Rabbit IgG	Rabbit monoclonal	1:3000	Vector Laboratories
Immunizing peptide	Rabbit monoclonal	1:100	AbCam

2.2.3 Image Capture

Twenty images were captured per testis using a four-point five fields of view compass method with a Nikon Eclipse 80i microscope (Nikon Instruments Inc, Melville, Ney York, USA) (Fig 2.1). Image capture used 20x magnification. A blank background image was captured for the analysis stage.

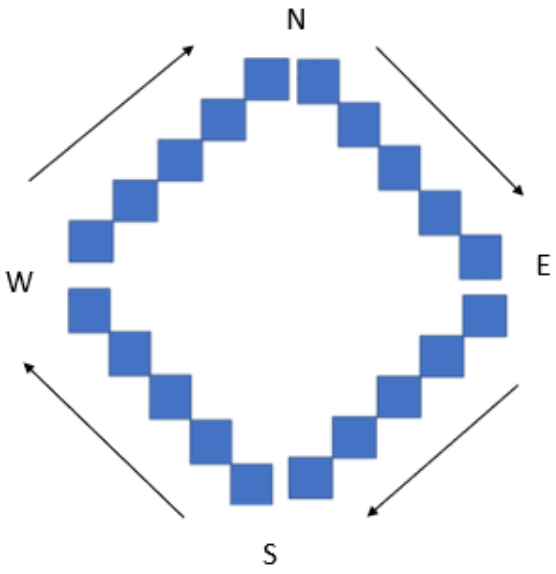

Figure 2.1: Compass method for image capture of a tissue section. Blue squares represent an image, arrows show direction of image capture.

2.2.4 Image Analysis

Images were analysed using Image-Pro Premier (Media Cybernetics, Maryland, USA) to quantify the percentage of nuclear or cellular staining for areas of interest.

2.2.4.1 Image-Pro Premier Set Up

All images had the blank background image subtracted before analysis to remove uneven shading from the microscope light.

2.2.4.2 Image Analysis

Multiple regions of interest (ROI) were selected using the polygon tool to draw around the periphery of specific regions of the tissue. The ROI for AMH was the seminiferous tubules and for CYP17A1 the adjacent interstitial area regions were selected. For PCNA, the ROI was separately drawn for the tubules and interstitial area to assess proliferation of Sertoli cells in the tubules and Leydig cells in the interstitial area. The threshold tool was used to allocate a colour to a particular shade of staining. For each marker, the stages of colour overlay are described below. After overlaying, the number of pixels per category, indicative of area overlaid, were quantified and the data was exported to Excel.

Selected stained areas include AMH positive cells (brown Dab stain) and AMH negative cells (blue ammoniated stain). Figure 2.2 illustrates the colour overlay phases.

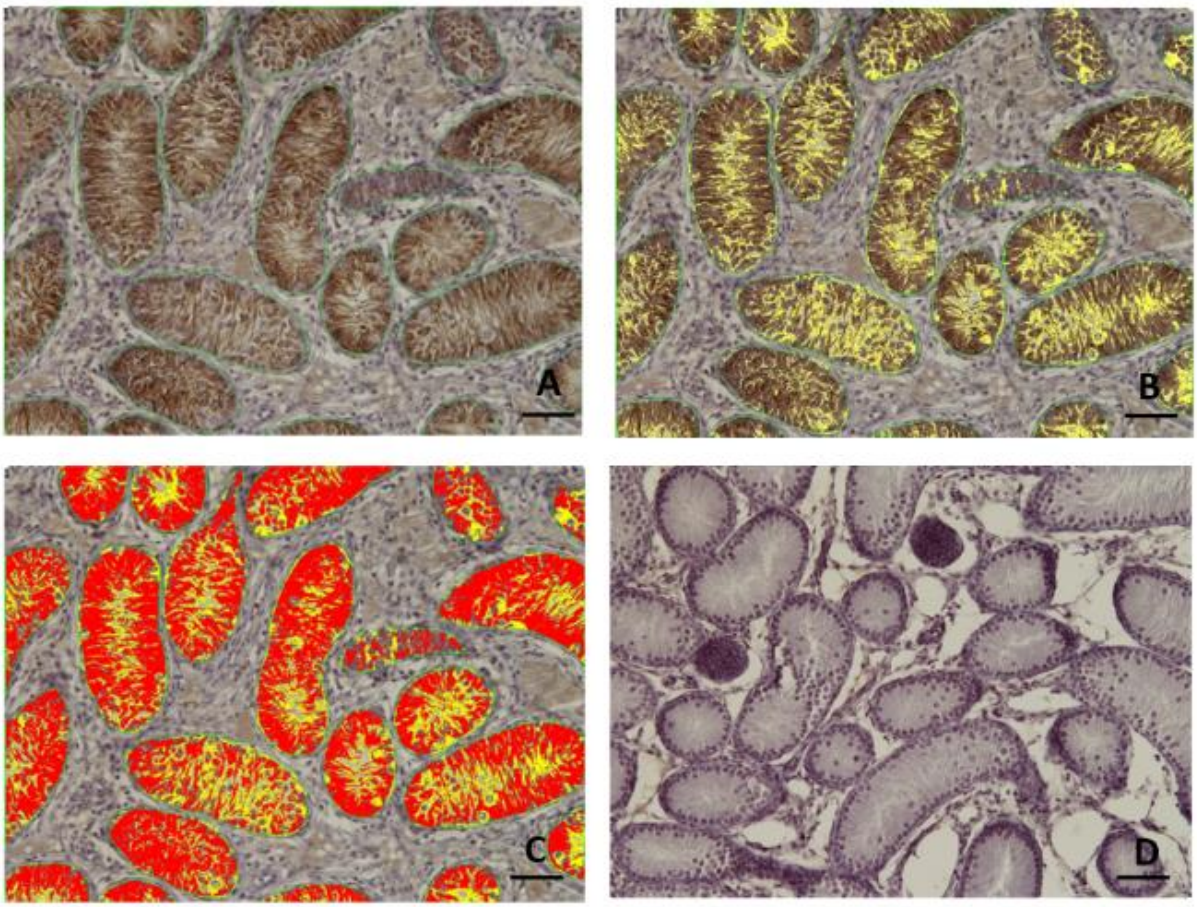

Figure 2.2: Stages of Image-Pro Premier colour overlay for AMH. (A) Outline of tubules drawn with polygon tool (green). (B) Ammoniated stained blue cells (AMH negative lumen) overlayed in yellow (C) Positive AMH cells stained brown overlayed in red. (D) Negative IgG control. Scale bar represents 200 µm.

Selected stained areas include cytoplasmic staining for CYP17A1 (brown Dab stain) and haematoxylin-stained nuclei (CYP17A1 negative). Figure 2.3 illustrates the colour overlay phases.

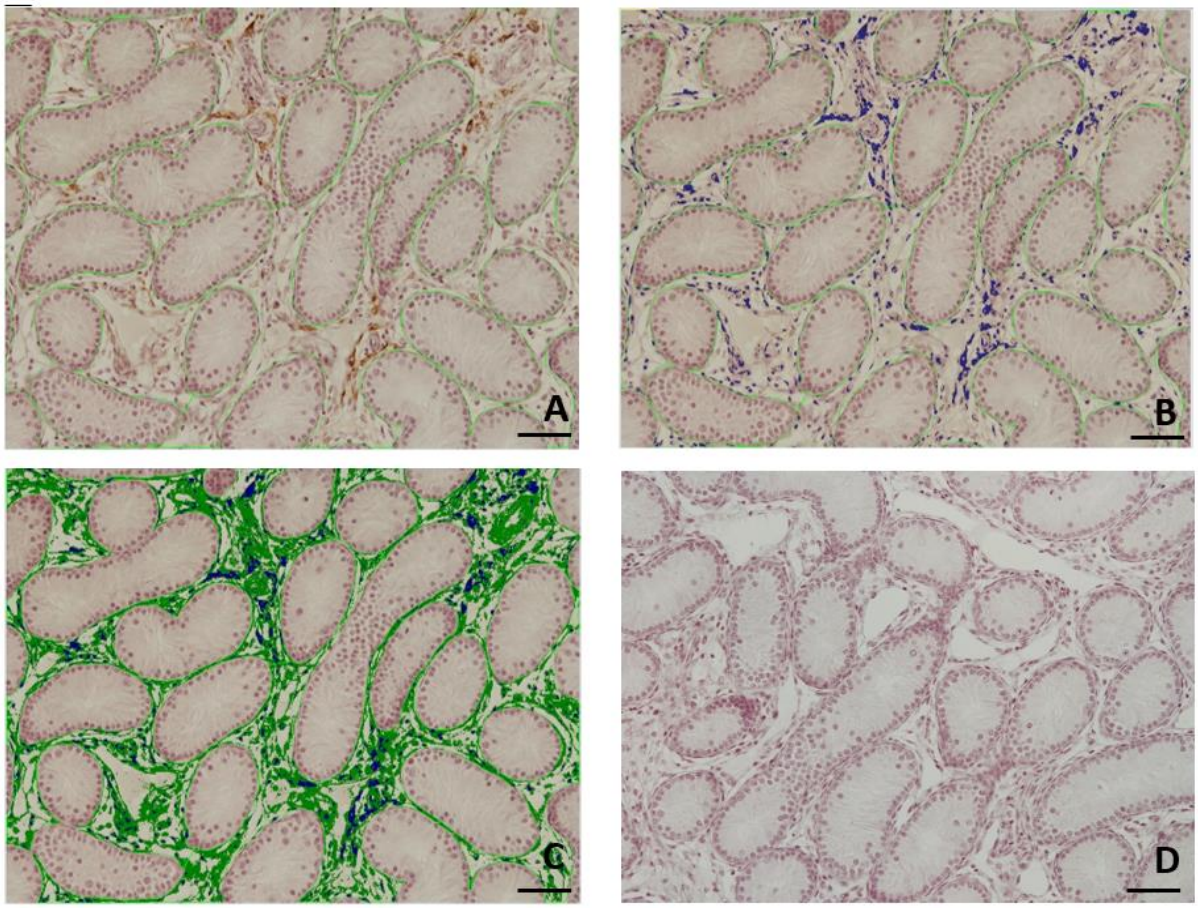

Figure 2.3: Stages of Image-Pro Premier colour overlay for CYP17A1 (green). (A) Interstitial areas outlined. (B) Positive CYP17A1 cells stained brown overlayed in dark blue. (C) Haematoxylin stained purple and overlayed in green. (D) Negative IgG control. Scale bar represents 200 µm.

Selected stained areas include PCNA positive nuclei (brown Dab stain) and PCNA negative nuclei (purple haematoxylin stain). Figure 2.4 and 2.5 illustrates the colour overlay phases for tubule and interstitial staining respectively.

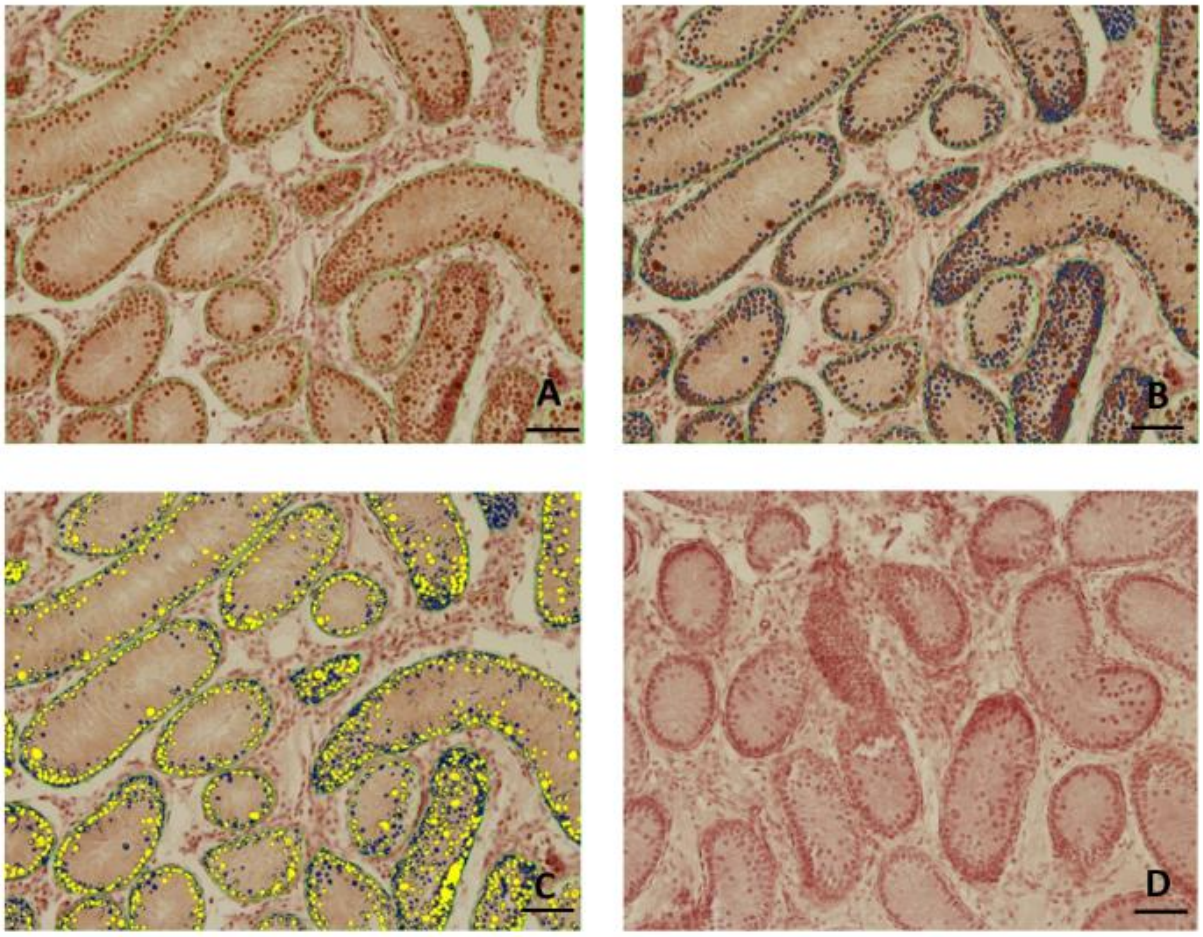

Figure 2.4: Stages of Image-Pro Premier colour overlay for tubule PCNA staining. (A) Outline of tubules drawn with polygon tool (green). (B) Haematoxylin-stained purple nuclei (PCNA negative) overlayed in dark blue. (C) Positive PCNA nuclei stained brown and overlayed in yellow. (D) IgG negative control stained with haematoxylin. Scale bar represents 200 µm.

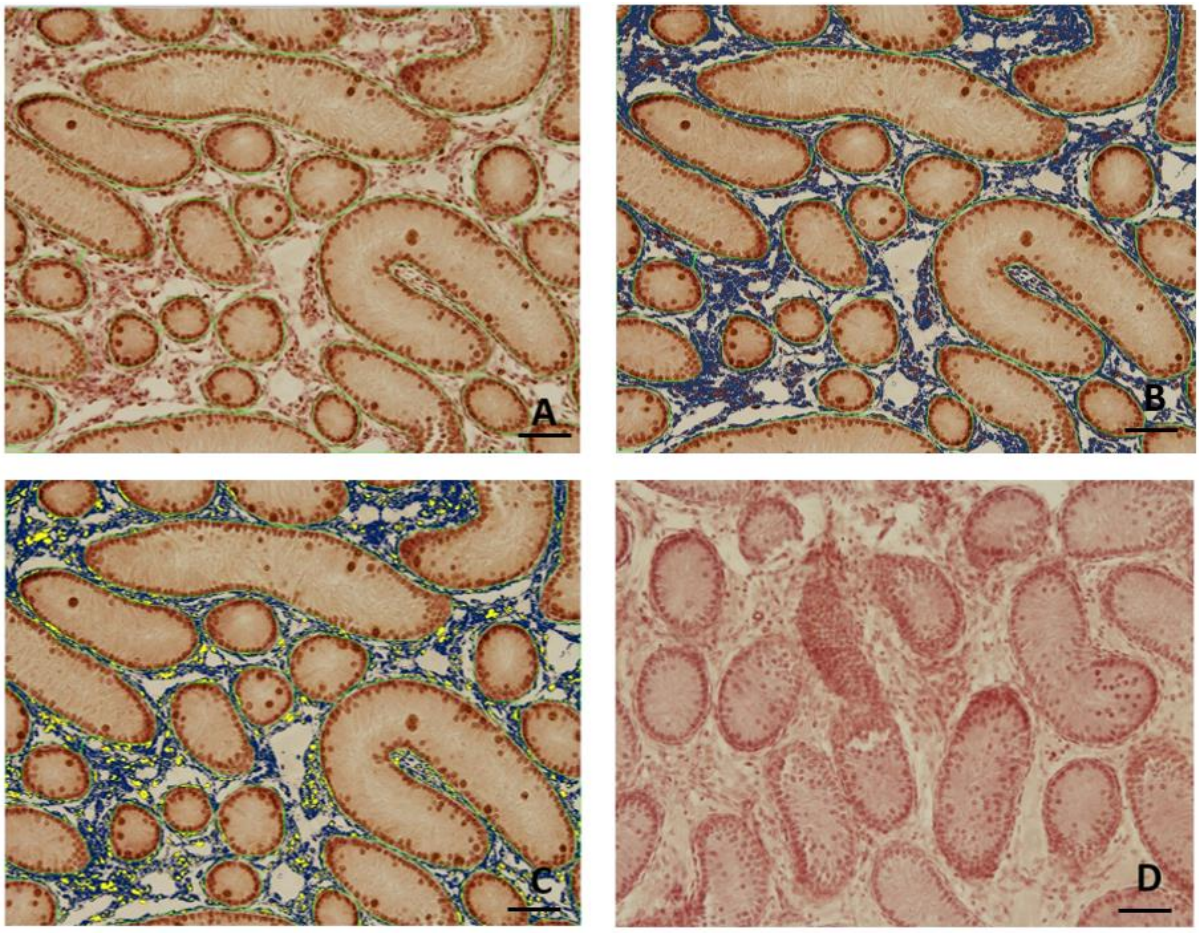

Figure 2.5: Stages of Image-Pro Premier colour overlay for interstitial PCNA staining. (A) Outline of interstitial drawn with polygon tool (green). (B) Haematoxylin-stained purple nuclei (PCNA negative) overlayed in dark blue. (C) Positive PCNA nuclei stained brown and overlayed in yellow. (D) IgG negative control stained with haematoxylin. Scale bar represents 200 µm.

2.5 Statistical Analysis

The percentage of positive immunohistochemical staining data were analysed using Generalized Linear Models that assumed binomial errors with logit-link functions (GenStat 21st Edition, 2020, VSN International Ltd, Hemel Hempstead, UK). Terms fitted to these models were ‘F0 Sire’ (of which there were four), F1 offspring ‘Birthweight’ (to account for any putative effect of *in utero* growth), ‘Age’ at necropsy (to account for variable date of birth) and 'Treatment’ (Biosolids vs Control). Data are presented as adjusted means with SEMs.

2.3 Results

2.3.1 AMH Positive Staining

Within the seminiferous tubules, there was no difference (P=0.104) in the proportion of positive AMH staining in Sertoli cells between Control and Biosolids testes tissues (Fig 2.6C and Fig 2.7).

2.3.2 CYP17A1 Positive Staining

Within the interstitial area, there was no difference (P=0.910) in the proportion of positive CYP17A1 staining in Leydig cells between Control and Biosolids testes tissues (Fig 2.6D and Fig 2.8).

2.3.3 PCNA Positive Tubule Staining

There was no difference (P=0.528) in the proportion of positive PCNA tubule staining for Sertoli cells between Control and Biosolids testes tissues (Fig 2.6A and Fig 2.9). Age and Sire showed a significant effect (P= 0.043 and P=0.017 respectively) on the proportion of PCNA positive tubule staining.

2.3.4 PCNA Positive Interstitial Staining

There was no difference (P=0.169) in the proportion of positive PCNA interstitial staining for Leydig cells between Control and Biosolids testes tissues (Fig 2.6B).

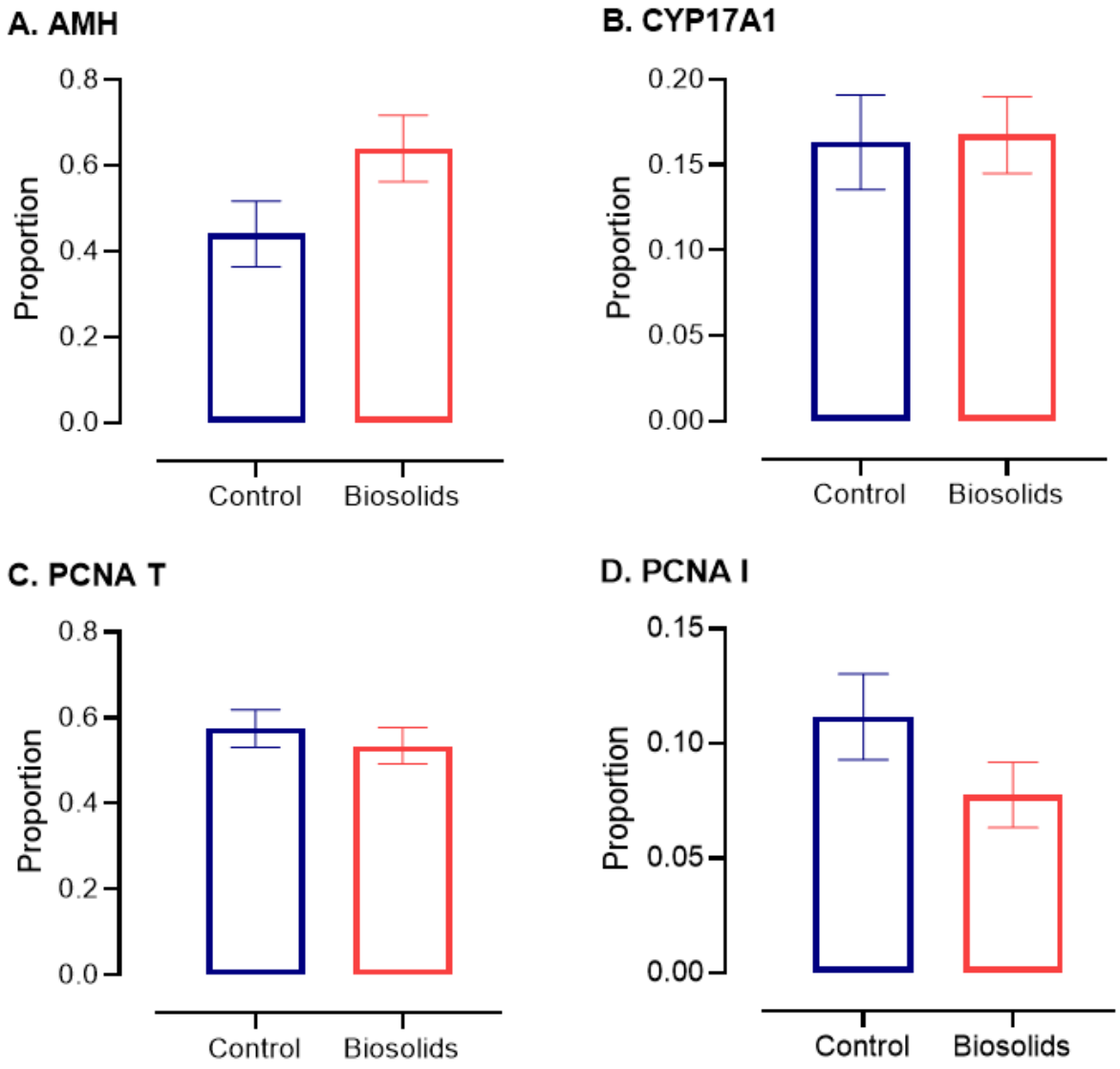

Figure 2.6: Bar graph depicting the proportions of positive immunohistochemical staining between Control and Biosolids prepubertal testes groups. A) Depicts the proportion of positive AMH staining. B) Depicts the proportion of positive CYP17A1 stainingC) PCNA T represents the portion of positive tubule PCNA staining. D) PCNA I represents the portion of positive interstitial PCNA staining.

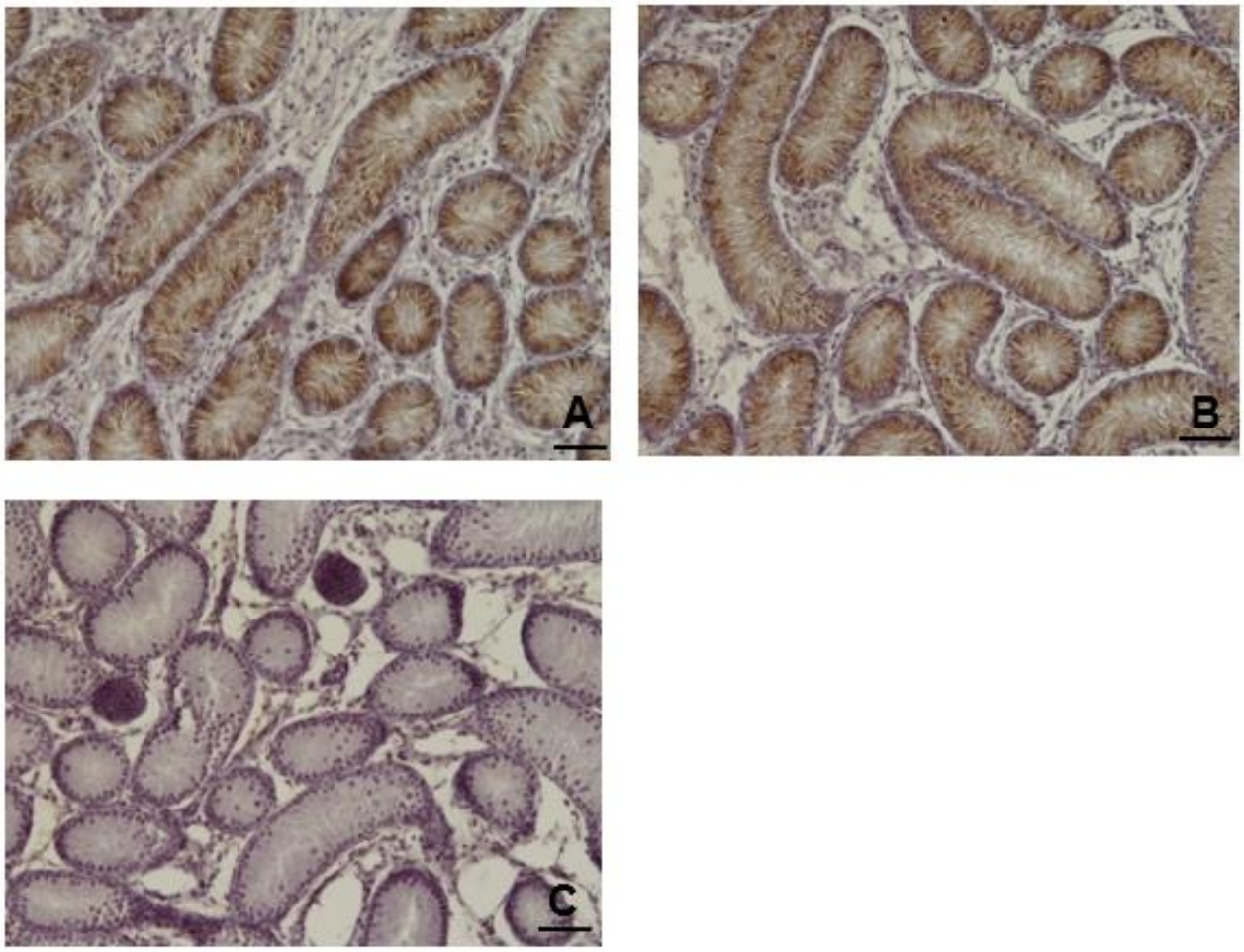

Figure 1.7: AMH Images for Control and Biosolids testes sections A) Control testis B) Biosolids exposed testis C) Negative IgG control. Scale bar represents 200µm.

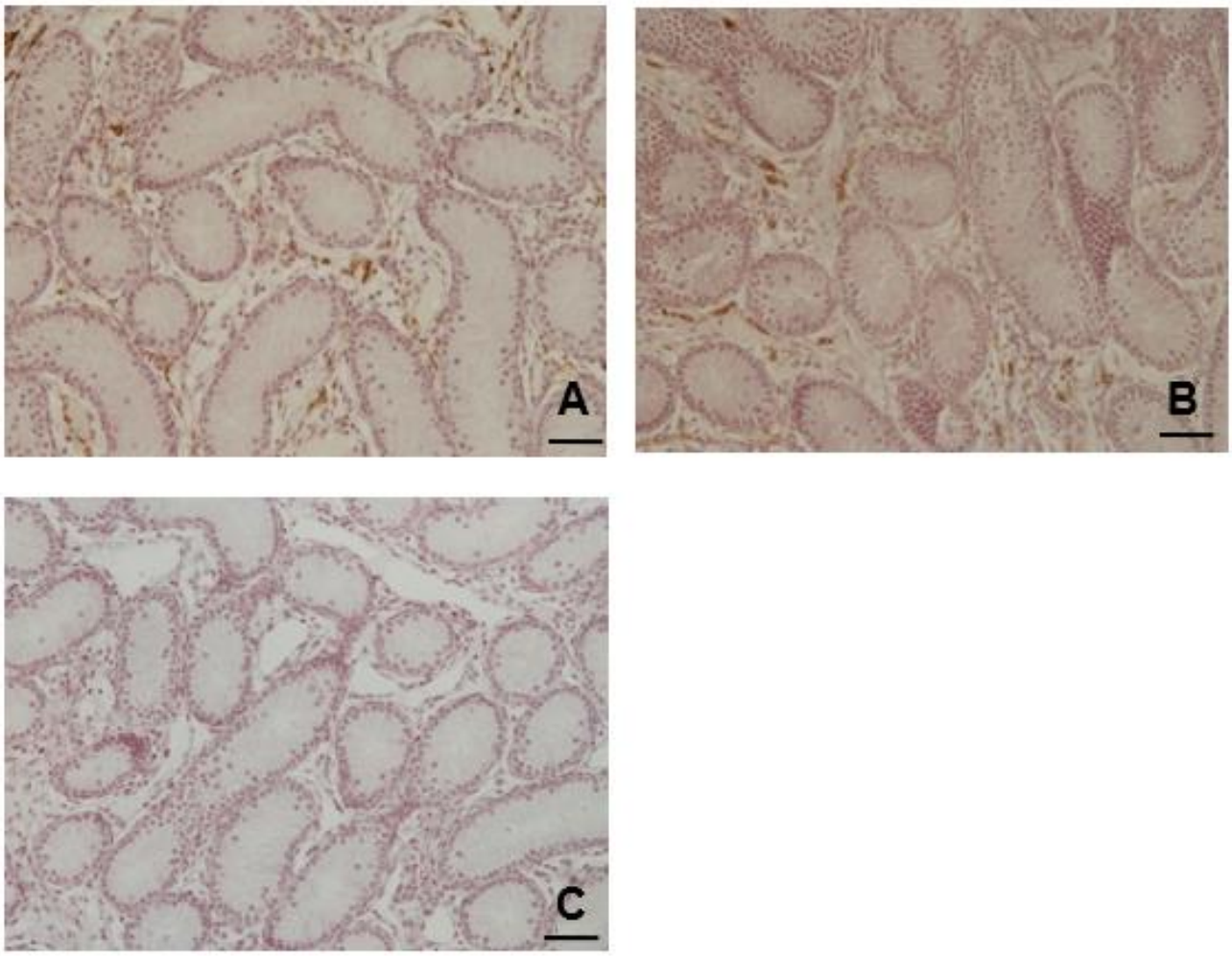

Figure 2.8: CYP17A1 Images for Control and Biosolids testes sections A) Control testis B) Biosolids exposed testis C) Negative IgG control. Scale bar represents 200µm.

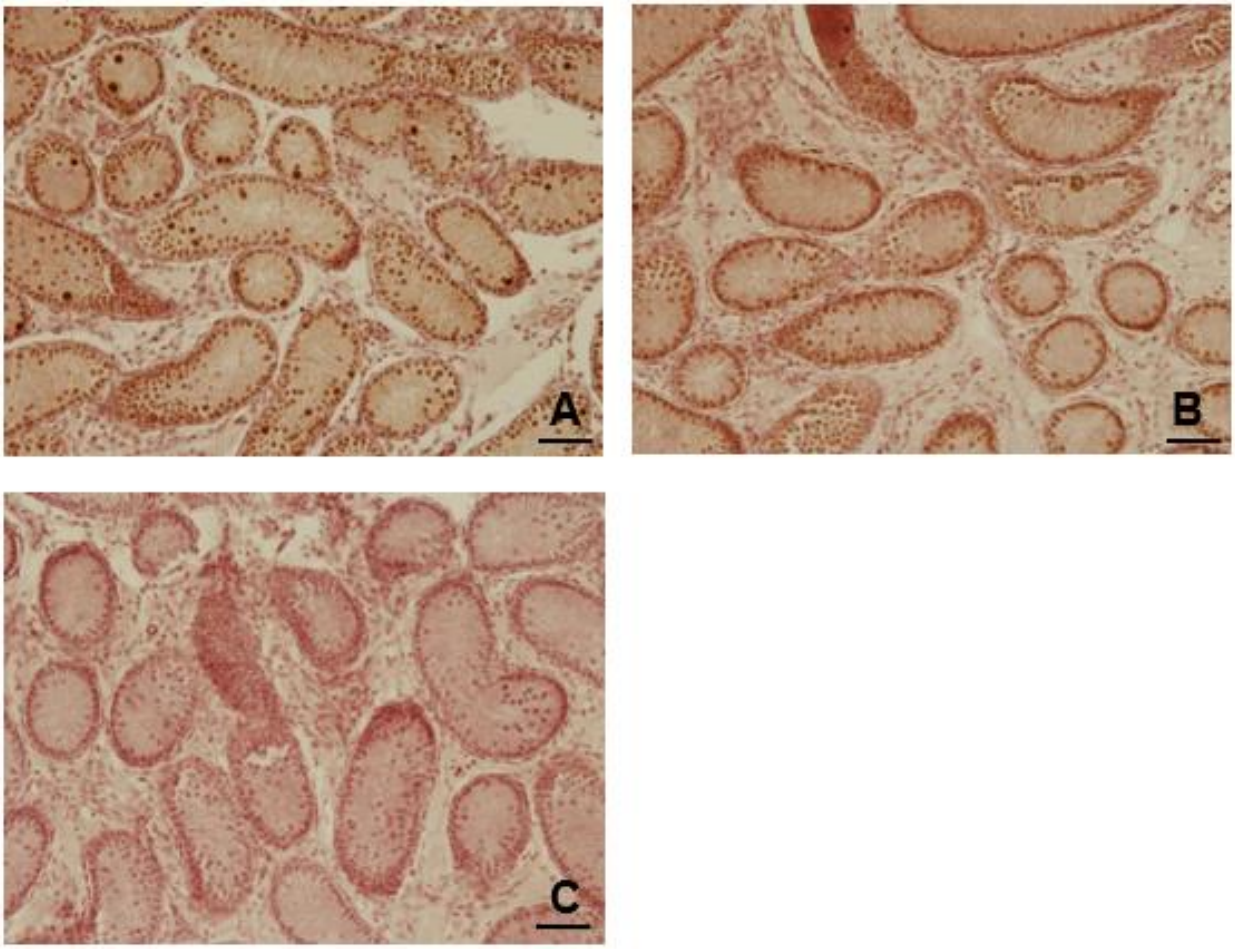

Figure 2.9: PCNA Images for Control and Biosolids testes sections A) Control testis B) Biosolids exposed testis C) Negative IgG control. Scale bar represents 200µm.

2.4 Discussion

Declining male fertility is an ongoing area of concern with a rising trend in incidences of testicular cancer, poor semen quality, undescended testis and hypospadias (Skakkebaek, 2003). Studies that have used biosolids exposed ewes as a real-life model for human exposure, have shown effects on the late gestation fetal male, day 1 and mature male offspring exposed in utero (Lea et al., 2022; Elcombe et al., 2021; Paul et al., 2005; Bellingham et al., 2012). At each timepoint, developmental effects and/or abnormalities are reported in the testis (Lea et al., 2022; Paul et al., 2005; Bellingham et al., 2012). This study has uniquely explored effects in 8-week prepubertal offspring exposed in utero from preconception till parturition. The key findings were that no significant treatment effect was observed for Sertoli and Leydig cell staining or for proliferation as assessed by PCNA, however , AMH and interstitial PCNA approached a significant increase in the treatment group (P=0.104 and P=0.169 respectively). It would appear therefore that at this developmental time point, there are no differences between Control and Biosolids testes for any of the cellular or steroidogenic markers.

2.4.1 AMH marker for Sertoli cells

Effective testis development requires controlled development of the key cellular components: Sertoli cells to support germ cell maintenance (Sharpe et al., 2003), Leydig cells for adequate steroid production (Svechnikov et al., 2010) and additional stromal cells (Kim et al., 2008) and peritubular myoid cells for maintenance of morphology (Yao et al., 2002). Although the dogma suggests exposure to biosolids reduces Sertoli cell numbers, we have previously reported that continuous exposure throughout pregnancy had no effect on Sertoli cell numbers in Day 140 fetuses (Lea et al., 2022). However, that study also reported that transient exposure periods limited to 80 Days in early, mid or late gestation were associated with a reduction in Sertoli cell numbers (Lea et al., 2022). Previously, biosolids exposure to Day 110 showed a numerical change in Sertoli cell number but stained area for AMH, a biomarker for Sertoli cells, did not differ between Control and Biosolids testes (Paul et al., 2005). Interestingly, Bellingham et al, (2012) reported that in utero exposure throughout gestation extended to seven months post

parturition in lambs, did not affect Sertoli cell numbers or area stained for this cell type (Bellingham et al., 2012). However testicular abnormalities were observed in a cohort of animals. The study reported here, examined eight week old prepubertal testes from lambs that were exposed in utero to biosolids eight weeks prior to mating until parturition. The study indicated no difference in staining area AMH between treatments however the value was approaching a significant increase in the treatment group (Pd=0.104). It is possible therefore that a potential positive effect may emerge. Although not evident from the current study, the counting of Sertoli cells as described by Lea et al., (2022), as well as AMH immunostaining may yield a more convincing and statistically significant result. Furthermore, prior studies that investigated biosolids exposure would have used different sheep (genetic effects) and biosolid EC composition and grazing patterns which may influence the results (Bellingham et al., 2012).

The study reported has shown similarities in AMH immunoexpression within Sertoli cells to studies conducted by Lea et al., 2022, Paul et al., 2005 and Bellingham et al., 2012, where lambs were exposed continuously throughout gestation to biosolids. However, there are stark differences when the exposure period is not prolonged throughout gestation. It has been hypothesised that continuous exposure could initiate compensatory mechanisms during fetal testicular development to recover Sertoli cell numbers and restore the normal testicular phenotype (Elcombe et al., 2021; Lea et al., 2022). In fetal and early postnatal life, Sertoli cells proliferate intensely and in rams, this period of “compensation” could extend to four months postnatal (Rebourcet et al., 2017; Bellingham et al., 2012). Transient exposure may not give the fetal testis sufficient time to adapt which might explain the variation in Sertoli cell numbers between continuous and periodical EC exposure (Lea et al., 2022).

2.4.2 CYP17A1 marker for Leydig cells

A key functional marker of the testis are the steroidogenic Leydig cells. CYP11A1 and CYP17A1 are microsomal enzymes that catalyse reactions in the gonadal steroid pathway to synthesise testosterone (Midzak et al., 2009). The steroid has a critical role in sexual

differentiation (testicular descent) and producing male sex characteristics (prostate and external genitalia) (Nassar and Leslie, 2018). Paul et al, (2005) had previously reported a significant reduction in the number of Leydig cells (biosolids exposure Day 110), however no change in P450scc (CYP11A1) immunohistochemical expression was shown (Paul et al., 2005). In contrast, Lea et al, (2022) showed less CYP11A1 staining in fetuses exposed continuously throughout gestation (Lea et al., 2022). Furthermore, this study also identified a reduction in CYP17A1 testicular area stained when exposure was limited to early, mid or late gestation: 0-80,30-110,60-140 days (Lea et al., 2022).

The current study does not show similar findings in post-natal animals exposed in utero. That is, there was no difference in immunoexpression of CYP17A1 in 8-week prepubertal offspring between Control and Biosolids lambs. In the eight-week period post parturition, lambs were grazed on non-treated pastures. As hypothesised before, a compensatory mechanism could have been implemented throughout gestation and the relief period after birth from ECs could reflect a recovery phase for the Leydig cell population. Therefore, differences in CYP17A1 may not have been detected. However, in this study the proportion of area stained was measured as opposed to numerical nuclei count so it is difficult to accurately evaluate Leydig cell populations, but we can give an estimate based upon enzymes associated with these cells. In future studies it would be of interest to count Leydig cell nuclei and measure serum levels of testosterone.

2.4.3 PCNA marker for proliferation in tubule and interstitial compartments

A reduction in cell numbers could reflect an increase in cell death and/or a reduction in proliferation. The current study utilised a commonly used marker of proliferation, PCNA, to assess the extent of proliferation. Sheep exhibit two periods of intense Sertoli cell proliferative activity; first after sexual differentiation, followed by a reduction between Day 110 gestation and birth and secondly, after birth proliferation is increased again (Hochereau-de Reviers et al., 1995). Sertoli cell proliferation (Ki67 marker) in Day 110 biosolids exposed male fetuses did not indicate any differences in area stained between treatments (Paul et al., 2005).

In the current study, PCNA immunopositive staining was analysed in the tubule and interstitial compartments of the prepubertal testes to measure Sertoli and Leydig cell proliferation. No differences were identified in immunopositive staining for either tubule or interstitial areas. This is interesting as we previously hypothesised that fetuses continuously exposed in utero may undergo compensatory mechanisms to recover Sertoli and Leydig cell populations. Therefore, once EC exposure is removed, proliferation of these cell types might increase as a catch up mechanism. On the contrary, the results of this study do not appear to support this theory. However, the testes were collected at one point in time and therefore represent a "snap-shot". Consequently, earlier gestational changes in proliferative activity would be missed. As mentioned before, proliferation increases immediately after birth as seen in Sertoli cells (Hochereau-de Reviers et al., 1995). The testes were collected at 8-weeks post parturition, so differences in proliferation if any, were more likely to manifest earlier. Furthermore, in this study PCNA was used as the proliferative marker, however PCNA has previously been criticised for showing less sensitivity in immunological detection when compared to other markers such as Ki67 (Bologna-Molina et al., 2013). The half-life in PCNA is long therefore cells that have recently left the cell cycle will still be immunologically detectable when immunostaining with PCNA (Hall et al., 1990).

Intriguingly, in this study age and sire showed a significant effect (P= 0.043 and P=0.017 respectively) in the proportion of immunopositive staining within the tubules. It is unclear as to why only proliferation within the tubule was affected by these factors. In future work, it would be of interest to use Ki67 as a proliferative marker and to investigate proliferation at different developmental periods, specifically during early post parturition.

2.4.4 Conclusions

Data presented here have not shown any treatment effects of in utero exposure on 8-week post-natal testes using immunostaining for markers of Sertoli, Leydig and proliferating cells.

Although no effects were shown in the current study, when other studies have included numerical evaluation of cells, there are noticeable differences in numbers of Sertoli and Leydig cells between Control and Biosolids testes. It is clear that the timing of exposure is a key factor during fetal testis development. The continuous exposure may have generated compensatory effects to restore normal testis function and therefore at 8-weeks post parturition, it is less likely differences would be seen. Other studies that have incorporated periodic exposure have established effects on Sertoli and Leydig cell populations. As mentioned, further work needs to be conducted which evaluates multiple exposure periods, including early post parturition, to provide more information on testicular cellular activity and potential compensatory mechanisms.

Chapter 3. Evaluation of iSperm Methodology and Comparison to IVOS II for Assessment of Ram Semen

3.1 Introduction

Evaluation of sperm motility and kinematic parameters provides a valuable insight into the energy status of mammalian sperm and imparts information of the fertilization ability of the animal (Palacín et al., 2013). Over the past four decades, semen evaluation techniques have evolved especially within animal andrology laboratories (Amann and Waberski, 2014). CASA platforms were developed to critically analyse sperm motion characteristics in a precise and timely manner (Contri et al., 2010). However, they are expensive and generally non-portable (Moraes et al., 2019). Cell counts conducted with a Neubauer chamber are considered the gold standard, although this method is laborious and time consuming, and subject to human error (Piccinini et al., 2014).

The iSperm (Aidmics biotechnology, Taipei, Taiwan) is a recent CASA platform in the form of a smartphone-based semen analyser that presents an alternative method for in-field semen analysis (Moraes et al., 2019). The affordable, portable device has algorithms optimised to measure concentration and motility of spermatozoa in several domestic species (Dini et al., 2019) including porcine (Matsuura et al., 2017), canine (Bulkeley et al.,2021) and equine (Moraes et al., 2019). The system has not formally been evaluated in sheep.

The iSperm is composed of an iPad mini (Apple Inc., San Jose, CA) that has sperm motility analysis software installed, an optical lens for sperm visualisation, attachable heater for temperature control, sample collector, disposable cover and base chips and measuring cups (Fig. 3.1A). There are three sampling methods (Dropper, Dipper and Pipette) that can be used for in-field semen analysis (Fig. 3.1B, 3.1C, 3.1D). The Dipper was the original sampling method (Moraes et al., 2019) and involved dipping a sample collector fitted with a base chip, into a diluted semen sample that is thoroughly mixed in a measuring cup. A cover chip is inserted over the base chip and attached to the warmed sampling chamber on the iSperm

case. The manufacturer introduced the Dropper and Pipette methods after issues arose with the Dipper method regarding improper mixing (Moraes et al., 2019). The Dropper and Pipette methods pipette a set volume of sample onto the cover chip (Dropper) or base chip (Pipette), and then proceed with sample collector attachment to the iSperm case.

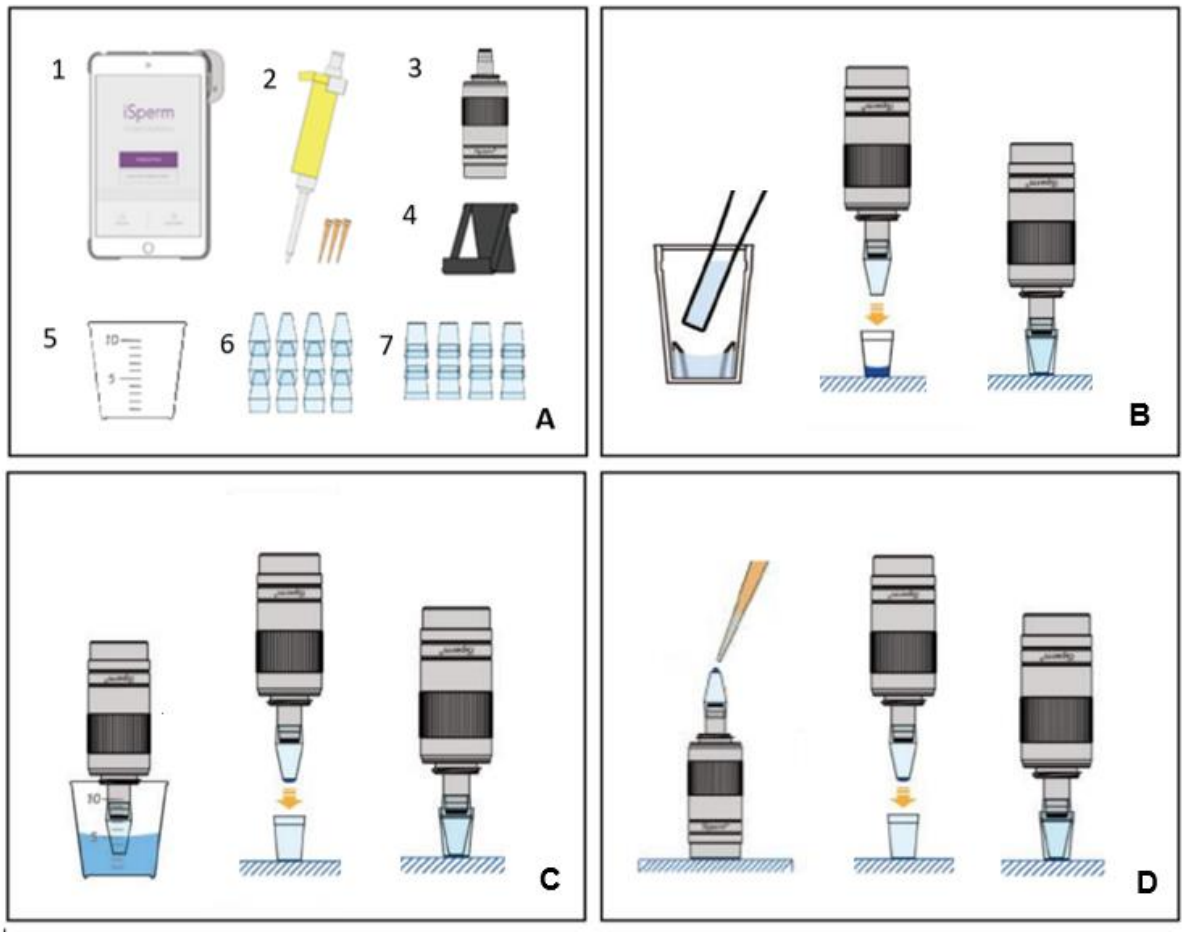

Figure 3.1: The iSperm product components and sampling methods. A) 1- iSperm software downloaded onto an iPad mini with a specialised case, heater and lens attached. 2- Pipette and tips. 3- Sample collector. 4 Adjustable stands. 5- Measuring cup. 6- Base chips. 7- Cover chips. B) Diagram of the Dropper method showing cover chip filling and sample collector attachment. C) Diagram of the Dipper method showing submersion of base chip and attachment to sample collector. D) Diagram of the Pipette method showing base chip coverage and sample collector attachment. Figure adapted from Aidmics Biotechnology, (2020).

The IVOS II (Integrated Visual Optical System), (Hamilton Thorne Ltd, Massachusetts, USA) is a laboratory based CASA platform with optimised semen analysis features for a diverse range of species including but not limited to: avian (Gloria et al., 2019; Lenicky et al., 2019; Mussa et al., 2021), bovine (Bastos et al., 2021; Duracka et al., 2021), equine (Serafini et al., 2019; Cavalero et al., 2019), ovine (Bernecic et al., 2021; Rickard et al., 2014) and porcine

(Santos et al., 2021; Yeste et al., 2018). This system is comprised of specialist features such as integrated phase contrast optics, stroboscopic illumination system, video camera, automated internal heated specimen stage and software optimised for tail detection, adaptive smoothing and smart tracking (Mortimer et al., 2015; Hamilton Thorne, 2020)

The study aimed to evaluate the iSperm for repeatability and accuracy in ram semen analysis. The three iSperm sampling methodologies were assessed initially to determine a suitable standardised method to take forth into the sequential experiments. To validate the iSperm for ram semen analysis, a comparison was made to the IVOS II. Sperm motility and kinematic parameters values were compared between the systems and statistical methods determined the influence of system on these parameters. Within the CASA comparison, the effect of diluent type on sperm motility and kinematic values was also evaluated.

3.2 Materials and methods

3.2.1 Animals, Semen Collection and Cryopreservation

Semen for the two experiments described in this chapter were provided by AB Europe (East Mains, Ormiston, East Lothian, UK: https://www.abreeds.co.uk/). Semen was collected with use of an artificial vagina from six sexually mature Texel rams from the AB Europe portfolio and cryopreserved according to the protocol of Evans et al. (1987). Briefly, the extender for cryopreservation consisted of a mixture of egg yolk (10 mL), IMV diluent (8 mL) and 32 mL distilled water. Warmed (30°C) diluent was added to semen to provide a dilution of approximately 1 in 5 in 15 mL Falcon tubes held in a beaker containing 200 mL water at 30°C. This was placed in a refrigerator at 4°C and cooled for approximately 3 h. Aliquots of 0.25 mL diluted semen were dispensed onto a block of dry ice containing appropriately sized indentations and allowed to freeze (several minutes), before dispensing into liquid N.

3.2.2 Thawing Sperm Pellet

Test tubes were warmed to 37°C in a water bath before being suspended in a beaker filled with water at 37°C. Pellets were stored in a liquid nitrogen Dewar and one pellet was selected then transferred to the pre-warmed glass test tube. A record of time was made then the test tube was agitated in the beaker for one to two minutes before being placed in the water bath.

3.2.3 Haemocytometer Sperm Count

Eppendorfs were warmed in a 37°C heating block and 6 mL INRA 96 extender (IMV Technologies, France) was warmed in a 37°C water bath. Working at an initial 1:50 dilution, 20 µL of sperm was transferred to an Eppendorf with 980 µl of extender and homogenised. An immotile sample at a 1:200 dilution sperm was made using 20 µL of sperm-extender solution and 60 µL of water. The immotile sperm sample was homogenised then 10 µL was pipetted into both ends of a Neubauer counting chamber slide (Weber Scientific, England) and viewed under a microscope (Leica, Wetzlar, Germany) at x40 magnification. Figure 3.2 illustrates the Neubauer counting grid and the red overlay indicates the regions where sperm

counting was undertaken. The counts were compared to test consistency between samples and an average was made when both samples had values within a 10-point range of each other. Values outside of this range indicated poor sample mixing and the protocol was repeated until the optimised range was met. The average sperm count was calculated using the total sperm counted within these five regions for both samples and divided by two.

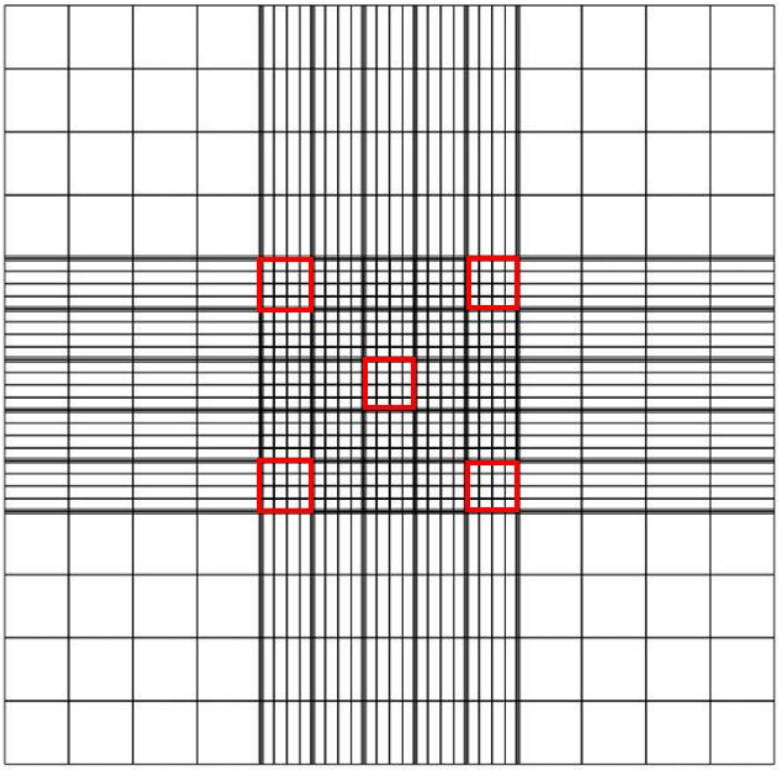

Figure 3.2: Neubauer chamber grid with five red squares overlayed to indicate where sperm were counted. Each red square contains 16 squares in total. An average count was calculated by the total number of sperm in the five red squares for both samples then divided by two. Figure adapted from Barbedo (2013).

3.2.4 Experiment 1: Comparison of Three Recommended Methods of Semen Presentation for iSperm Analyses (i.e., Dropper, Dipper and Pipette Methods)

3.2.4.1 Experimental Design and Procedures

Three pellets from each of three rams (anonymously identified as A, B and C) were analysed in triplicate to compare the three iSperm methods (i.e., Dropper, Dipper and Pipette).

The following parameters measured by the iSperm in this study were defined by Aidmics Biotechnology, (2020) and Sloter., et al. (2006). Motility is the percentage of sperm with VAP≥ 20 µm/s and VSL≥ 0 µm/s. Progressive Motility is the percentage of sperm with STR ≥ 70% and VAP ≥ 50 µm/s. VCL is the average velocity measured across the total point to point distance followed by the sperm. VAP is the average velocity of a sperm head along a smoothed trajectory path. VSL is the average velocity of a sperm measured in a straight line between its first and last track point. LIN measures the straightness of curvilinear trajectory calculated as VSL/VCL x 100.STR measures the straightness of the average path calculated as VSL/VAP x 100.

A working concentration of 30 million sperm/mL and average sperm count determined the dilution factor and volume of INRA 96 to use.

The iSperm was calibrated and a heater was attached around the lens prior to the experiment. Measuring cups, cover chips and base chips were warmed to 37°C in a heating block. The calculated volume of INRA 96 was pipetted into an Eppendorf and homogenised with 20 µL of sperm.

The homogenised sample was divided between the three methods. Dropper method pipetted 80 µL into a cover chip, pipette method pipetted 7 µL onto a base chip and dipper method pipetted the remaining sample into the measuring cup, then a base chip attached to the sample collector was dipped in the homogenised sample. The sample collector was attached onto the iSperm lens port. Five measurements were taken at minute intervals over a five-

minute period to give a total of five measurements. The order of methods is as followed: Dropper, Pipette then Dipper.

3.2.4.2 Experiment 2: System and Diluent Comparison on Sperm Motility Parameters

One pellet from each of the three remaining rams (from the group used in experiment 1: anonymously identified as C,D and E) was analysed by both the iSperm and IVOS II (Hamilton Thorne Ltd, Massachusetts, USA) in each of three diluents: unfiltered INRA, filtered INRA and PBS. The process involved thawing of a single pellet from each ram then counting sperm using a Neubauer chamber as stated in Section 3.2.3. A final concentration of 30 million sperm/mL was formulated using each of the diluents. The filtered INRA was prepared using a 0.22 µm syringe filter to remove fat globules from the milk-based diluent (Sartorius Stedmim Biotech, Germany).

For the IVOS system, 7 µL diluted sample was loaded into a prewarmed (37°C) counting chamber (20 µm depth, 2 chamber slide) and a total of 30 frames captured at 60 frames per second. This was repeated three times per sample. The IVOS objective was 10x negative phase; analysis software was Animal motility 11 and programme parameters were: sperm head area = 5 to 50 μm^2, sperm elongation= 23 to 100% and minimum sperm counted= 200. The software cut off values were as follows: VAP threshold for motility, ≥ 20 µm/S; VSL threshold for motility, ≥ 30 µm/S; VAP threshold for progressive motility, ≥ 50 µm/S and STR threshold for progressive motility, ≥ 80%.

For the iSperm system, the pipette method was used (see Section 3.2.4.2) and three measurements were recorded over a three-minute period. Settings for the iSperm system were as follows: frames acquired, 210; frame rate, 30 Hz; VAP threshold for motility, ≥ 20 µm/S; VSL threshold for motility, ≥ 0 µm/S; VAP threshold for progressive motility, ≥ 50 µm/S and STR threshold for progressive motility, ≥ 70%.

3.2.5 Statistical Analysis

Data were analysed by ANOVA (GenStat 21st Edition, 2020, VSN International Ltd, Hemel Hempstead, UK). For Experiment 1, analyses were based on three pellets from each of three rams. These terms (i.e., 'Ram' and 'Pellet' nested with Ram) were incorporated as blocks within the models which compared the three 'Methods' of semen preparation (i.e., Dropper, Dipper and Pipette) and time of image capture/analyses ('Time sequence'), together with interactions between these terms. Time sequence was of secondary importance in these analyses and therefore not treated as a repeated measure. Experiment 2 also involved analyses of semen from three rams, but single pellets per ram on this occasion. This experiment was arranged as a two ('System'; iSperm vs IVOS) by three ('Diluent'; INRA vs INRA-F vs PBS) factorial and analysed as such by ANOVA; with interactions between these two factors (i.e., 'System' and 'Diluent') forming an additional term in the model; which was also blocked by 'Ram'. In both experiments, residual plots confirmed normality and homogeneity of variances. Data are presented as means with SEDs.

3.3 Results

3.3.1 Experiment 1: Comparison of the Three iSperm Methods

The mean sperm concentration across all three methods gave similar values ranging from 30.68 to 32.62 x10^6 spermatozoa/mL (Table 3.1). This was expected due to the experimental design that established a set concentration to dilute semen to. However, there was a marginal but significant (P<0.001) decline in sperm concentration from minute one to five.

Percentage motility and progressively motile sperm were greater (P<0.001) for the Dropper than the other two methods (Table 3.1). Percentage motile sperm was similar for the first three readings but declined (P=0.016) at the 4th and 5th readings at around the 4-to-5-minute mark. With respect to sperm velocity parameters (i.e., VCL, VAP, VSL) readings for the Dropper method were lower (P=0.01) for percentage VAP and for percentage VSL compared to the other two methods (Table 3.1). No significant differences were observed between methods for VCL. Readings across each of the five time points were similar for all three sperm velocity parameters.

Values for motility pattern parameters (i.e., STR and LIN) were lower (P<0.001) for the Dropper than the other two methods, which were similar. Readings across each of the five time points were similar for all three sperm velocity parameters.

Table 3.1 Sperm parameter values for the three iSperm methods (i.e., Dropper, Pipette and Dipper) and minute interval-time sequences for readings

	Method (M)				Time-sequence (TS)						Significance (P)		
Parameter	**Dropper**	**Pipette**	**Dipper**	**SED**	**1**	**2**	**3**	**4**	**5**	**SED**	**M**	**TS**	**M x TS**
Replicate	3	3	3		3	3	3	3	3				
Concentration, (m/mL)	32.62	30.68	32.06	1.489	33.25^{x}	31.38^{y}	31.65^{y}	31.46^{y}	31.21^{y}	0.521	-	<0.001	-
Motility, %	36.5^{a}	20.4^{b}	20.7^{b}	2.01	27.8^{x}	$26.1^{x,y}$	$25.7^{x,y}$	25.2^{y}	24.5^{y}	0.99	<0.001	0.016	-
Progressive motility, %	23.4^{a}	15.2^{b}	16.1^{b}	1.65	19.4	18.4	18.1	18.2	17.1	0.85	<0.001	-	-
VCL, (µm. S^{-1})	135.0	130.9	128.2	3.23	132.5	131.4	131.3	132.2	129.4	4.90	-	-	-
VAP, (µm. S^{-1})	89.9^{a}	95.5^{b}	95.0^{b}	2.09	94.1	94.5	93.8	93.9	92.4	1.81	0.010	-	-
VSL, (µm. S^{-1})	70.9^{a}	82.2^{b}	84.9^{b}	2.05	79.5	79.7	79.8	80.0	77.5	1.89	<0.001	-	-
STR, %	76.4^{a}	83.1^{b}	84.9^{b}	1.12	81.1	81.4	82.1	82.3	80.3	1.00	<0.001	-	-
LIN, %	53.2^{a}	62.0^{b}	65.2^{b}	1.27	59.5	60.9	60.6	60.6	58.9	1.11	<0.001	-	-

Superscripts (a,b) denote differences between 'Method' means at P<0.05; Superscripts (x,y) denote differences between 'Time-sequence' means at P<0.05

Curvilinear velocity (VCL) (µm/S), average path velocity (VAP) (µm/S), straight line velocity (VSL) (µm/S), straightness (STR) (%) and linearity (LIN) (%)

3.3.2 Experiment 2: CASA System and Diluent Comparisons on Sperm Motility Assessments

Sperm concentration was uniform for both CASA systems and diluents due to the preestablished dilution factor used for each ram. Mean sperm concentration values ranged from 37.5 to 46.5 $x10^6$/mL and 32.4 to 37.3 $x10^6$/mL for the iSperm and IVOS II respectively.

Percentage motile sperm differed (P<0.001) between the two systems (33.1% vs 15.8% for IVOS II and iSperm respectively; SED = 1.96). However, the interaction (P = 0.01) with diluent (Table 3.2) indicated that readings were dependent on the diluent used, and that these readings were not consistent between the two systems. A broadly similar pattern was observed for the percentage progressive motile sperm. Values were higher (P = 0.005) on average for the IVOS compared to the iSperm system (15.3% vs 10.9%; SED = 1.24) but, as for percentage motile sperm, the interaction (P = 0.003) with diluent indicated that these values were sensitive to method of sample preparation (Table 3.2). With respect to the three sperm velocity parameters (i.e., VCL, VAP, VSL), readings for the former two were greater for the IVOS compared to the iSperm system (VCL, 202.0 vs 123.3 $\mu m.S^{-1}$ [SED = 8.99; P < 0.001]; VAP, 107.7 vs 91.9 $\mu m.S^{-1}$ [SED = 4.94; P = 0.01]), whereas VSL readings did not differ between the two systems (82.1 vs 79.4 $\mu m.S^{-1}$ [SED = 4.38]). In contrast, readings for motility pattern parameters (i.e., STR and LIN) were lower for the IVOS compared to the iSperm system (STR, 75.2 vs 81.7% [SED = 1.87; P = 0.006]; LIN, 43.2 vs 63.1% [SED = 2.52; P < 0.001]). However, as for motility, there was evidence that these readings differed between diluents (Table 3.2).

Table 3.2 System and diluent comparison of sperm concentration and motility parameters

System (S)	**IVOS**			**iSperm**				**Significance (P)**		
Diluent (D)	**INRA**	**INRA-F**	**PBS**	**INRA**	**INRA-F**	**PBS**	**SED**	**S**	**D**	**SxD**
Replicate	3	3	3	3	3	3				
Concentration (m/mL)	32.4	34.5	37.3	46.5	39.7	37.5	6.08	0.095	-	-
Motility, %	40.0^{a}	30.5^{b}	28.9^{b}	12.9^{c}	22.2^{b}	12.2^{c}	3.40	<0.001	0.056	0.01
Progressive motility, %	14.7^{a}	11.6^{a}	19.5^{b}	9.1^{c}	14.9^{a}	8.8^{c}	2.14	0.005	-	0.003
VCL (µm. S^{-1})	211^{a}	212^{a}	183^{a}	119^{b}	124^{b}	127^{b}	15.6	<0.001	-	-
VAP (µm. S^{-1})	107^{a}	106^{a}	109^{a}	89^{b}	90^{b}	97^{ab}	8.6	0.01	-	-
VSL (µm. S^{-1})	76	76	94	79	75	84	7.6	-	0.067	-
STR, %	72.2^{a}	69.9^{a}	83.4^{b}	84.6^{b}	78.4^{b}	82.1^{b}	3.23	0.006	0.012	0.036
LIN, %	40.5^{a}	36.8^{a}	52.3^{b}	65.1^{c}	58.9^{bc}	65.4^{c}	4.36	<0.001	0.016	-

For each parameter, values with a different superscript are different at P<0.05. Curvilinear velocity (VCL) (µm/S), average path velocity (VAP) (µm/S), straight line velocity (VSL) (µm/S), straightness (STR) (%) and linearity (LIN) (%)

3.4. Discussion

In this study, two novel findings emerged from the evaluation of the iSperm for ram semen analysis. Firstly, it was shown that there was significant variation of sperm motility and kinematic parameters depending upon the iSperm method used. The Dipper and Pipette methods were in agreeance for all parameter values and the Dropper method readings significantly deviated when compared to the Dipper or Pipette. Secondly, the most important novel finding of this study emerged from the first formal comparison of the iSperm to the IVOS II, a validated CASA system. The data showed no agreement between the systems across all sperm motility and kinematic values aside from VSL.

3.4.1 Comparison of the Three iSperm Sample Methods

The iSperm system has three dispensing methods available (i.e., Dropper, Dipper and Pipette). It was, therefore, important to compare these methods to establish a standardised method that could be used is subsequent experiments.

In this study the 'Dropper' method consistently gave the highest values for both percentage motility and percentage progressive motility. It is hypothesised that a swim up effect could be responsible for the disparity in motility readings between this and the other two methods. The dropper method used 80 µL of sample, more than ten times that used for either the 'Dipper' or 'Pipette' methods. With more medium, this could provide an opportunity for motile sperm to aggregate towards the surface and adhere to the base chip upon attachment (Amman and Waberski, 2014). However, the period between sample application and chip attachment was a matter of seconds. Arguably this is not long enough to incur separation of sperm, although it is a factor that should be considered. Also, during chip attachment, excess media was dispersed around the sides of the chip, out of the field of capture therefore a high proportion of the sample was not analysed which could have influenced the results.

Regarding the 'Dipper' and 'Pipette' methods, percentage motility was statistically similar. For these methods, sample coverage was restricted to the area of the base chip, which minimised

the total volume of medium and limited excess dispersal. Therefore, it could be inferred that the medium volume was likely to be the variable that resulted in these similarities. Sperm motion kinetic parameters (i.e., VCL, VSL, VAP, LIN, STR) were evaluated, and results were consistent for the ‘Dipper’ and ‘Pipette’ methods; which were both higher than for the 'Dropper’ method. Once again, the volume of medium could be the factor that influenced these findings, as hypothesised before, by affecting the tracking ability of the software. Moreas et al. (2019) have previously criticised the Equine iSperm application with regard to velocity measurements stating iSperm velocity values were significantly different to the comparative CASA system (CEROS II) and recommended algorithms for sperm tracking to be improved.

An additional factor that was evaluated was ‘time sequence’ of readings; i.e., five sequential readings each around 1 minute apart. In this study it was found that the first reading gave a percentage motility value that was higher than the 4 to 5 minute-values. At the time of this experiment, neither the iSperm User Manual nor website provided a guide on sample repetition or frequency (Aidmics Biotechnology, 2020; Aidmics Biotechnology, 2022). It is now known that samples should not be tested multiple times and require to be analysed within the first minute to avoid semen aggregation caused by the small space between the chips (Aidmics Biotechnology, 2017).

To the best of our knowledge, there are no studies that have evaluated all three iSperm dispensing methods, therefore the current study represents the first formal comparison. The ‘Dipper’ and ‘Pipette' methods have previously been compared separately to other CASA systems (canine semen evaluated by SpermVision SAR (Bulkeley et al.,2019), equine semen evaluated by Androvision (Dini et al., 2019) and CEROS II (Moraes et al., 2019). With respect to motility, both methods agreed with the other CASA systems and the authors concluded that the iSperm was a suitable alternative software to measure motility (Moraes et al., 2019; Dini et al., 2019; Bulkeley et al., 2021). From the current study, on account of statistically similar results for motility, progressive motility, VAP, VSL, STR and LIN, it would be acceptable to use either the ‘Dipper’ or ‘Pipette’ methods. However, because the 'Pipette’ method uses a

controlled volume of sample, in the second experiment in this chapter it was selected as the most suitable method to take forward.

3.4.2 System and Diluent Comparisons on Sperm Motility Parameters

The recent addition of an ovine 'module' to the iSperm application offered an opportunity to evaluate this system for use in sheep. Previous studies have evaluated the iSperm for use in a variety of other domestic species.

In this study the effect of system and diluent were considered in terms of sperm motility parameters. Across all parameters, except for VSL, there were significant differences in readings between the two systems, with the IVOS II consistently providing higher values for motility, progressive motility, VCL and VAP. One reason for the motility disparity could be due to the difference in measurement ranges between the systems. O'Meara et al., (2022) evaluated IVOS II parameter ranges for bovine semen analysis and adjusted the settings to low (STR ≥ 20%, VAP ≥ 20μm/S), medium (STR ≥ 40%, VAP ≥ 60μm/S) and high (STR ≥ 80%, VAP ≥ 120μm/S). These authors concluded that the higher cut off values for STR and/or VAP resulted in a proportion of progressive motile cells being classed as motile and not progressive (O'Meara et al., 2022). The higher motility values for the IVOS II (INRA and PBS) could be the result of a larger STR cut off value when compared to the iSperm. In support of this, a study that evaluated the iSperm equine application identified a stronger linear relationship for motility when parameters were synchronised (Dini et al., 2019). Another aspect considered for the motility differences was the use of a planar capillary chamber in the IVOS II. Previous literature has shown that fluid dispersed by capillary conditions will be under the influence of Laminar Poiseuille flow (Del Gallego et al., 2017). Del Gallego et al., (2017) showed that sperm motility differentiated greatly and was dependent upon the microscopic field with exaggerated readings shown at the outermost fields (Del Gallego et al., 2017). Particulates suspended in this fluid will be exposed to a velocity gradient and will gather at the leading edges (Kruster, 2005). However, it is unlikely that the IVOS II motility results were due to this effect because the readings were captured along a central plane.

Frame rate is also a factor to consider for both motility and kinematic parameters. Bompart et al. (2018) correlated increased velocity parameters with the increase in frame rate. The higher frame rate of the IVOS II would provide more information to reconstruct sperm trajectory pathways with greater accuracy (Rijsselaere et al., 2003). In comparison, the lower frame rate of the iSperm could risk the loss of trajectory information, in particular higher velocity sperm (Rijsselaere et al., 2003). The iSperm gave significantly lower VCL values which could be a consequence of the limited frames analysed per second.

Previous studies indicated that fat globules could obstruct microscopic sperm evaluation (Pickett and Amman, 1987). Therefore, it was hypothesised that unfiltered INRA, a milk-based extender, could result in the misidentification of sperm and lead to inaccurate results. In this study, there was limited effect on sperm motility, progressive motility, VCL, VAP and VSL with the exception of LIN and STR. There was non-significant trend between motility values (P=0.056). Although the diluent alone did not influence motility or progressive motility, when system was factored in alongside the diluent a significant difference was present (P=0.01 and 0.003 respectively). The filtered INRA diluent, with respect to motility and progressive motility, gave statistically similar values. Therefore, in future studies that involve both systems it is recommended to use filtered INRA.

3.4.3. Conclusions

Until now, the iSperm has not been compared to the IVOS II CASA system for ram semen. The current data indicate that the iSperm system does not agree with a validated CASA system for all sperm motility and velocity measurements. Interestingly, and in contrast to the results from this study, other studies that have evaluated the iSperm system, using semen from other species, arrived at the conclusion that the device is suitable for motility measurements and that the iSperm does not deviate significantly from the comparative CASA systems (canine semen evaluated by SpermVision SAR (Bulkeley et al.,2019), equine semen evaluated by Androvision (Dini et al., 2019) and CEROS II (Moraes et al., 2019). Moraes et al. (2019) did, however, recommend that the iSperm should not be used in research due to

the inconsistency in velocity parameter measurements when compared to CASA which is consistent with the present study (Moraes et al., 2019). The differences between systems regarding algorithms, software parameter cut offs, frame acquisition rates, video capture length, camera resolution quality and microscopic optics are all factors that could limit the effectiveness of iSperm.

Chapter 4. iSperm evaluation of fresh and frozen-thawed semen from F1 rams exposed *in utero* to environmental chemicals from biosolids-treated pastures.

4.1 Introduction

It has been established in global studies that human male fertility has decreased, and there is a decline in semen quality (Dissanayake et al., 2019). EC exposure has, at least in part, been attributed to this decline and prompted investigations into the effects of EC exposure in various species across different developmental periods including gestation and into adulthood (canine, (Lea et al., 2016b), human (Jurewicz et al., 2009; Li et al., 2011), mouse (Rahman et al., 2017), rat (Aly, 2013), sheep (Bellingham et al., 2012). The need to understand the biological impacts of these chemicals led to the development of the Sheep-Biosolids Model (Chapter1, Section 1.2). The model aims to approximate human exposure to complex chemical mixtures providing an insight into the potential consequences of continuous low dose exposure on reproduction and fertility.

In Chapter 2, the developmental effects of *in utero* exposure to biosolids on aspects of testis development were explored. Sections of prepubescent F1 ram testicles were stained for AMH, CYP17 and PCNA to identify histological changes. The detection of these markers can inform on the extent of Sertoli and Leydig cell populations and degree of cell proliferation. The density of the cell populations and extent of cell proliferation can directly impact upon spermatogenic capacity in adulthood (Bellingham et al., 2012).

In Chapter 3, a new CASA platform, the iSperm, was evaluated as an alternative device to use for in-field semen analysis. In these scenarios sperm concentration and motility are often evaluated by subjective methods such as simple visual inspection (Moraes et al., 2019). These methods are laborious and results can vary from person to person depending on experience (Amann and Waberski, 2014). Laboratory-based CASA platforms can more accurately determine sperm concentration and sperm motility parameters but are expensive and non-

portable (Moraes et al., 2019). An affordable, portable CASA device like the iSperm may offer an alternative method to analyse semen in the field.

This study built upon those reported in Chapters 2 and 3. The study used the iSperm system to analyse fresh and cryopreserved semen from F1 sexually mature rams that had been exposed *in utero* to a complex mixture of chemicals arising from biosolids-treated pastures on which their mothers grazed.

4.2 Materials and methods

4.2.1 Animals, semen collection and cryopreservation

Semen used in these experiments were collected from an ongoing NIH-funded study at the University of Glasgow (Cochno farm & Research Centre) in collaboration with the Universities of Michigan (USA) and Nottingham. The treatment protocol used to prepare Control and Biosolids pastures and the period of EC exposure are each described in Section 2.2.1. Semen was collected from sixteen sexually-mature 'Easycare' rams of approximately 18 month of age using an artificial vagina. Semen cryopreservation followed the protocol of Evans et al. (1987) summarised in Chapter 3 (Section 3.2.1).

4.2.2 Experiment 1: Motility and velocity parameters of fresh ram semen from Control and Biosolids-exposed F1 rams using the iSperm

Experimental work described here was conducted by our group prior to the validation of the iSperm methodologies. Two 50ul aliquots were collected per ejaculate and diluted initially 1:100 with water (aliquot 1) or 1:100 INRA-96 (aliquot 2). The former was used to kill/immobilise the sperm which were then counted using the iSperm. The second aliquot was maintained at 37 °C before being assessed for motility, progressive motility and other motility parameters (VCL, VAP, VSL, STR, LIN) using the Dropper method, described in 3.2.4.2. A further 1:10 dilution was needed for some samples giving a final dilution of 1:1000.

4.2.3 Experiment 2: Motility and velocity parameters of frozen-thawed ram semen from Control and Biosolids exposed F1 rams using the iSperm

One pellet from each of the sixteen rams was analysed by the iSperm in triplicate to assess sperm quality. The procedure commenced by thawing a single pellet followed by a cell count using a Neubauer chamber, as described in Chapter 3 (Section 3.2.3), to generate a working concentration of 30 million sperm/mL for subsequent analyses. Previous work by our group used the iSperm Dropper method to analyse fresh semen. Therefore, it was appropriate to

include both the Dropper method and validated Pipette method to analyse each pellet as described previously (Chapter 3, Section 3.2.4.2).

4.2.4 Statistical analysis

Data were analysed by ANOVA (GenStat 21st Edition, 2020, VSN International Ltd, Hemel Hempstead, UK). For Experiment 1, analyses were based on single ejaculates from each of 16 F1 rams representing each of the two treatment groups. These 16 rams, in turn, were derived from four F0 rams who inseminated ewes on each of the two treatment groups. Consequently, 'F0 ram' served as a blocking factor in these analyses.

For Experiment 2, which assessed the quality of frozen-thawed semen from each of the 16 rams that donated fresh ejaculates for evaluation in Experiment 1, a comparison was also made between the Dropper and Pipette methods for the iSperm. This was undertaken to assist with interpretation of outputs in line with those of Experiment 1 (this chapter) and those of Chapter 3. F0 ram also formed a block in these analyses, and F1 Sire formed the stratum that permitted a comparison between the two dispensing methods for the iSperm (as these were undertaken for frozen-thawed semen from each of the 16 rams). The data was therefore analysed as a 2 x 2 factorial arrangement (i.e., Biosolids vs Control and Dropper vs Pipette; with interactions between these terms).

In both experiments, residual plots confirmed normality and homogeneity of variances. Data are presented as means with SEDs.

4.3 Results

4.3.1 Experiment 1: Motility and velocity parameters of fresh ram semen from Control and Biosolids-exposed F1 rams using the iSperm

There was no significant difference in sperm concentration between the Control and Biosolids groups and the mean values were 54.1 m/mL and 80.8 m/mL respectively (Table 4.1). However, percentage motility differed (P=0.006) between the Control and Biosolids treatment groups (56.6% vs 75.1% for Control and Biosolids respectively: SED = 5.40). With regard to progressive motility, there was no significant difference between the two groups. Data on sperm velocity and motion pattern parameters also did not differ between the two experimental groups.

4.3.2 Experiment 2: Motility and velocity parameters of frozen-thawed ram semen from Control and Biosolids exposed F1 rams using the iSperm

Mean sperm concentration did not differ between the two treatment groups or method of analyses; values ranged from 29.9 m/mL to 32.1 m/mL (Table 4.2). Percentage motile sperm did not differ between Control and Biosolids groups. However, motility differed (P<0.001) between the Dropper and Pipette Methods (Mean Dropper = 11.53% and Pipette = 4.35%SED = 1.731). A broadly similar pattern was observed for the percentage progressive motile sperm. Values were higher (P<0.001) for the Dropper compared to the Pipette (Mean Dropper = 9.03 % and Pipette = 2.95%, SED = 1.458. No difference was observed for percentage progressive motility between the Control and Biosolids groups.

With respect to sperm velocity and motion pattern parameters, there was no difference between Control and Biosolids. In contrast, there were differences between the Dropper and Pipette methods for all velocity and motion pattern parameters (Table 4.2). The three sperm velocity parameters (i.e., VCL, VAP, VSL), were greater (P = 0.003) for the Dropper method than the Pipette (VCL, Control 110 vs 96.5 $\mu m.S^{-1}$, Biosolids 111.1 vs 88.1 $\mu m.S^{-1}$ [SED 9.33; P=0.002]; VAP, Control 80.8 vs 71.8 $\mu m.S^{-1}$, Biosolids 83.8 vs 64.6 $\mu m.S^{-1}$ [SED = 6.23;

P=0.003]; VSL, Control 72.3 vs 63.3 µm.S^{-1}, Biosolids 73.7 vs 56.5 µm.S^{-1} [SED = 5.85]). Also, the sperm motion parameters (i.e., STR and LIN) were greater (P = 0.009) for the Dropper than the Pipette method.

Table 4.1 Treatment comparison of sperm concentration and motility parameters using the iSperm (Dropper method) in fresh semen collected from Control and Biosolids exposed (*in utero*) F1 rams.

Treatment (T)	**Control**	**Biosolids**		**Significance (P)**
Parameter			**SED**	
Number rams	8	8		
Mean Concentration (m/mL)	54.1	80.8	19.59	-
Motility, %	56.6	75.1	5.40	0.006
Progressive motility, %	48.1	63.0	7.03	0.057
VCL (µm. S^{-1})	150.1	151.2	9.45	-
VAP (µm. S^{-1})	128.7	132.4	8.78	-
VSL (µm. S^{-1})	120.0	120.7	10.02	-
STR, %	90.0	87.8	3.04	-
LIN, %	77.3	77.0	3.71	-

Table 4.2: Treatment comparison of sperm concentration and motility parameters using the iSperm dropper and pipette methods for cryopreserved semen collected from Control and Biosolids exposed (*in utero*) F1 rams.

Treatment (T)	**Control**		**Biosolids**			**Significance (P)**		
Method (M)	**Dropper**	**Pipette**	**Dropper**	**Pipette**	**SED**	**T**	**M**	**TxM**
Number rams	8	8	8	8				
Mean Concentration (m/mL)	29.94	32.12	31.62	30.66	1.499	-	-	-
Motility, %	10.51	4.23	12.55	4.47	3.020	-	<0.001	-
Progressive motility, %	8.51	2.90	9.55	3.00	2.480	-	<0.001	-
VCL (µm. S^{-1})	110.0	96.5	111.1	88.7	9.33	-	0.002	-
VAP (µm. S^{-1})	80.8	71.8	83.8	64.6	6.23	-	0.003	-
VSL (µm. S^{-1})	72.3	63.3	73.7	56.5	5.85	-	0.003	-
STR, %	81.8	74.3	81.8	66.5	4.81	-	0.005	-
LIN, %	62.4	58.6	63.9	50.2	3.60	-	0.009	-

4.4 Discussion

The current study evaluated fresh and frozen-thawed semen from F1 rams that were exposed *in utero* to a complex mixture of ECs originating from biosolids-treated pastures. In contrast to fresh semen, where an effect of Biosolids exposure was observed on sperm motility, no treatment effect was observed in frozen-thawed semen, where sperm motility was generally lower. In keeping with the results of Chapter 3, differences in readings for motility and other sperm parameters were observed between the two methods of sample deposition (i.e., Dropper vs Pipette).

4.4.1 Motility and velocity parameters for fresh ejaculates Control from F1 rams

In this study there was no difference in sperm concentration between the Control and Biosolids groups. Sperm concentration reflects the spermatogenic output of the testes and is governed by germ cell production of spermatogonia through the support of Sertoli cells (Nishimura and L'Hernault, 2017). Sertoli cells are core regulators of germ cell maintenance and define the population of germ cells present in the adult testes (Rebourcet et al., 2017). In a previous study by our group, Lea et a., (2022) concluded that Sertoli cell numbers of fetal male sheep were unaffected by continuous (140 days) *in utero* exposure to biosolids. The absence of effect to Sertoli cell populations supports the findings of the current study because a change in Sertoli cell numbers would be reflected in sperm concentration.

Sperm motility from F1 rams exposed *in utero* to ECs from biosolid-treated pastures was increased compared to that from F1 Control rams. This is interesting because it is inconsistent with previous studies (Species Mouse Rahman et al., 2017; Species Mouse Barakett et al., 2019; Species Canine Lea et al., 2016). F1 mice that were exposed *in utero* to the lowest observed adverse effect level BPA presented a decrease in sperm ATP regulatory proteins and decreased motility percentages (Rahman et al., 2017). Mice prenatally exposed to a mixture of phthalates at environmentally relevant levels (i.e., 20 µg/kg/day) produced sperm with reduced motility (Barakat et al., 2019). Furthermore, a study that analysed natural

exposure of ECs in dogs reported a decline in motility over a 26-year period (Lea et al., 2016b). On evaluation of the remaining sperm parameters, neither the percentage of progressively motile cells nor sperm kinematic parameters differed between the Control and Biosolids groups. Studies that involved low exposure doses of individual ECs in humans found negative effects between progressive motility and exposure chemical (Jager et al., 2013; Hernández-Ochoa et al., 2005). Li et al., (2021) conducted a human *in vitro* study to assess different doses of BPA exposure (0, 10^{-3}, 10^{-2}, 10^{-1}, 10, 10^{3} nM) on sperm quality. The authors concluded that although 0.1 nM BPA induced a decline in progressive motility, the value was within the range of the WHO criteria for normal sperm quality, therefore motility decline would not significantly affect fertility (Lie et al., 2021). Furthermore, in support of our study, the authors concluded that there was no effect of exposure on any of the kinematic parameters of sperm movement (Lie et al., 2021).

These studies are inconsistent with our findings which identified no effect of EC exposure on progressive motility but a significant increase in motility. In this study, fresh semen was analysed from rams that were exposed pre-conceptionally and throughout gestation to a complex mixture of ECs originating from biosolids. These factors should be taken into consideration as EC interactions and perturbation of reproductive cells and hormones varies between species, and is dependent on the exposure dose, the age exposure occurred, duration of exposure, type of EC and whether the exposure includes an individual or mixture of ECs (Cescon et al., 2020; Guo et al., 2000; Magnusson and Ljungvall, 2014; Birnbaum and Staskal, 2004).

4.4.2 Motility and velocity parameters of frozen-thawed semen from Control from F1 rams

The present study revealed no effect of Biosolids on sperm motility, progressive motility, or kinematic motion parameters in frozen-thawed semen. Apart from motility, this is in agreement with the readings for fresh ejaculates. The percentage motile sperm and percentage

progressive motile sperm assessed by the Dropper method were lower in cryopreserved compared to fresh ram semen.

Cryopreservation is the more likely factor that produced the substantially lower motility readings. Several studies have reported deteriorated sperm quality through cryodamage to cellular membranes, mitochondria, axonemes and acrosomes (O'connell et al., 2002; Keskin et al., 2020). Ram sperm is more susceptible to cryodamage than other species due to its membrane composition; a higher polyunsaturated fatty acid to saturated fatty acid ratio leaves the membranes vulnerable to oxidative damage (Sangeeta et al., 2015). The axoneme, a microtubule unit in the sperm's mid-piece, utilises mitochondrial ATP to facilitate movement (Satir, 1989). Oxidative stress depletes ATP resulting in insufficient axonemal phosphorylation and motility decline (Bansal and Bilaspuri, 2011). With reference to Chapter 3, the cryopreserved semen in those studies was notably higher in motility and progressive motility than this study. A broadly similar cryopreservation protocol was used in both cases, but the studies used two different ram populations therefore a ram effect is likely a contributing factor in these differences. An alternative reason for the disparities could stem from human errors during sample preparation or loading of samples into the iSperm cap or base. To investigate that further, it would be of interest to repeat with a different technician for both Chapter 3 (Experiment 3.2.4) and Chapter 4 (Experiment 4.2.3) to rule out a human effect. Furthermore, this could provide more data towards the reproducibility of results using the iSperm.

It is important to note, fresh semen analysis was conducted prior to the iSperm methodology comparison. At the time of analysis in Glasgow, the Dropper method was selected as the most appropriate sampling method to use as it was recommended by the manufacturer. Therefore, both the Dropper and Pipette methods were included in the evaluation of the cryopreserved F1 ram semen. There were significant differences between the Dropper and Pipette readings for all motility and kinematic parameters for the Control and Biosolids groups. The Dropper consistently produced higher readings than the Pipette which strongly agrees with the work conducted in Chapter 3. As previously hypothesised, the differences could be due to the

different sample volumes used and effect the tracking ability of the software (Moraes et al., 2019).

4.4.3 Conclusions

To the best of our knowledge, this is the first study to evaluate the impact of continuous *in utero* exposure to ECs from biosolids on F1 ram semen quality from fresh and cryopreserved samples from the same ram population. The main outcome highlighted from these studies was the lack of an effect of biosolids on the measured parameters; except for increased motility in fresh semen from Biosolids rams. The findings in adjacent studies in humans (Jager et al., 2013; Hernández-Ochoa et al., 2005; Lie et al., 2021), mice (Rahman et al., 2017; Barakett et al., 2019) and dogs (Lea et al., 2016b) showed negative effects to semen quality when exposed to low levels of environmental chemicals. The findings from the current study as whole were at odds with those of other studies (Rahman et al., 2017; Barakett et al., 2019; Lea et al., 2016b; Jager et al., 2013; Hernández-Ochoa et al., 2005; Lie et al., 2021). As discussed, there were a variety of factors which could have contributed to these disparities. Potentially the most significant changes stemmed differences in species, exposure duration and dosage, the biosolids' chemical formulation and protocol effect. Furthermore, in future studies that involve the iSperm, it would be of interest to analyse fresh semen with the Pipette method as the Dropper method has repeatedly shown to give inflated results.

Chapter 5. General Discussion and Conclusions

5.1 Key findings

This novel study has followed the reproductive development of a population of F1 rams that were exposed in utero to biosolids. The histological key findings of the study showed no changes to immunostaining of biomarkers associated with Sertoli cells or Leydig cells in the prepubertal treatment group. Semen analysis of pubertal F1 rams differed between the fresh and cryopreserved samples. An increase in percentage motility was identified in the fresh biosolids sample. In contrast, no differences in sperm motility or kinematic parameters were associated with treatment when the cryopreserved semen was analysed. A new CASA platform, iSperm, was used to analyse these samples. An evaluation of the three iSperm methodologies revealed stark differences in motility and kinematic readings for the Dropper method compared to the Pipette or Dipper. Finally, when compared to the IVOS II, a verified CASA platform, sperm motility and velocity parameters did not agree between the two systems.

5.2 General Discussion

This study used the Sheep Biosolids Model, a paradigm for real life human exposure to a mixture of environmental chemicals, to establish the effects gestational exposure had on ovine testes. In Chapter 2, histological analysis of 8-week prepubertal testes was conducted to evaluate biomarkers of Sertoli and Leydig cell staining and the extent of proliferation. AMH, a biomarker for Sertoli cells, and CYP17A1, a biomarker for Leydig cells, did not show significant changes in immunostaining between the Control and Biosolids testes. Furthermore, PCNA, used as an index for proliferation within the tubules and interstitial area, also did not differ in immunostaining between the Control and Biosolids testes. The data from this study reflects similarities to previous studies that have used the Sheep Biosolids Model. Continuous in utero exposure to biosolids has been shown by Paul et al, (2005) (Day 0-110), Bellingham et al, (2012) (Day 0 – 7 months post parturition) and Lea et al, (2022) (Day 0-140) to have no

effect on Sertoli cell immunostaining (Paul et al., 2005; Bellingham et al., 2012; Lea et al., 2022). However, upon assessment of Sertoli cell nuclei numbers, Paul et al, (2005) did report a significant decrease in the exposed fetal testes (Paul et al., 2005). In addition, Lea et al, (2022) reported transient exposure periods restricted to either early, mid or late gestation decreased Sertoli cell numbers (Lea et al., 2022). With regard to CYP17A1, the findings of the current study did not agree with Lea et al, (2022) who indicated a reduction of CYP17A1 immunostaining in biosolids exposed fetuses (Lea et al., 2022).

It was hypothesised that a compensatory mechanism during fetal testicular development could be, in part, responsible for the differences shown between the current study and previous studies. The cAMP signalling pathway has a critical role in normal testicular development, spermatogenesis and the regulation of differentiation gene expression (Don and Stelzer, 2002). Elcombe et al, (2021) noted changes in the cAMP and cAMP-response element binding protein (CREB) signalling pathway in lambs (Day 1 post parturition) exposed gestationally to biosolids (Elcombe et al., 2021). The authors suggested the changes could be an underlying compensatory effect to maintain normal testicular morphology (Elcombe et al., 2021). A decline in either Sertoli or Leydig cells during fetal life may have initiated compensatory mechanisms to recover these cell types (Elcombe et al., 2021).

In the gestational exposure studies conducted by Paul et al, (2005) and Lea et al, (2022), the testicular histological staining was performed on fetal testes and showed changes in immunostaining (Paul et al., 2005; Lea et al., 2022). In the current study, histological staining was performed on 8-week post parturition testes and lambs were grazed on control pastures after birth, providing an EC relief phase. Evidence from rat studies have demonstrated that severe depletions in Sertoli cell numbers at birth, can be recovered and compensated for postnatally, when exposure to ECs ceased (Auharek et al., 2010). Fetal testes in Paul et al, (2005) and Lea et al, (2022) studies did not experience a relief phase; therefore, it is difficult to predict whether the same immunostaining, a reflection of a change in cell number, would emerge if there was a period of EC cessation (Paul et al., 2005; Lea et al., 2022). The EC

relief phase may have initiated Sertoli and Leydig cell recovery by an increase in proliferation. In the current study, no changes were shown in PCNA staining for tubule or interstitial areas. The age of the lambs in this study could have been a contributing factor. Sertoli cells proliferate intensely immediately after birth so at 8-weeks of age, if there was an increase in proliferation, it would have already occurred (Hochereau-de Reviers et al., 1995).

Histological changes during testicular development may trigger consequential effects to semen quality in adult life. The extent of AMH immunostaining can give an indication of the Sertoli cell population within the seminiferous tubules (Grinspon and Rey, 2010). Sertoli cells have a fundamental role during the formation of the fetal gonad, triggering seminiferous tubule formation and initiate the differentiation of fetal Leydig cells (Mackay, 2000). In adulthood, Sertoli cells nourish and maintain GCs to support the cells throughout the phases of spermatogenesis (Bellingham et al., 2012). Each Sertoli cell can support a finite number of GCs therefore it is important to have sufficient proliferation during fetal life to maintain a normal spermatogenic output in adulthood (Sharpe et al., 2010). CYP17A1 is another biomarker used to assess Leydig cell populations. In the fetal gonad, fetal Leydig cells synthesise testosterone and INSL3 which are key components in the development of the male phenotype (Bellingham et al., 2012). Testosterone is a regulating factor for the inguinoscrotal phase of testicular descent; inadequate testosterone production may result in cryptorchidic testicles (Niedzielsk et al., 2015). Undescended testicles experience heat stress which could impair the development of GCs and sequentially spermatogenesis (Nakai et al., 2000). Disruption within the spermatogenic pathway could ultimately result in a lower sperm count and sequential reduce fertility (Rehman et al., 2018; Sharpe, 2012).

In Chapter 4, it was important to analyse sperm motility and kinematic movements due the fundamental roles those parameters play in fertilization (Turner, 2005). Sperm that enter the female reproductive tract will encounter biological obstacles, such as the cervical mucus, designed to filter out immotile or low motile sperm (Lee et al., 2021). The viscosity of the cervical mucus increases fluid resistance (Kirkman-Brown and Smith, 2011). Therefore, for

sperm to successfully reach the oviduct, the cells must have adequate velocity and follow a relatively linear trajectory (Cox et al., 2006). Sperm that have abnormal trajectory pathways (low LIN and STR) and/or slow swimming velocity (low VCL and VAP) are unlikely to progress to the oviduct to fertilise the oocyte (Suarez and Pacey, 2006).

Chapter 4 therefore, used a CASA platform to analyse semen from pubertal F1 rams exposed in utero to biosolids, to determine whether an effect on semen quality would be reflected in adulthood. When fresh semen from pubertal F1 rams was analysed, there was an increase in the percentage of motile sperm in the Biosolids group. It is unclear as to why an increase was shown because previous studies have established a negative association of ECs with motility (human Jager et al., 2013; human Hernández-Ochoa et al., 2005). Differences in species, research models, exposure period, dosage and chemical formulation could all have contributed to the variation reported in the current study. The immunohistological data presented in this study indicated no differences in Sertoli or Leydig cell populations. A rise or decline in either of these cell types would alter the spermatogenic capacity of the testis and would be reflected in the sperm concentration (Sharpe et al., 2010). An increase in sperm concentration might link to a higher percentage of motile sperm (Park et al., 2018) but in this study, no changes in sperm concentration were reported.

On review of cryopreserved semen from the same F1 rams, there were no changes in sperm motility or kinematic parameters between Control and Biosolids rams. However, there was a noticeable decrease when compared to the fresh samples. Cryopreservation would have contributed significantly to that decline because it is a very damaging process and can inflict oxidative stress upon sperm cells (O'connell et al., 2002). Studies have identified ram sperm to be very vulnerable to cryodamage due to the high polyunsaturated fatty acid (PUFA) to saturated fatty acid ratio within the membranes (Sangeeta et al., 2015). Destabilization of membranes by oxidative stress can inflict significant functional changes to sperm organelles like the mitochondria (Keskin et al., 2020). Mitochondrial ATP synthesis provides the energy

required for flagella movements and dictates the swimming velocity of the sperm and ultimately motility (Bansal and Bilaspuri, 2011).

To undertake the type of experiments reported in Chapter 4, a portable instrument to measure semen quality in real time was required. In Chapter 3, the iSperm, a new portable CASA device, was evaluated. The iSperm had three methodologies (Dropper, Dipper and Pipette) to analyse sperm and to date, no other study had evaluated all three methods. This study showed a significant deviation in sperm motility and kinematic parameters. The Dropper consistently produced higher motility and progressive motility percentages than either the Pipette or Dipper, which broadly agreed with one another. Furthermore, the Dropper showed lower kinematic parameter readings than either the Pipette or Dipper. It was hypothesised that a difference in sample volume could account for the dissimilarities. The larger volume used in the Dropper method may have interfered with the tracking ability of the software and skewed the findings (Moraes et al., 2019). This study established a standardised method which could be used in the sequential CASA comparison to the IVOS II. Due to the consistency in results and controlled volume required, the Pipette method was selected.

Previous studies that have evaluated the iSperm using a comparative CASA system, found statistically similar readings for semen concentration and percentages of sperm motility and progressive motility (canine semen evaluated by SpermVision SAR (Bulkeley et al.,2019), equine semen evaluated by Androvision (Dini et al., 2019) and CEROS II (Moraes et al., 2019). The results from the CASA comparison to the IVOS II contradicted these previous findings since data showed significant disparities in sperm motility, progressive motility and kinematic readings between the systems. Accurate analysis of semen concentration and sperm motility parameters is vital and imparts valuable information of the fertilization ability of the animal (Palacín et al., 2013). Under or over estimation of these values could result in the misclassification of semen samples. The iSperm showed significantly lower motility and progressive motility percentages than the IVOS II. This might result in normal semen samples being classed abnormal with poor motility and discarded.

5.3 Limitations

The testes used for immunohistological staining were collected and evaluated from one age of lamb. Similarly, the semen analysed in the adult F1 rams was also collected from one age group. This represents a “snap-shot” in development therefore it is difficult to fully evaluate the effects shown in the study without the knowledge of earlier and/or later postnatal developmental ages. In addition, analysis of Sertoli and Leydig cell population involved only immunostaining and did not include nuclei count. Due to time limitations, it was not feasible to conduct work with the size of the samples on multiple animal ages. This does not invalidate the results presented in the studies, but it would have provided a broader understanding of biosolids in utero exposure across an extend period of post parturition development.

5.4 Future Work

In order to understand in greater depth, the effects of gestational exposure on testicular histology and semen quality, and the long-term consequences of said exposure, there are several areas of work that could be pursued. Future studies that incorporate the Sheep-Biosolids Model could investigate several periods throughout early post parturition, to analyse Sertoli and Leydig cell immunostaining. Examination of Sertoli and Leydig nuclei numbers during this window would provide a better understanding of the populations of both cell types. Furthermore, to begin to see whether a compensatory mechanism is initiated during an extended period of EC relief, it would be of interest to measure the level of proliferation within Sertoli and Leydig cells throughout early postnatal periods. In addition, measuring serum testosterone levels throughout gestation and into adulthood would provide more information on the endocrinological effects ECs have on this critical male steroid. The implications of environmentally relevant mixtures of ECs on semen quality, has not been explored a great deal using the Sheep-Biosolids Model. It would be of interest to analyse semen from F1 rams over an extended period of adult life and the sequential F2 and F3 generations. In addition, it would be of interest to expand the CASA comparison work conducted with the iSperm and IVOS II with the use of larger sample sizes in both fresh and frozen semen.

5.5 Conclusions

In conclusion, the data presented in this study demonstrated that continuous in utero exposure to an environmentally relevant mixture of ECs had no significant implications on 8-week prepubertal testis immunohistology. This study, for the first time using the Sheep-Biosolids Model, demonstrated that adult F1 ram semen quality was not negatively affected by in utero exposure to ECs. It is hypothesised that a compensatory effect may have been initiated during fetal testicular development to combat against environmental insults and maintain normal testicular phenotype. This study also reviewed the iSperm, a new portable CASA system. The data presented uncertainty in the accuracy of measuring sperm motility and kinematic parameters with the iSperm when compared to a validated CASA system. Collectively, the data presented from the biosolids exposure studies, highlights the need for further work to be conducted in order to broaden the understanding of environmental chemicals on male reproductive development into adulthood. The new knowledge gained from the studies will add to the growing body of evidence of the male reproductive effects that are linked to environmental chemical exposure.

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