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Master of Research Dissertation

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Title: The Impact of Environmental Endocrine Disrupting Compounds on Ovine Fetal Adrenal Gland Development and Function

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Abstract

Humans and wildlife are constantly exposed to ubiquitous environmental chemicals which have the capability to disrupt the endocrine system. These endocrine disrupting compounds (EDCs) are found within everyday items including food cartons, baby-milk formula and can even be inhaled in dust particles. *In utero* exposure to EDCs has been found to disturb normal fetal hypothalamic, pituitary, gonadal and thyroid function in both genders. In contrast, despite increasing evidence of correlation between EDCs and metabolic diseases, chemical effects on the adrenal gland have received little attention. This study examined ovine fetal adrenal glands available from pregnant domestic sheep (*Ovis aries*) exposed to a real-life cocktail of EDCs found within human sewage sludge fertiliser (normal agricultural practice). Ewes were exposed for 80 day windows (0-80, 30-110, 60-140), or continually throughout gestation (0-140 days) and fetal adrenals subjected to immunohistochemistry for specific markers (CYP17A1 and AGTR1) or to real time PCR for the target genes (CYP11A1, CYP17A1, 3βHSD, MC2R, AGTR1 and ER1). Adrenal zone thickness stained for AGTR1 and CYP17A1 protein were significantly reduced ($p=0.037$ and $p=0.031$ respectively). AGTR1 and CYP17A1 gene expression exhibited a significant treatment by gender interaction ($p=0.027$ and $p=0.032$ respectively) in the females from the mid and late gestation treatment groups. ER1 gene expression was also significantly reduced in mid and late treatment goups compared with controls ($p=0.042$). These data illustrate that *in utero* exposure to EDCs within sewage sludge dysregulates the developing ovine fetal adrenal gland. It is hypothesised that this fetal adrenal dysregulation may contribute to the increasing incidence of human metabolic disease.

Contents

1. Introduction

Since the publication of Rachel Carson's "Silent Spring" in 1962 (Carson, 1962), there was a period of increased awareness and concern over environmental contaminants. These pollutants have the profound capability to harm human and wildlife populations. These chemicals are associated with increased incidences of specific adult diseases as well as perturbed reproductive function, and reduced biodiversity. Even Joni Mitchell sang "Hey farmer, farmer put away that DDT now, give me spots on my apples, but leave me the birds and the bees, please!" referring to the organochloride pesticide dichlorodiphenyltrichloroethane (DDT), which is now banned in the USA and Europe, but still widely used globally to combat malaria-spreading mosquitos. However, in popular culture, references to environmental pollutants have been put aside, and from personal experiences, the general population is very naïve to the possible consequences.

Many of these environmental chemicals induce their effects by adversely interacting with the endocrine system, particularly during pregnancy. This problem posed by such contaminants, collectively termed the Endocrine Disrupting Compounds, or EDCs, due to their capabilities to disrupt the endocrine system, is highlighted by two disturbing observations:

1. Increasing endocrine related disease in humans, for example, reduced fertility and increased metabolic diseases, including obesity;
2. Decreasing biodiversity and increasing abnormalities observed within aquatic and terrestrial ecosystems.

Worryingly, the USA EDC screening program estimated there to be approximately 10,000 ubiquitous chemicals with the potential to interfere with the endocrine system (EPA, 2014).

Normal physiology is controlled by an abundance of response-producing molecules, or hormones, derived from cholesterol, which are secreted throughout the body into the venous circulation by glandular organs: the Endocrine System. Briefly, the hypothalamus of the brain produces many stimulating and inhibiting hormones, which bind with specific receptors to cause a cascade of events resulting in a response. Hypothalamic hormones bind to receptors within the pituitary gland, to cause the release of further hormones, which then specifically act upon their target organs, for example, the adrenal gland to produce cortisol, or the gonads to produce sex hormones. These end step target gland hormones negatively feedback to the hypothalamus and pituitary gland to regulate the production of the initiating hormones. A summary of the endocrine system can be seen in Figure 1. The endocrine system enables homeostasis to be maintained; therefore any compound with the capabilities of disrupting this finely balanced system, has the potential to cause widespread physiological harm by dysregulating homeostatic mechanisms.

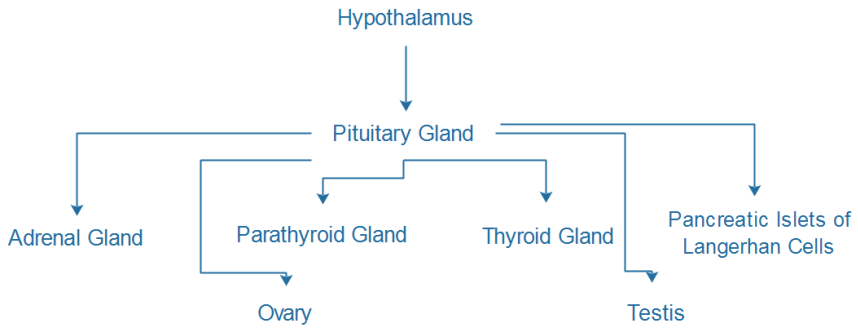

Figure 1: Summary of the main organs involved within the Endocrine System.

1.1: Endocrine Disrupting Compounds

In 2001, McLachlan defined EDCs as chemicals with the ability to mimic or block vertebrate steroid hormones due to interaction at the receptor site (McLachlan, 2001). The following year, the International Program on Chemical Safety (IPCS) produced the following definition (IPCS, 2012):

"An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations.

A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations."

Anthropogenic pollutants consisting of numerous heavy metals and synthetic organic compounds are ubiquitous and persistent in the environment due to their widespread use in agricultural practice as pesticides and fertilisers, as pharmaceuticals and as constituents of everyday items. It is well documented that low environmental levels of these chemicals can disrupt the endocrine systems in a wide range of animal species. Studies have shown there are hundreds of environmental chemicals with oestrogenic properties (Nishihara et al., 2000). The developing fetus is therefore the most susceptible to harm, and EDCs have the ability to alter the structure and function of organs in future generations who were not directly exposed (Steinberg *et* al., 2008). Global chemical production has now increased to become worth over €3,127 billion annually (The European Chemical Industry Council, 2013) which relates to 400 million tons (Casals-Casas et al., 2008) and the US Environmental Protection Agency "is considering 87,000 substances as potential candidates for testing" as EDCs (EPA, 2014).

Humans and animals are exposed to chronic low concentrations, or doses, of these EDCs in their everyday environment; low dose exposure is defined as "a dose below the lowest dose at which a biological change (or damage) for a specific chemical has been measured in the past, i.e. any dose below the lowest observed effect level or lowest observed adverse effect level (LOAEL)" (Welshons et al., 2006). However, at environmentally relevant low levels, EDCs are still able to cause adverse physiological effect, especially when present in mixtures as found in the environment. Notably, this complex mixture can be antagonistic, synergistic or additive in effect (Rhind et al.,

2010a, Kortenkamp, 2007). Furthermore, EDCs do not follow normal toxicological rules: an increased dose does not necessarily result in an increased effect. Instead EDCs have the capacity to follow the rules of non-monotonicity: this means the dose response increases and decreases with increasing dose (Vandenberg et al., 2012).

1.1i: Routes of Chemical Exposure

Routes of human exposure to EDCs include consumption of meat and dairy products, ingestion of water, inhalation and absorption through the skin (Fowler et al., 2008). The main means of exposure for animals and humans is oral consumption, either through food or contaminated water sources, the latter becoming increasingly problematic (Stuart et al., 2012). However, there is also increasing evidence of inhalation of volatile chemicals, which are on the rise in industrial centres, and can be absorbed through the skin (Rhind et al., 2010a). One major oral source of contamination for livestock is through pesticides, herbicides and fertilisers that are regularly used to treat pastures. Traditional organic manure and sewage fertiliser had been replaced with inorganic man-made nitrate fertilisers. However, the economic and environmental cost of using and producing these synthetic fertilisers has resulted in a resurgence in sewage sludge usage (Swanson et al., 2004). Sewage sludge fertiliser, or biosolids, is a bi-product of human wastewater treatment, and is often available free of charge from the large wastewater management centres. It contains high concentrations of nitrogen and phosphorus required for grass production; however, it contains relatively high concentrations of EDCs (Rhind et al., 2005b, Stevens et al., 2003).

1.2: The Importance of EDCs

It is estimated that 24% of all human disease can be attributed, at least in part, to environmental factors (Pruss-Ustun and Corvalan, 2007). Fifteen to 20% of human couples have difficultly conceiving, and there are clear links between environmental contaminants and human infertility (Hruska et al., 2000). Studies have shown EDCs have negative effects on fetal gonads as well as affecting the hypothalamic pituitary gonadal (HPG) axis (Paul et al., 2005, Fowler et al., 2008, Bellingham et al., 2013), thyroid (Hombach-Klonisch et al., 2013), adrenal and the hypothalamic pituitary adrenal (HPA) axis (Hinson and Raven, 2006, Kaludjerovic and Ward, 2012). In addition, there is an increased incidence of metabolic diseases, including obesity and type 2 diabetes, which correlates with changes in the industrial and agricultural chemical procedures initiated over the past 40 years (Casals-Casas et al., 2008).

It is not only humans that suffer the consequences of EDC exposure. Global biodiversity is declining at a terrifying rate for numerous reasons: poaching, deforestation and global warming to name but a few. EDCs are also contributing to this diminishing pool of wildlife. For example, Lake Apopka in North America is famous not only for alligators (*Alligator mississippiensis*), but also for high EDC burdens including the now banned DDT. The alligators in this lake, and others containing high ions of organochloride pesticides, have low egg clutch viability compared to controls, leading to lower population numbers of juvenile alligators (Rauschenberger et al., 2007). EDC contaminated lakes alter plasma hormone concentrations, steroiodenesis and gonadal morphology (Guillette et al., 2000). Avian species are also in decline, and DDT has been shown to reduce egg hatchability due to eggshell thinning (Bernanke and Kohler, 2009).

1.2i: Cancer

A number of lines of evidence suggest that exposure to EDCs may be related to an increase in the incidence of various cancers, particularly those associated with the reproductive system. Epidemiological studies have demonstrated an increased risk of prostate cancer, for example in agricultural workers in the United States. A prospective study of over 55,000 individuals from the American Agricultural Health Study Cohort demonstrated that prostate cancer appears to be associated particularly with the use of organochlorine pesticides, including DDT and dieldrin in Caucasians (Meyer et al., 2007).

Studies of animal models of breast cancer have identified at least 216 chemicals, including many EDCs, which are associated with an increase in mammary gland tumours (Rudel et al., 2007). Human epidemiological studies have linked various EDCs with breast cancer, including the organochlorine 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (DDE), levels of which were found to be significantly higher in women with breast cancer than those without (Wolff et al., 1993). However, not all studies have managed to fully substantiate a link. Knower and colleagues reviewed a number of known EDCs, and even though epigenetic effects seem apparent, the link between EDCs and, in this case, mammary cancers, are incredibly varied and complex, and further investigations are required (Knower et al., 2014). One potential mechanism is the modification by EDCs of known risk factors for the development of breast cancer. For example, certain EDCs have been shown to reduce the age of menarche; early menarche is an accepted risk factor for breast cancer.

1.3: Environmental EDCs

Environmental chemicals with the capacity to induce hormonal effects include solvents, pesticides, heavy metals, bi-products of the pharmaceutical industry, cosmetics and plasticisers to name a few. Different families of chemicals have different hormonal properties. Some environmental xenobiotics have the capacity to disrupt the endocrine system in their original form, however, others have to be converted and transformed within the body before they become hormonally active (IPCS, 2012).

1.3i: Persistent Organic Pollutants

Persistent organic pollutants (POPs) are chemicals found within the environment with the capacity to persist for long periods of time without degrading, and with the potential to bio-accumulate in animals. POPs can be detected in fat stores of animals and are generally found at higher concentrations in those species at the top of the food chain (IPCS, 2012). One example of a POP is the polychlorinated biphenyl group of chemicals.

Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are lipophilic, synthetic chemicals with two-linked benzene rings and a combination of hydrogen and chloride atoms, which were used until the 1980s for insulating and sealing buildings (Annamalai and Namasivayam, 2015). PCBs have both oestrogenic and anti-oestrogenic activity at the level of the oestrogen receptor (Connor et al., 1997), and a number of PCB congeners have the capacity to bio-accumulate (Annamalai and Namasivayam, 2015). Even though PCBs were banned decades ago due to their high environmental persistence, waste disposal and

incineration has resulted in continuing contamination today (Steinberg et al., 2008). PCBs have a history of concern due to their capacity to alter reproductive function (Cillo et al., 2007) as well as having carcinogenic properties (National Toxicology Program, 2010).

Due to their lipophilic nature and the increasing fat mass in people, interactions between PCBs and disease is likely to be on the increase in modern day populations (Kim et al., 2015). POPs including PCBs, have been correlated with altered thyroid function, lower birth weights, altered neural development and reproductive disturbances (Annamalai and Namasivayam, 2015). An environmentally relevant mixture of PCBs (Aroclor-1254) has been found to severely disrupt rat prostate cell morphology and cause DNA damage (Cillo et al., 2007). PCBs have also been associated with hypertension, however, this was only shown in cohorts of the population with an above average body mass index due to increased potential PCB storage in adipose tissue (Arrebola et al., 2015).

1.3ii: Phthalates

Phthalates are esters of phthalic acid and are added as plasticisers to plastics used in windows, toys and even food containers. Unlike PCBs, phthalates are not persistent in the environment, and act as anti-androgens resulting in perturbations of male genitalia (Annamalai and Namasivayam, 2015). Indirect maternal exposure to monobutyl phthalate (MBuP) during late pregnancy in rats increased rates of cryptorchidism, lowered birth weights, decreased anogential distance in male offspring and caused an overall increase in pregnancy loss (Ema and Miyawaki, 2001). However, it is important to distinguish this as a toxicological study, as high concentrations of phthalates were used rather than environmentally relevant concentrations. Other male reproductive perturbations caused by anti-androgen phthalates include reduced sperm quality, suppression of fetal androgen levels and internal and

external genital malformations (Schug et al., 2011). However, it is not only males that are affected by phthalates. A statistically significant association between elevated female periconception urine concentrations of monoethylhexyl phthalate and pregnancy loss has been found in a cohort of 430 Danish couples (Toft et al., 2012). *In utero* exposure to PCBs causes most harm. Maternal exposure to phthalates have been associated with early thelarche, endometriosis, reduced semen quality and reduced anogenital distance in offspring (Diamanti-Kandarakis et al., 2009). *In utero* exposure to the phthalate di-(2 ethylhexyl) phthalate (DEHP) (which has been found in amniotic fluid) induced epigenetic changes and hotspots of dysregulated gene expression in the offspring, primarily targeting the immune system (Martinez-Arguelles and Papadopoulos, 2014). Worryingly, not only are metabolites of DEHP found in consumer cow milk, but also human milk and baby formula (Mortensen et al., 2005).

1.3iii: Brominated Flame Retardants

Halogen-containing flame-retardants are used widely for preventing fires in electrical equipment and, particularly, in soft furnishings, carpets and furniture. Wide reaching effects of flame retardants have been investigated, and include lung inflammation, ovarian malformations, hepatomegaly and disruption of thyroid hormones (Annamalai and Namasivayam, 2015).

Bisphenol A

The widespread domestic use of flame-retardants including bisphenol A (BPA), has resulted in constant human exposure from everyday household items including food tins and even within contaminated dust (Brandsma et al., 2013). BPA has been found in human urine (Christiansen et al., 2014). Similarly to phthalates, *in utero* exposure is more harmful than adult exposure

and BPA causes widespread reproductive dysfunction due to its oestrogenic properties (Schug et al., 2011). Pregnant rats exposed to environmentally relevant concentrations of BPA gave birth to male offspring with reduced spermatogenesis and fertility and female offspring with early onset puberty and decreased luteinising hormone (LH) concentrations in adult life (Salian et al., 2011). In another rat study where pregnant dams were exposed to varying concentrations of BPA by gavage (0, 0.025, 0.250, 5 or 50 mg/kg body weight per day), anogential distance was significantly reduced in male and female offspring compared to controls. No other significant change in reproductive function was reported, for example there was no difference in reproductive organ weights seen in offspring by day 16 when they were euthanised. However, reproductive dysfunction is more likely to be evident at puberty and into adulthood, which was not investigated in this study (Christiansen et al., 2014).

1.4: EDC Mechanism of Action

EDCs can interact in many ways at numerous sites in hormonal pathways and have the capability to perturb function or the metabolism of hormones by mimicking natural hormones with respect to transport, mode of action and storage (Schug et al., 2011). EDCs can act on nuclear and non-nuclear steroid hormone receptors, the aryl hydrocarbon receptor, and on receptors for a variety of other physiological mediators. EDCs are capable of wider physiological interference than just endocrine disruption, including on neurotransmitters as well as directly affecting enzymatic pathways of steroid biosynthesis and/or metabolism (Diamanti-Kandarakis et al., 2009).

1.4i: Oestrogen Receptors

Numerous chemicals with oestrogenic properties and the ability to mimic hormones have been found in the environment (Nishihara et al., 2000). Due to the promiscuity of the ligand binding region of oestrogen receptors (ERs) (Hamlin and Guillette, 2011, Swedenborg et al., 2010), EDCs can act as oestrogens, binding with receptors (agonists), or by blocking the receptor (antagonists) (Swedenborg et al., 2010). EDCs can also partially bind to the receptor, resulting in a variable response (Hamlin and Guillette, 2011), and many EDCs also have the ability to interact with numerous hormonal pathways simultaneously (Swedenborg et al., 2010).

There are two oestrogen receptors: ER1 and ER2, which have distinct physiological roles (Gustafsson, 2003). Oestrogen receptors are located throughout the body and therefore interference by EDCs can have widespread consequences. Oestrogens are steroid hormones produced, like all steroid hormones, by a pathway of enzyme reactions from cholesterol. Activation of ER1 and ER2 can be by natural oestrogens including plant phytoestrogens, or chemicals which were never designed to modulate oestrogen receptors, for example DDT (Schug et al., 2011).

The use of molecular dynamic simulations to investigate structure-activity relationships between oestrogen ER1 and a number of EDC xenoestrogens suggest that van der Waals interactions are the principle driving force in their binding to the oestrogen receptor by hydrogen bonding (Li et al., 2012, Zhuang et al., 2012). Given the apparent similarity of receptor interactions between endogenous oestradiol-17β and xenoestrogens, as well as the high biological potency of many identified EDCs, it is surprising that there are often only limited structural similarities between an EDC and the endogenous hormone, the activity of which it is mimicking (Acerini and Hughes, 2006). For example Figure 2 compares the structures of a number of oestrogenic EDCs with the naturally occurring endogenous 17β-oestradiol, and it can be seen that there is very limited molecular commonality.

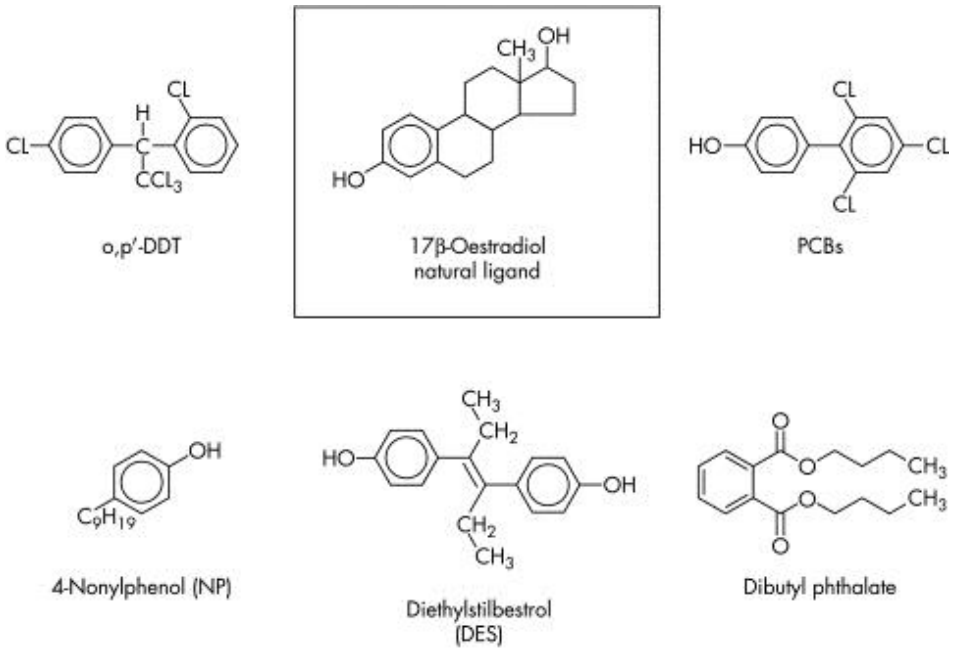

Figure 2: Examples of the chemical structures of certain EDCs with known oestrogenic effects (xenoestrogens), compared with the endogenous hormone 17β-oestradiol (Acerini and Hughes, 2006).

However, not all xenoestrogens are true oestrogen mimics. Some have oestrogenic properties by causing different signal transductions than true oestrogen, and have differing oestrogenic potencies (Watson et al., 2007). In addition, certain oestrogenic EDCs, for example BPA as discussed above, not only have the ability to bind to oestrogen receptors, therefore being an oestrogen agonist, but also binds to thyroid receptors, acting as an antagonist (Schug et al., 2011).

1.4ii: Aryl Hydrocarbon Receptor

EDCs also interact through the aryl hydrocarbon receptor (AhR), which is a key receptor in xenobiotic defences (Swedenborg et al., 2010). The AhR is ubiquitously distributed in mammalian tissues. Activation of cytosolic AhR by EDCs, including xenoestrogens such as PCBs, results in its translocation to the nucleus. Following interaction with specific dioxin-responsive DNA sequences,

the transcription of cytochrome P450 steroidogenic enzymes (CYPs) is modulated (Xiao et al., 2014), thereby potentially influencing levels of both sex steroids, mineralocorticoids and glucocorticoids which heavily rely on CYP enzymes.

1.4iii: Epigenetics

One mechanism whereby EDCs may affect the fetus is via modification of epigenetic mechanisms (Skinner et al., 2011). “An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence” (Berger et al., 2009). This is an adaptive response to enable changes in organogenesis to optimise survival in a changing environment (Crain et al., 2008, Skinner et al., 2011). Gene expression can be influenced by a variety of histone and DNA modifications, in particular DNA methylation. In order for gene transcription to take place, the DNA has to be loosely coiled to enable access for the transcriptional machinery including RNA polymerases. Methylation results in tightly coiled DNA, resulting in an inactive gene, and therefore increased methylation results in a reduction in gene expression (Bird, 2002). The cells in different tissues have unique patterns of gene expression, and this is a result of epigenetic modifications (Bird, 2002). During development, the pattern of epigenetic modifications may change, a process called epigenetic re-programing, affecting both germ cells (cells that produce the gametes) and somatic cells (all other cells) (Morgan et al., 2005). This presents a window of vulnerability to environmental signals that may lead to epigenetic modifications influencing the health of the offspring as well as resulting in the potential for trans-generational transmission. Alterations by EDCs to somatic cells will cause the adult onset of disease; however, alterations by EDCs to germ cells will result in altered transgenerational phenotypes (Skinner et al., 2011).

1.4iv: Fetal Origin of Adult Disease, Transgeneration Consequences and Windows of Susceptibility

There is increasing evidence suggesting *in utero* environmental changes can result in variations in fetal programming, leading to altered gene expression and the onset of adult chronic disease (Gluckman and Hanson, 2004). Changes to the epigenome of offspring can be caused by maternal hormone and nutrient exposure *in utero*, and this alteration can result in altered gene expression and subsequent altered physiology in later life (Franklin et al., 2010). The majority of EDCs do not alter DNA sequences, and it is thought developmental effects caused by EDCs encompasses changes to the epigenome (Skinner et al., 2011), for example by the phthalate DEHP (Martinez-Arguelles and Papadopoulos, 2014). In a rat model, *in utero* exposure to the PCB mixture Aroclor-1221 not only caused abnormalities in gender skew and LH concentrations in the daughters (F1), but even greater perturbations in the next generation, the grand-daughters (F2), which showed supressed LH and progesterone concentrations, and reduced ovarian and uterine weights (Steinberg et al., 2008). The authors hypothesised this generational effect to be either direct genotoxicity, altered methylation pattern, or altered hormonal concentration during development. More worrying still, EDCs have the potential to alter not only F1 and F2 generations, but also F3 generations. For example, in rodent models of diethyhexyl phathalate and dioxin exposure during pregnancy, F3 generations were affected both in males (lower sperm counts, lower germ cell function, abnormal seminiferous tubules) and females (reduced fertility) (Rissman and Adli, 2014).

Fetuses are most sensitive to the actions of EDCs due to their undeveloped defences, especially to chemicals with steroidogenic activity. As many EDCs are lipophilic in nature, they accumulate within the developing fetus (Gluckman and Hanson, 2004, Hamlin and Guillette, 2011). Further sensitive

windows of exposure are during other critical developmental times including childhood and puberty (Fudvoye et al., 2014). In addition, recent studies suggest a transient period of exposure during fetal development is more detrimental than exposure throughout the entire gestation, for example, on ovine ovarian health (Bellingham et al., 2013).

1.5: EDCs and Female Reproductive Dysfunction

Female reproductive health has decreased over the past half a century, however, it is far more difficult to assess than male fertility as the gametes are inaccessible (Crain et al., 2008). EDCs are hypothesised to interfere with the timing of puberty in the human including earlier breast development in females, altered hormone concentrations including LH and progesterone, and reduced ovarian and uterine weight (Steinberg et al., 2008, Mouritsen et al., 2010). In one study, Buttke and colleagues demonstrated a significant inverse correlation between urinary levels of certain phenolic EDCs (including 2, 5-dichlorophenol and 2, 4-dichlorophenol) as an index of environmental exposure, and the age of menarche in adolescent girls, the average of which has now decreased to less than 13 years of age (Buttke et al., 2012). In addition, there has been a decrease in the proteins that are associated with endometriosis (MMP-9, ELNE and FAM49B), and ELNE and FAM49B levels are inversely correlated with the concentration of EDCs including PBDE #183, γ-HCT and PCBs (Williams et al., 2015).

A decreasing age of menopause has also been linked to EDC exposure. Eskenazi and colleagues concluded that 2, 3, 7, 8-Tetrachlorobenzo-p-dioxin (TCDD) was associated with an increased risk of early menopause (Eskenazi et al., 2005). In addition to this investigation, a cross-sectional study completed on 219 Hispanic women within the Health and Nutrition Examination Survey concluded that exposure to, and serum levels of, organochlorine pesticides was associated with a younger age of menopause, and this was concentration

dependent. Woman in the highest exposure categories (determined by serum concentrations of pesticide) reached menopause up to 5.7 years earlier than women with undetectable levels of pesticide (Akkina et al., 2004).

1.5i: Diethylstilbestrol

Diethylstilbestrol (DES) while not strictly an EDC, provides a good indication as to what might be expected from ingestion of exogenous oestrogenic compounds. It is an orally active synthetic oestrogen that was developed in 1938 as the first easily synthesised and usable oestrogen (Laronda et al., 2012). DES was subsequently used in the 1940s-1970s in the United States and Europe to treat infertility, and to prevent miscarriage during "high risk" pregnancies (Bamigboye and Morris, 2003, Laronda et al., 2012, Crain et al., 2008). The theory was that oestrogen is required to maintain pregnancy, and miscarriage is linked to low urine concentrations of oestrogen, therefore increasing the concentration of oestrogen, using an antenatal dose of synthetic DES, will help maintain pregnancy and reduce miscarriage incidences (Smith, 1949). Dr George Van Siclen Smith strongly advised the use of DES in pregnancy (Smith, 1949) and his work led to the FDA approval of DES to be used to prevent miscarriage in 1947, as reviewed by Laronda and colleagues (Laronda et al., 2012).

However, later studies not only showed that DES was of no benefit to pregnancy outcome, but also there were numerous harmful consequences for the developing fetus. When the female fetuses reached puberty, they tended to have increased genital tract cancers, including rare young-onset clear-cell adenocarcinomas (Herbst et al., 1971). DES was the first transplacental carcinogen reported (McLachlan, 2006). Female offspring of DES treated mothers also experienced significant primary infertility caused by adenosis of the vagina and cervix. *In utero* exposure to DES increased the risk of developing endometriosis by 80%, and 5 weeks or more of DES exposure

tripled the chance of developing endometriosis compared with those who were exposed for less than 5 weeks (Missmer et al., 2004).

In addition, reproductive system problems were discovered in men who had been exposed to DES *in utero*: high incidence of cryptorchid testes, epidermal cysts, hypoplastic testes, abnormal sperm and an increase in testicular cancers (Gill et al., 1979, Bamigboye and Morris, 2003). DES was also linked to low birth weight in both genders (Bamigboye and Morris, 2003, Laronda et al., 2012).

In a rodent model, male mice exposed to DES *in utero* experienced a higher incidence of cryptorchidism, retained Müllerian ducts, abnormal spermatogenesis and testicular cancers (McLachlan, 2001). Pregnant rats exposed to DES suffered from increased stillbirth, prolonged gestation, increased labour time, decreased birth weight, decreased survival post birth and an increase in dam adrenal gland weight (Zimmerman et al., 1991).

1.6: EDCs and Male Reproductive Dysfunction

1.6i: Declining Sperm Densities

Male reproductive health is a great concern as human fertility is reported to be on the decline. A meta-analysis between 1938 and 1990 published by Carlsen and colleagues demonstrated a 19% reduction in semen volume and a 42% decrease in sperm count (Carlsen et al., 1992). However, the validity of these data have been much criticised for numerous reasons including inappropriate statistical techniques, differing analysis methods and poor data sources, and no accounting for varying study populations (Olsen et al., 1995). However, further re-analysis by Swan and colleagues, using linear regression models of the original data, and the addition of 47 more studies, suggests that mean sperm densities are indeed decreasing in Europe and the United States of America, and in fact this new analysis suggests declines have been more

rapid than Carlsen and colleagues originally suggested (Swan et al., 1997, Swan et al., 2000). Swan and colleagues also interestingly noted the wide variation in the geographic distribution of differing sperm densities, suggesting a similar aetiology and the requirement for further investigation into temporal and environmental variations in sperm health. Additional studies have also highlighted the declining sperm densities: Rolland and colleagues suggested a significant and continuous decrease in sperm concentrations of 32.2% in their French study population over a 17-year period (Rolland et al., 2013). However, again, these data were criticised due to changes in laboratory practice during the study period (Pacey, 2013). Contrasting this, a more recent study suggested a rise in sperm counts, worryingly however, only 23% of the young Danish (mean age: 19 years old) "general population" participants had optimal sperm concentration and morphology (Jørgensen et al., 2012).

Schiffer and colleagues tested the effects of 96 ubiquitous EDCs on human sperm and concluded that EDCs had the capability to impact the CatSper channel (within the sperm tail plasma membrane), therefore affecting sperm motility due to an increased calcium influx, with possible consequences for human fertility (Schiffer et al., 2014). There is a negative impact of EDCs on sperm morphology as shown by Zhang and colleagues who discovered a positive correlation between DEHP concentration and spermatozoa malformation (Zhang et al., 2006). In a study of healthy men aged 21-40, grouped as "infertile" (failure to impregnate their partners within one year with regular, unprotected intercourse) or "control" (partners successfully conceived within one year), dibutyl phthalate (DBP) and DEHP were detected in more than 80% of infertile samples. In addition to this, the concentration of DEHP in the fertile men was found to be 0.77µg/mL, compared to a concentration of 5.73µg/mL within oligoasthenospermic men (Pant et al., 2011). A significant negative association was found between DEHP levels and sperm motility, and both DEHP and DBP caused a decrease in sperm viability in a time and concentration dependent manner (Pant et al., 2011).

A review of bovine artificial insemination (AI) records from 1960-1995 have demonstrated a reduction in sperm density in bovine semen over the years 1970 – 1980/5, followed by an improvement thereafter (Wahl and Reif, 2009). Although it appears that the overall success of bovine AI is declining, it is particularly difficult to interpret data such as these in terms of environmental factors because there are so many confounding issues involved in cattle breeding, particularly selective pressures and genetic effects (Karoui et al., 2011).

1.6ii: Testicular Dysgenesis Syndrome

Following the increased awareness of declining sperm quality, widespread studies have been completed on the physiological causes of this problem. Poor semen quality has now been linked with wider male reproductive perturbations, primarily testicular cancer, crypotorchidism, altered anogenital distance and hypospadias. The grouping of these conditions is referred to as Testicular Dysgenesis Syndrome (TDS) (Skakkebaek et al., 2001). Many of these conditions are increasing and there is growing evidence to suggest an environmental aetiology (Main et al., 2010). One aspect of TDS is testicular cancer, and the most important tumour in this syndrome is germ cell neoplasia. Germ cell tumours consist of abnormal primordial germ cells or gonocytes, termed *carcinoma in situ* (CIS) cells, and eventually develop into malignant tumours in adolescent and young men (Rajpert-De Meyts et al., 2003). Indeed, as most of these human male reproductive pathologies can be mimicked in experimental animal models by exposure to EDCs *in utero* or during the perinatal period, Skakkebaek and colleagues (2001) suggest that TDS is caused by environmental factors, which are affecting embryonic and gonadal development. Hu and colleagues reviewed the effects of phthalates on Leydig cell development and concluded that although animal studies of phthalate exposure show an association with TDS, a human epidemiological link between phthalates and TDS is difficult to obtain (Hu et al., 2009). In

addition, *in utero* exposure of rat fetuses to the phthalate diisononyl phthalate (DINP) caused fetal Leydig cell aggregation and reduced expression of steroidogenic enzymes (Li et al., 2015).

Despite these observations, a recent review of published epidemiological studies has revealed that there is only a weak association between individual EDCs and TDS (Kortenkamp et al., 2014). Based on reported tissue levels of 22 different EDCs, these authors investigated the effects of EDC mixtures in an *in vitro* anti-androgenicity assay system directed at androgen receptor (AR) antagonism. Kortenkamp and colleagues failed to show effects at the average serum EDC concentrations found in European serum. This study has a number of limitations including only investigating 22 out of many thousands of potential EDCs, the relevance of adult tissue levels to the *in utero* situation, and the relevance of the *in vitro* assay endpoint to the *in vivo* pathophysiological situation. For example, the cellular response to selected EDCs *in vitro* is far removed from *in vivo* responses to an environmentally relevant mixture of contaminants. To overcome this problem, more recent studies now focus on *in vivo* models of fetal development and EDC exposure.

1.7: EDCs and Animal Models of Human Exposure

1.7i: Rodent Models

There are no human models of everyday EDC exposure, and therefore the real life actions of EDCs have been questioned (Schiffer et al., 2014). However, in reality, it is not possible to complete fetal studies of EDC exposure on humans for ethical reasons. Limited human studies that have been carried out have focussed on first trimester fetal testis tissues (Fowler et al., 2007) and ovary tissues (Fowler et al., 2014) obtained from social abortions. Consequently, many studies investigating the effects of EDCs have focused on rodent models and a single chemical entity, over relatively short time periods at higher than

environmentally relevant concentrations (pharmaceutical doses) (Fowler et al., 2008). This is very important to enable a broader understanding of the mechanisms by which EDCs act; however, these experiments do not follow a normal everyday pattern of human or animal exposure. Looking at the effect of exposure on whole organisms provides a far greater understanding of endocrine disruption. The complexity of a whole organism cannot be replicated *in vitro*, for example, in cell cultures. One example of this complicated interaction is that EDCs may interact with steroidogenic pathways at each level of the hypothalamic pituitary adrenal axis (Harvey et al., 2007). In addition, it is important to address whether or not environmental concentrations of chemicals, rather than pharmaceutical or toxicological concentrations, elicit negative physiological effects.

AGTR1

Angiotensin II hormone interacts with the Angiotensin II Receptor, Type 1 (AGTR1) to regulate aldosterone secretion. Aldosterone, a mineralocorticoid, acts upon the distal tubules and collecting ducts within the kidney to retain sodium and secrete potassium, and therefore retain fluid, increasing blood volume. Maternal EDC exposure during pregnancy has been investigated in a variety of animal models using a number of different pathophysiological endpoints, with rodents being a commonly used experimental species. For example, the offspring of pregnant rats treated with DEHP *in utero* (gestational day 14 until parturition) exhibit a decrease in circulating aldosterone but not corticosterone concentrations, and a reduction in adrenal AGTR1 expression (Martinez-Arguelles et al., 2011). The authors conclude from their results that *in utero* DEHP exposure causes reduced adult aldosterone concentrations due to a reduction in adrenal AGTR1, and this reduction is sufficient to prevent normal physiological angiotensin-II induced aldosterone responses. However, these studies only showed a significant effect on AGTR1 expression following treatment of pregnant females with at

least 100mg/kg/day, and in some cases 500mg/kg/day of DEHP (Martinez-Arguelles et al., 2011). Since a final human equivalent dose would be approximately 10-40mg/kg/day (Reagan-Shaw et al., 2008), this would not appear to be environmentally relevant; the human of average weight is unlikely to be consuming 2800mg/day DEHP. Effects of maternal exposure to phthalates on the fetal development of the male reproductive system has also been investigated *in vivo* in rodent models with the conclusion that rats and mice, and indeed different strains of mice, have a different response to maternal phthalate exposure (Johnson et al., 2012).

While there is a substantial literature relating to the effects of maternal exposure to EDCs on various aspects of the endocrine system, these studies can be criticised as not reflecting the real life situation because (a) they investigate a single chemical entity rather than the complex mixtures found in the environment, (b) they have used far higher concentrations of EDCs than result from normal environmental exposure, and (c) some of the resulting effects appear to be species specific and therefore this approach may be a poor model of human exposure.

1.7ii: *In vitro* Models

In addition to studies on intact animals, a number of *in vitro* cell or tissue culture methods to investigate EDC exposure have been developed. The strength of such an approach is that effects of EDCs on specific cell types or tissues can be investigated in isolation; the weakness is that such an approach does not take into account the interactions that occur between different organ systems *in vivo* and of course such a cell or organ based approach cannot be used to investigate the effect of maternal exposure on fetal development. Furthermore, the concentrations of EDCs that are utilised in such *in vitro* approaches are generally far greater than the levels of exposure of tissues *in vivo.* For example, Habert and colleagues report significant

effects using concentrations of 10^{-6} and 10^{-5}M of the plasticiser mono-(2-ethylhexyl) phthalate. These workers also demonstrated that cultured rat, mouse, and human fetal testis implants showed quite different responses to individual EDCs according to species (Habert et al., 2014), calling into question the usefulness of this approach as a predictor of the effects of the same chemicals on different species or as a model of human exposure.

1.7iii: Ovine Models

Agricultural animals provide a realistic model for human exposure to numerous toxins and EDCs. They are housed within close proximity to people, resulting in similar, but not identical, levels of exposure. In addition, ruminants have similar reproductive anatomy, are more similar in weight to humans than rodents, and fetal development follows a similar pattern to the human (Padmanabhan and Veiga-Lopez, 2014). Ruminant models of exposure therefore show insight into the potential perturbations of EDCs on human hormonal pathways. Sheep are an "exceptional animal model to study developmental programming of adult reproductive disorders" (Padmanabhan and Veiga-Lopez, 2014). Given this breadth of application of ovine models to the human, the use of such a model for the investigation of environmental contamination is likely to be of significant relevance to human health.

In addition to sheep being exceptional models of development for humans, it is important to create a model representative of environmental EDC exposure, as unlike many investigations of a single chemical entity, the environment is contaminated with a complex mixture of EDCs at varying concentrations. One useful model in this regard is a real-life ovine model of exposure defined by Fowler and colleagues as "exposure through natural route, not via specific application such as injection" (Fowler et al., 2008). British sheep management systems using human sewage sludge fertiliser have been exploited as a highly relevant animal model of human exposure to an

environmentally relevant cocktail of EDCs. This has a number of advantages: like humans, sheep are long-lived, have a long gestation period (145 +/-5 days), and organogenesis is similar to humans (Hombach-Klonisch et al., 2013). They are also relatively available, and act as a human source of EDC exposure as humans eat their meat, along with the EDCs found therein.

As mentioned previously, sewage sludge biosolids are increasingly being used in animal keeping systems throughout the world as a source of pasture fertiliser. It is a by-product of human wastewater treatment, contains a cocktail of EDCs and heavy metals, and prolonged usage causes a build-up of EDC concentration in the soil (Rhind et al., 2013). Prior to use, it is processed to remove some environmental contaminants and pathogens. Using sewage sludge as a fertiliser is a preferred alternative method of human waste disposal than the alternatives: landfill or deposition into waterways (Rockefeller, 2002). However, due to their EDC content, sewage sludge fertiliser is now banned for use in certain agricultural practices in Switzerland (Wager, 2007).

An experimental model has been established in which pregnant ewes are maintained either on pastures fertilised with sewage sludge, or pastures fertilised with an inorganic, EDC-free fertiliser, reflecting the reality of environmental contamination. This model also enables the investigation of the effects of EDCs within sewage sludge on different windows of embryological development of fetuses. The sewage sludge model used in this study is currently the only available real-life model of the exposure to an everyday cocktail of EDCs.

In addition to investigating ruminants as a model for human exposure, they are also a source of human EDC exposure via their meat. Many EDCs are lipophilic and therefore have the capacity to accumulate in adipose tissue; for example, EDCs have been detected in beef from cows grazed on contaminated pastures (Petro et al., 2010). Furthermore, EDCs are found within ruminant milk (Rhind et al., 2007), another source of human exposure,

and elevated concentrations of PCBs are linked with higher yielding dairy cows compared to lower yielding cows (Petro et al., 2010). This is therefore an increasing source of human exposure due to the trend to breed for high milk production in the UK dairy industry. Interestingly, breeding for such traits has been linked with the increasing infertility issues that are having major negative impacts on the UK dairy industry. Fertility in the UK dairy industry is decreasing at an alarming rate as indicated by the increasing inter-calf interval. Decreasing fertility is partially attributed to increased yields and a widespread negative energy balance; however, it is reasonable to assume that exposure to environmental EDCs is an additional factor adding to the increased infertility seen (Rhind, 2005).

Studies of maternal and fetal tissues from ewes maintained on EDC containing sewage sludge-treated pastures show significant effects on reproductive potential in male and female fetuses (Paul et al., 2005). Levels of certain EDCs and heavy metals have also been measured in fetuses from the sewage sludge model and significant concentrations of copper, lead, zinc and DEHP were found (Rhind et al., 2002, Rhind et al., 2005a, Rhind et al., 2005b, Rhind et al., 2007). In addition to human EDC exposure from meat consumption, the sewage sludge model broadly reflects direct human EDC exposure (Rhind et al., 2005b, Rhind et al., 2002).

Ovine Models of Female Pathologies

Ovarian gametogenesis requires a precise combination of oestrogen, inhibin and activin locally within the developing fetus, and any interference in this could impact upon normal ovarian development. This is concerning as many EDCs are known to have oestrogenic properties. The pool of primordial follicles, which constitute the reproductive lifespan of the mammalian female, is completed during fetal development *in utero*. Any disturbance of fetal ovarian development could drastically reduce this oocyte pool and, therefore,

female fecundity, in contrast to the male, which undergoes spermatogenesis throughout adult life. Indeed, the ovine sewage sludge models of EDC exposure have shown that fetal ovarian development is detrimentally affected, as shown by significantly decreased oocyte density compared to controls, and significant female fetus growth restriction has been recorded (Fowler et al., 2008). In addition, Fowler and colleagues found a trend, although not significant, for decreased expression of ovarian CYP17A1 and consequently a lower serum oestradiol concentrations in these fetuses, suggesting EDCs have the ability to reduce steroidogenesis (Fowler et al., 2008). Further fetal sheep ovary studies concluded that a change in the uterine environment during gestation, even if from a higher than normal EDC exposure to zero EDC exposure, is more detrimental than a continual exposure to EDCs throughout gestation (Bellingham et al., 2013). In support of this, ovaries from sheep fetuses exposed both continuously or transiently to sewage sludge during gestation showed fewer ovarian follicles regardless of the treatment group (Lea et al., 2014b).

Ovine Models of Male Pathologies

Using the previously mentioned ovine sewage sludge model, Paul and colleagues demonstrated marked perturbation in fetal testes development (Paul et al., 2005). During this study, testes from 110-day-old fetuses exhibited both reduced Leydig and Sertoli cell numbers, which is likely to negatively impact spermatogenesis in later life. As described in the female, more recent studies indicate that a short transitory period of exposure during gestation may be more detrimental than continual EDC exposure throughout gestation (Lea RG et al., 2010, Lea et al., 2014b). These alterations were seen in further studies, which extended sewage sludge exposure to include post-natal contact. Bellingham and colleagues saw reduced testicular germ cell numbers and increased numbers of abnormal tubules in a subset of male lambs from the exposed groups (Bellingham et al., 2012).

1.8: The Hypothalamic Pituitary Gonad and Hypothalamic Pituitary Adrenal Axes

As previously mentioned, EDCs have the capacity to affect reproductive function in male and female sheep fetuses within the ovine models of exposure described above. Although initial studies focussed on the gonads, more recent studies have also shown effects on the hypothalamic and pituitary components of the HPG Axis. Reproduction in mammals is driven by a complex system of hormonal interactions and feedback loops from the hypothalamus, pituitary gland and the gonads creating the HPG axis. However, the endocrine system operates as an integrated whole, and reproduction involves systems other than the HPG axis, including the hormones derived from the adrenal gland and therefore the Hypothalamic Pituitary Adrenal (HPA) axis. The endocrine system in general, including reproductive aspects, is particularly vulnerable to disruption by a variety of synthetic environmental EDCs that can interfere particularly with steroid hormones, their synthesis, metabolism and receptor binding (Harvey et al., 2007). Whereas the HPG axis and the influences of EDCs thereon are relatively well understood, environmental and toxicological studies on the HPA axis have been less well studied. Indeed, Harvey and co-workers stressed an urgent need for an "adrenal toxicology assessment strategy" (Harvey et al., 2007) and the adrenal zona fasciculata, key for glucocorticoid production, is reported to be particularly vulnerable (Plechner, 2004).

Normal mammalian reproduction relies on a complex neuroendocrine pathway illustrated in Figure 3. Hypothalamic Gonadotropin Releasing Hormone (GnRH) results in the release of Luteinising Hormone (LH) and Follicle Stimulating Hormone (FSH) from the Anterior Pituitary Gland. These then act upon the gonads to stimulate testicular and ovarian maturation, steroidogenesis and gametogenesis (Bellingham et al., 2009). Figure 3

illustrates the numerous possible sites of EDC interaction from brain to gonadal level.

1.9: The HPG Axis

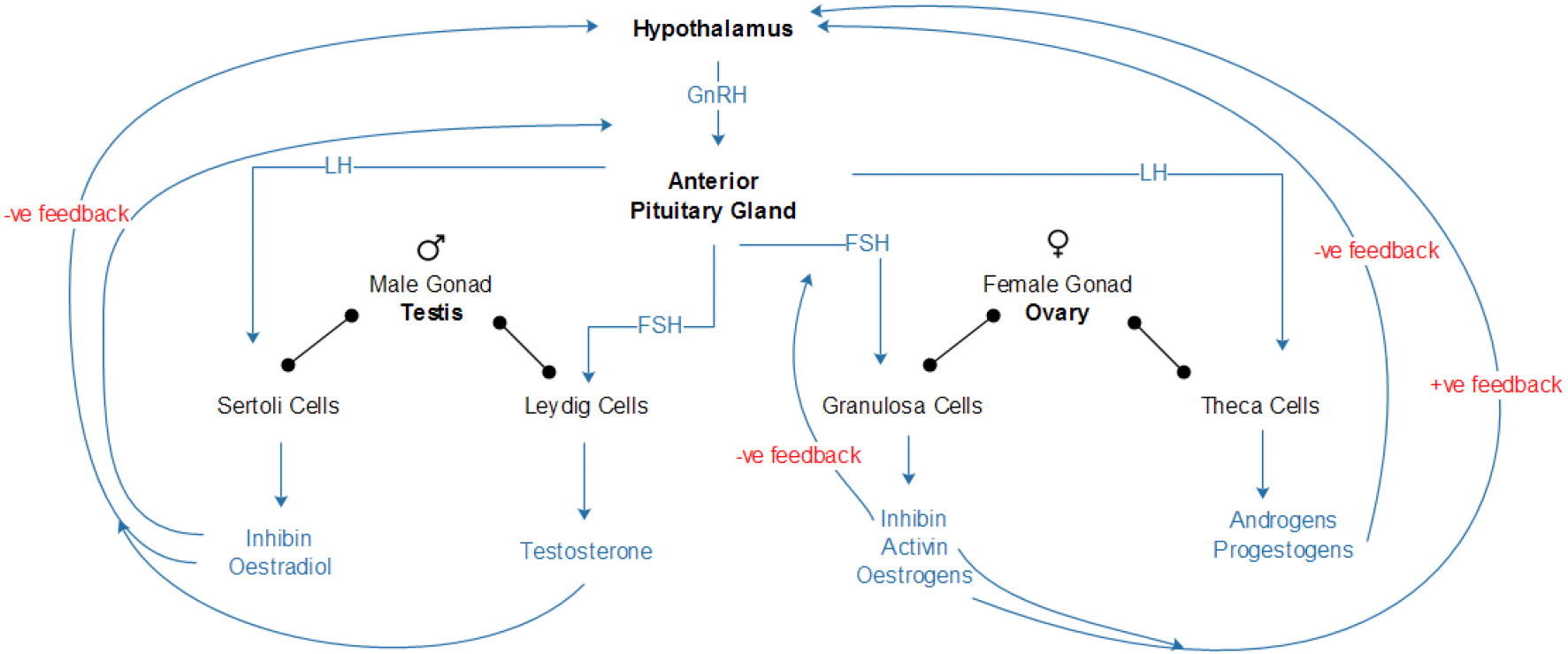

Figure 3: A schematic of the hypothalamic pituitary gonadal axis, EDCs have the potential to act upon multiple receptors, or as multiple hormones within this axis.

1.9i: The Hypothalamus and Pituitary Gland

As outlined in Figure 3, the HPG axis is regulated by an abundance of hypothalamic inputs and is highly sensitive to the activating effects of endogenous steroids during fetal life (Robinson et al., 2002). Bellingham and colleagues investigated the impact of EDC exposure on ovine fetal hypothalami and pituitary glands. In this study, sheep were maintained on pastures treated with human sewage sludge fertiliser up to day 110 of gestation. This resulted in reduced expression of fetal hypothalamic Gonadotropin Releasing Hormone 1 (GnRH1) mRNA and pituitary gland GnRH receptor (GnRHR) mRNA (Bellingham et al., 2010).

The neuropeptide kisspeptin and its receptor, G-protein-coupled receptor 54 (GPR54), are obligatory for GnRH secretion and therefore normal reproductive function (Roa et al., 2008). GnRH levels can therefore be affected by alterations in the kisseptin/GPR54 system, and this system may be one way EDCs perturb normal reproduction (Navarro and Tena-Sempere, 2008). Kisseptin expression is regulated by oestradiol (Navarro and Tena-Sempere, 2008) highlighting another location of possible oestrogenic chemical interaction. Indeed Navarro and colleagues showed neonatal injection of oestradiol benzoate resulted in reduced expression of hypothalamic KiSS-1 mRNA in both male and female rats (Navarro et al., 2009).

Bellingham and colleagues also showed that fetal exposure to sewage sludge resulted in reduced expression of both kisseptin and GPR54 mRNA expression (Bellingham et al., 2009). Early exposure to environmental chemicals with sex steroid activities may disrupt gonadotropin secretion in later life, and therefore alter reproductive function, due to direct interactions of EDCs and the kisspeptin system (Tena-Sempere, 2010).

In addition, Bellingham and colleagues have shown EDCs can affect the fetal pituitary gland. In an ovine study of sewage sludge exposure, fetal pituitary

glands showed a significant decrease in the number of luteinising hormone beta (LHβ) immunopositive cells, and also a significant decrease in double labelled LHβ and kisspeptin cells (Bellingham et al., 2009). In addition the fetal pituitary showed significantly fewer oestrogen receptor 1 (ER1) positive cells and LHβ/ER1 positive double labelled cells (Bellingham et al., 2009). Furthermore, ovine fetal hypothalamic and pituitary glands had lower GnRH receptor (GnRHR) and galanin receptor (GALR) mRNA expression, and fetal hypothalami had lower GnRH mRNA expression, when exposed *in utero* to sewage sludge compared with controls (Bellingham et al., 2010).

1.10: Endocrine Disruption and the HPA Axis

1.10i: Ovine Fetal Adrenal Development and Anatomy

Adrenal glands develop bilaterally at the cranial pole of the mesonephros (Tangalakis et al., 1992). During embryonic development, intermediate coeleomic mesoderm cells from the urogenital ridge migrate to the gonads to produce steroidogenic tissue. The same mesoderm cells also migrate to surround the immature adrenal medulla to form the adrenal cortex (Dyce et al., 2002). The resulting steroid-producing cells in the gonads and adrenal cortex therefore have the same ontogeny (Kaludjerovic and Ward, 2012). In addition, the expression of steroidogenic genes appear to be controlled by the same transcriptional regulatory promoter (SF1) in adrenal, testis and ovary (Buaas et al., 2012). It follows therefore that like gonads, the adrenal glands are also likely to be negatively affected by EDCs.

The anatomy of the adult and fetal adrenal gland is illustrated in in Figures 4 and 5. Both include a central medulla, which produces catecholamines surrounded by a steroid-rich cortex. The adult mature adrenal cortex is split into three distinct zones, each producing different classes of steroid hormones.

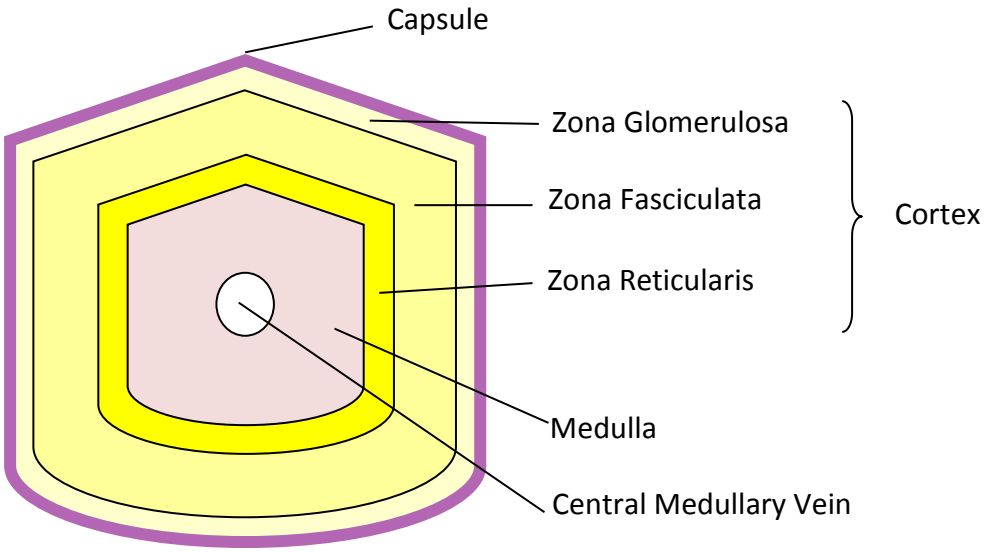

Figure 4: A Schematic of basic adult adrenal anatomy: the outer fibrous capsule encasing the cortex, the three layers of the cortex: zona glomerulosa, zona fasciculata and zona reticularis, the central medulla and central medullary vein.

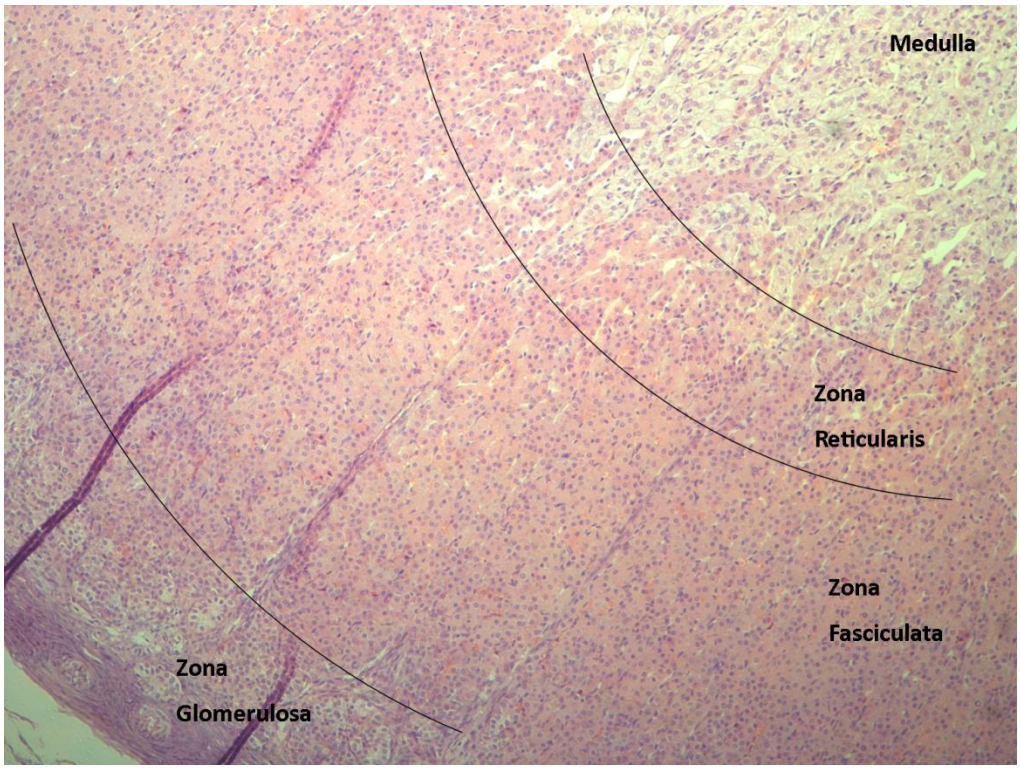

Figure 5: Histological Section (H&E Stain) of an ovine fetal adrenal gland at day 140 of gestation demonstrating the adrenal layers, including the location of the zona reticularis which will not develop until post birth (100x magnification).

Ovine adrenal gland development is shown as a timeline in Figure 6. Fetal pituitary adrenocorticotropic hormone (ACTH) secretion stimulates the development of the fetal adrenal cortex (Matthews and Challis, 1996). Subsequently, adrenal epigenesis is as follows: coelomic mesothelial epithelium proliferates at the cranial edge of the mesonephros resulting in the initial adrenal cortex (Aly, 1978). The cortical area is then invaded by migrating sympathochromaffin cells from the sympathetic ganglia, which migrate into the centre of the dividing mass, creating the fetal adrenal medulla (Boshier and Holloway, 1989). Both the developing medulla and cortex are encased by the developing capsule (Aly, 1978). Within the developing cortex, wide sinusoids develop and cortical zonation begins from the periphery, starting with the outermost zona glomerulosa, followed by the zona fasciculata (Boshier et al., 1989), and finally the central section of the cortex. The zona reticularis develops after birth; there is no evidence of the zona reticularis at two days post-partum (Tangalakis et al., 1992). Differentiation of the cortex and medulla is complete by day 100 of gestation (Boshier et al., 1989), followed by maturation of the cortical zones (Wintour, 1984).

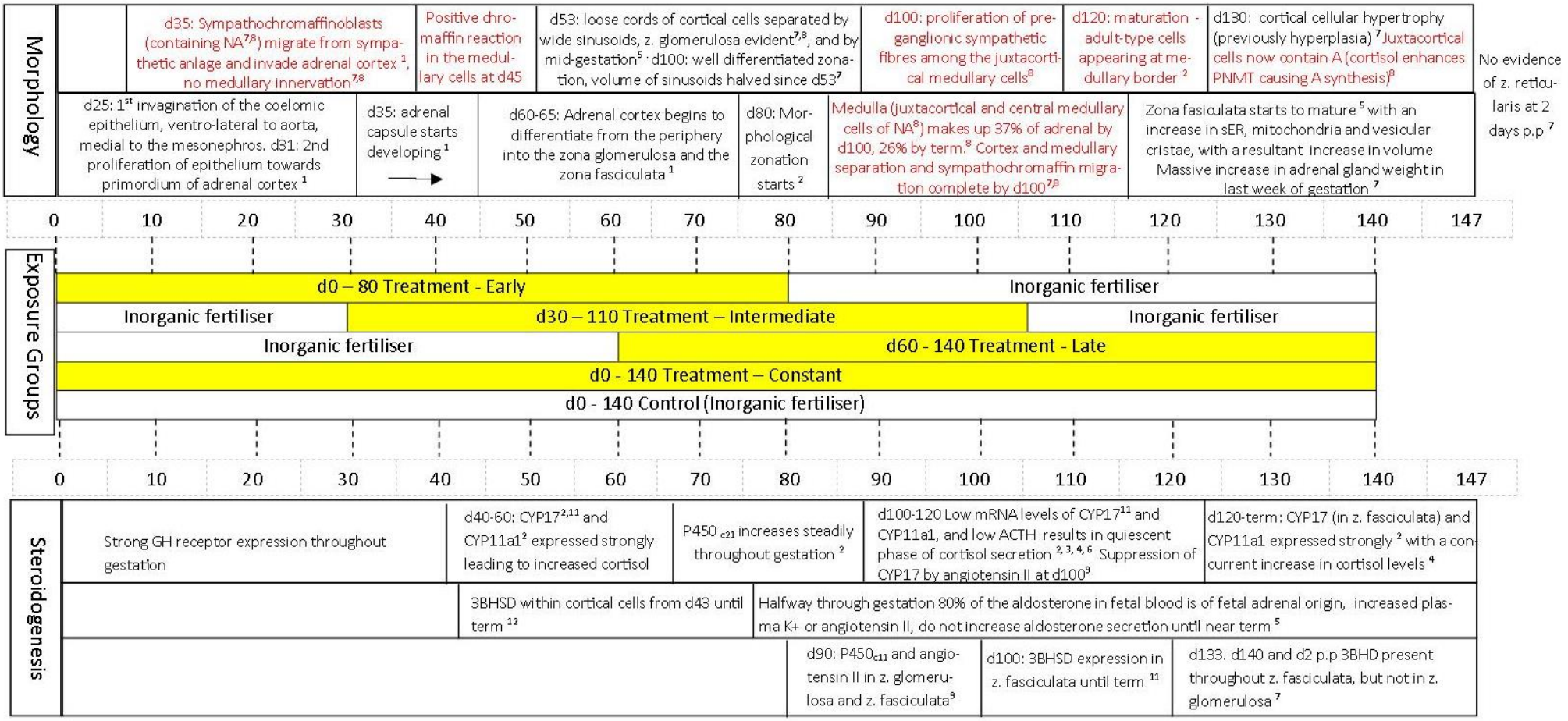

Figure 6: Timeline of ovine fetal adrenal gland development. Conception at day 0. Black: cortex, Red: medulla development. 1. (Aly, 1978), 2. (Tangalakis et al., 1989), 3. (Glickman and Challis, 1980), 4. (Tangalakis et al., 1990), 5. (Wintour, 1984), 6. (Tangalakis et al., 1992), 7. (Boshier and Holloway, 1989), 8. (Boshier et al., 1989), 9. (Coulter et al., 2000), 10. (Buckley, 2012), 11. (Han et al., 1997), 12. (Riley et al., 1992).

The principle regulators of the HPA axis are corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) from the parvocellular division of the paraventricular nucleus (PVN) which act synergistically and are released into hypophyseal portal circulation (Papadimitriou and Priftis, 2009) to initiate ACTH release (Figure 7). In the sheep, CRH mRNA increases throughout gestation within the PVN, with a significant increase between day 100/120 and day 140/143 of gestation. AVP also increases throughout gestation, and AVP mRNA levels within the magnocellular region of the PVN significantly increases between 60/80 and 140/143 days of gestation (Matthews and Challis, 1995). Matthew and Challis also discovered that hypoxemia, a common *in utero* stressor, caused a significant increase in CRH mRNA, and an increase, however not significant, of AVP mRNA within the fetal PVN (Matthews and Challis, 1995).

ACTH is released as the pro-hormones pre-ACTH and pro-opiomelanocortin (POMC) from corticotropes within the pars distalis of the anterior pituitary gland. POMC cleavage products include α-melanocyte stimulating hormone (α-MSH) and β-endorphin, and POMC mRNA increases within the ovine fetal pituitary gland late in the second half (days 100-135) of gestation, followed by a plateau before the increase for active labour. CRH mRNA also dramatically increases just before term, followed by decline post-partum (Matthews and Challis, 1996). Prohormone convertase I (PHC I) is found within the pars distalis of the pituitary gland, which further breaks down POMC to active ACTH (Papadimitriou and Priftis, 2009). ACTH acts directly on the adrenal cortex to elicit the release of glucocorticoids.

1.10ii: The HPA Axis

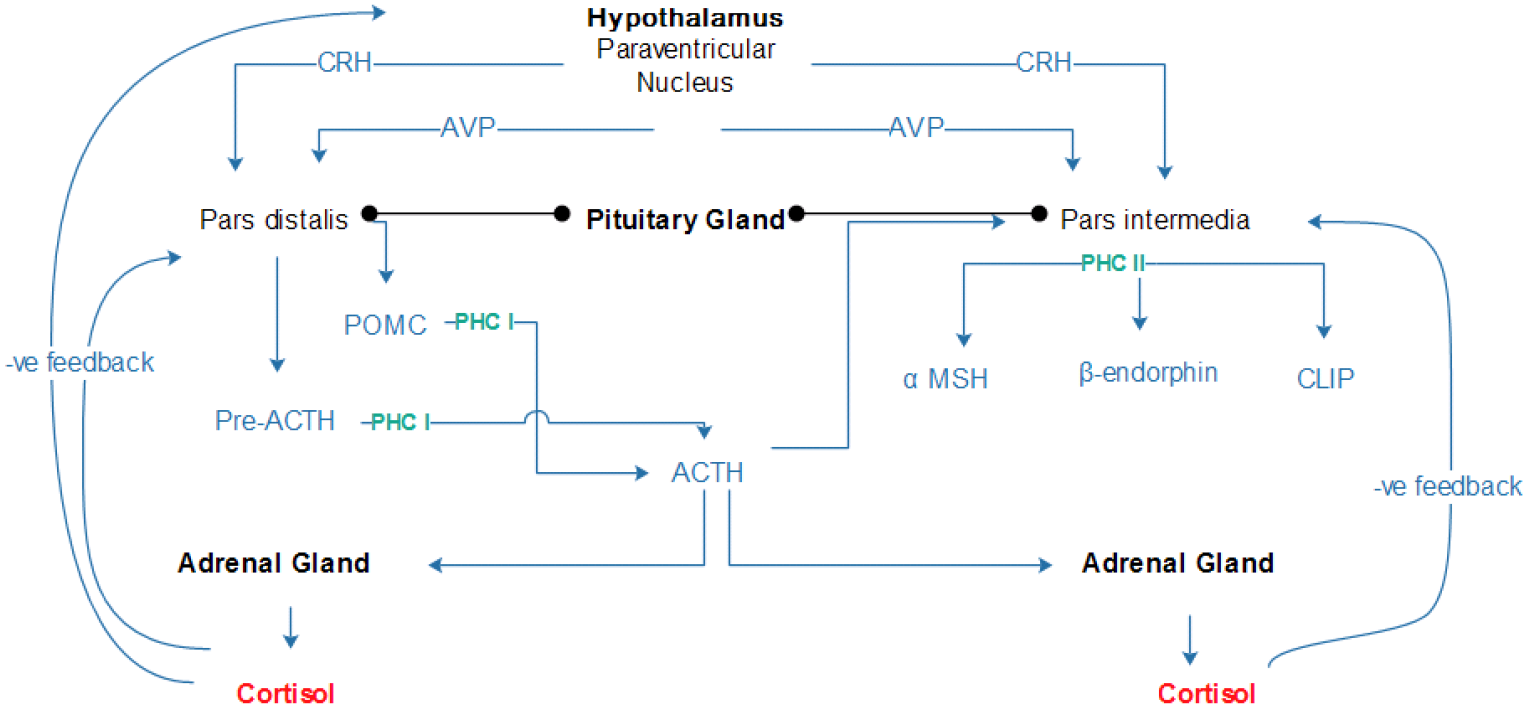

Figure 7: Schematic of the hypothalamic pituitary adrenal axis.

1.10iii: The Fetal HPA Axis and Parturition

The fetal HPA axis and adrenal gland, and subsequent production of glucocorticoids, is essential for brain development, stress responses, homeostasis and for the development, maturation and growth of the fetus *in utero,* and parturition (Kaludjerovic and Ward, 2012, Challis et al., 2000). A key factor in the reproductive success of mammalian species is this complex inter-relationship between the fetal adrenal gland and the maternal reproductive system (Kaludjerovic and Ward, 2012). Indeed, disruption of the ovine fetal pituitary, adrenal or PVN *in utero* resulted in prolonged gestation (Challis et al., 2000). Therefore, any factor that has a detrimental effect on the development of the fetal HPA axis has the potential to cause widespread alterations in adult phenotypes, supporting the concept of the fetal onset of adult disease.

Prior to parturition there is a rapid increase in the levels of the glucocorticoid cortisol in the fetus in many mammalian species including the human, although in rodents the principal glucocorticoid is corticosterone (Matthews and Challis, 1996). In sheep there is an exponential increase in fetal plasma cortisol levels in the last ten days of gestation following an increase in ACTH: the pre-parturient rise. In a negative feedback loop, cortisol reduces the production of CRH, AVP and therefore ACTH, however, immediately prior to parturition, there is an increase in cortisol, ACTH and CRH, indeed, CRH mRNA levels within the PVN is at its highest at late gestation (Matthews and Challis, 1995). These increases are somewhat counter-intuitive given the known negative feedback control of ACTH/CRH/AVP by glucocorticoids.

However, there are a number of factors that are thought to attenuate this negative feedback control, as can be seen in Figure 8 below. During gestation there is an opposing effect of ACTH, which is stimulatory in regards to cortisol production, and the larger molecular weight ACTH precursors POMC and pre-ACTH, which are inhibitory on the fetal adrenocortical cells. The secretion rates of these inhibitory factors decrease relative to ACTH by a factor of two

during late gestation, potentially contributing to the increased cortisol production during this time (Schwartz and McMillen, 2001, Matthews and Challis, 1996, Challis et al., 2001). The increase in fetal plasma cortisol reduces the expression of glucocorticoid receptors in the hippocampus, PVN (site of CRH and AVP release) and the anterior pituitary (site of ACTH and pro-hormone release) (Kapoor et al., 2006). The mRNA of pituitary receptors for CRH also decreases by approximately 40% from gestational days 102/105 to 137/139 (Schwartz and McMillen, 2001). Factors such as these may allow an increase in ACTH in the presence of increased cortisol during this period, maintaining the high cortisol level required for parturition.

In addition to fetal CRH production, the cortisol surge in both sheep and humans may be sustained by the additional placental CRH (Hamlin and Guillette, 2011). There are other factors that influence ACTH production including oxytocin (inhibitory), opiates (which supress CRH release), urocortins (which bind to CRH receptors), endocannabinoids (reduce basal stimulation of ACTH), and suppressor of cytokine signalling-3 (SOCS3) (inhibits POMC production and ACTH secretion), as reviewed by Papadimitriou and Priftis (Papadimitriou and Priftis, 2009).

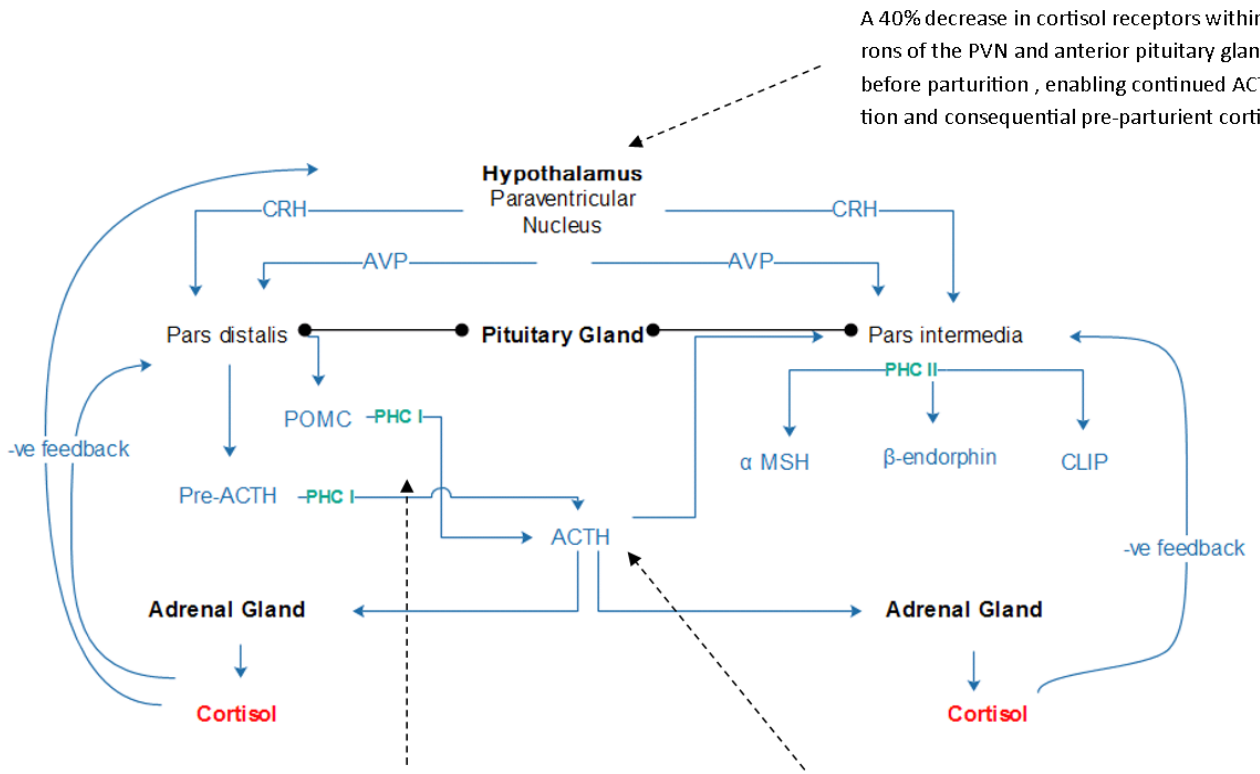

Figure 8: The Role of the hypothalamic pituitary adrenal axis in ovine parturition

In addition to pre-term activation of the fetal HPA axis, and the consequential cortisol surge, the maternal body has to be transformed for the impending labour. Simultaneously to the cortisol surge, the placenta increases the expression of CYP17A1 enzyme, which promotes placental progesterone conversion to androstenedione, therefore reducing progesterone levels (required for pregnancy maintenance) immediately prior to the commencement of labour, and increasing oestrogen production, which leads to the transformation of the uterus and cervix into the labouring state (Challis et al., 2001).

In addition to the glucocorticoids, the fetal HPA axis is controlled by a number of other factors including prostanoids (including prostaglandins) synthesised in the fetal brain. Hippocampal glutamate receptors stimulate prostaglandin endoperoxide synthase (PGHS2), also termed cyclooxygenase 2 (COX2), leading to an increase in prostaglandin-E2 (PGE2) which increases fetal HPA axis activity and subsequent cortisol levels (Reimsnider and Wood, 2006). This is hypothesised to be an additional mechanism to overcome negative feedback of cortisol in late gestation. In addition, fetal glucocorticoids up regulate PGH2S mRNA within the placenta, further activating the fetal HPA axis (Challis et al., 2001). Inhibition of PGHS2, for example by using non-steroidal anti-inflammatory drugs (NSAIDs), delays the start of labour (McKeown et al., 2000). The myometrium, which becomes increasingly activated by stretching caused by growth of the fetus, remains quiescent until the time of birth by the action of increased levels of progestagens (Fowden et al., 2008).

Studies have shown that the developing HPA axis is sensitive to environmental factors, including prenatal stress, and either experimental or natural stress situations such as exposure to predators in the wild, leading to raised cortisol levels during pregnancy. Exposure to excess levels of cortisone during fetal development can result in exacerbated stress responses subsequently in adult life (Lund et al., 1988). The adrenal gland itself is particularly vulnerable to environmental and toxic insults due to it being so

highly vascularised (Hinson and Raven, 2006). In addition, certain PCBs reduce plasma cortisol levels (Zimmer et al., 2013), and Zimmerman and colleagues showed that exposure of rats to DES caused delayed parturition and a prolonged labour time (Zimmerman et al., 1991) demonstrating gestational effects from oestrogenic EDCs.

1.10iv: Ovine Pregnancy Maintenance

The maintenance of pregnancy depends upon progesterone. Sufficient production of oestrogen by the placenta is required near term, which in turn depends upon the synthesis of dehydroepiandrosterone (DHEA) and its sulpho-conjugate DHEAS, ultimately from cholesterol via pregnenolone. The 17alpha-hydroxylase (CYP17A1) enzyme which catalyses the conversion of pregnenolone to DHEA and then DHEAS, while absent from the placenta, shows a high level of expression in the primate fetal adrenal cortex (Kaludjerovic and Ward, 2012, Mesiano and Jaffe, 1997, Labrie, 1991). Indeed, DHEA and DHEAS are the most abundant adrenal hormone in humans (Ikeda et al., 2012). Fetal adrenal glands from lower mammals, including sheep, produce relatively less DHEA and DHEAS than humans (Labrie, 1991). However, the fetal adrenal gland is essential in maintaining the required oestrogen levels for pregnancy maintenance by supplying the majority of the DHEA that the placenta requires for oestrogen synthesis. Some DHEA is also provided by the mother, but to a lesser extent, as can be seen in Figure 9 below (Kaludjerovic and Ward, 2012). There is a negative feedback system whereby increasing levels of plasma oestrogen suppresses the responsiveness of the fetal adrenal to ACTH (Albrecht and Pepe, 1995), lowering the production of DHEA and thereby reducing placental oestrogen synthesis, thus generating the potential for interference by EDCs, particularly oestrogenic compounds, in this feedback control system. However, placental oestrogen also can exert a positive feedback response, up-regulating 11-beta-hydroxysteroid dehydrogenase (11βHSD) promoting conversion of cortisol to

(inactive) cortisone, which stimulates fetal pituitary ACTH production and consequently an increase in DHEA (Albrecht and Pepe, 1999). 11βHSD mRNA within the pars distarlis of the fetal pituitary gland increases near term (Matthews and Challis, 1996). It follows therefore that environmental factors that inhibit CYP17A1 and/or 11βHSD enzymes will impact on essential pregnancy maintenance pathways dependent on the regulation of oestrogen and glucocorticoids.

Although the phytoestrogens genistein and daidzein (found in soy protein) have been reported to increase production of DHEA and DHEAS via inhibition of 21-hydroxylase in post-natal and adult adrenal cells (H295) cultured *in vitro*, there was no effect of these compounds on DHEA or DHEAS production by cultured fetal adrenal cells (Mesiano et al., 1999). The relevance of such cell-based *in vitro* culture systems to the physiology of the intact animal is uncertain as previously mentioned.

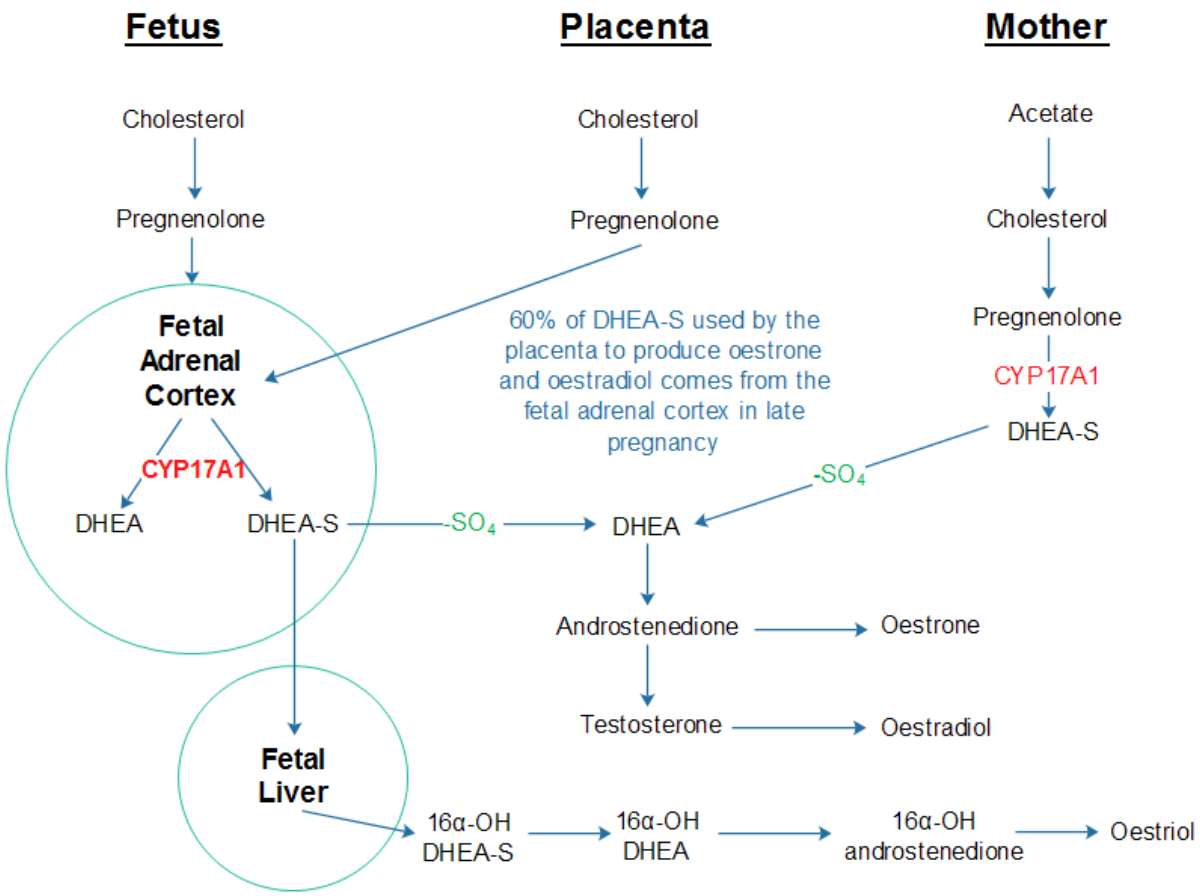

Figure 9: The inter-relationship between fetus and pregnancy maintenance. The placenta lacks the necessary CYP enzyme to produce the precursors of oestrone, oestradiol and oestriol, which are essential for pregnancy maintenance. Adapted from (Kaludjerovic and Ward, 2012).

Although there are clear endocrine communication pathways between the fetal adrenal and the placenta, there are some important species differences. The sheep fetal adrenal contains a high level of CYP steroidogenic enzymes and is steroidogenically active in early pregnancy as well as from 15-20 days before parturition (Zimmerman et al., 1991, Tangalakis et al., 1990). Effective sheep fetal adrenal gland steroidogenesis is linked to fetal catecholamine secretion since fetal cortisol is important for differentiation of the medulla and subsequent epinephrine secretion (Boshier et al., 1989, Boshier and Holloway, 1989). In early pregnancy, the sheep fetal adrenal cortex secretes lower levels of the oestrogen precursor dihydroepiandrosterone (DHEA) than its human counterpart, due to the lack of a prominent 'fetal' zone (undeveloped fetal adrenal cortex found in primates) and low levels of CYP17A1 (Labrie, 1991). However, preterm activation of the ovine fetal HPA axis stimulates CYP17A1 expression and thus adrenal cortisol production and placental progesterone conversion to androstenedione and oestrogen, which together transform the uterus and cervix to the labouring state and coordinate fetal organ system maturation with birth (Han et al., 1997, Tangalakis et al., 1989). Lambs adrenalectomised *in utero* display delayed parturition and post-natal over maturation (Drost and Holm, 1968). The similarities between the sheep and human adrenal development, including the pre-natal cortisol surge make the sheep a relevant model for HPA toxicity, and therefore EDC studies (Zimmer et al., 2013), especially as both sheep and humans produce cortisol, whereas rodents produce corticosterone due to the lack of DHEA and DHEAS (Challis et al., 2001, Martinez-Arguelles et al., 2014).

1.11: EDCs and Steroidogenesis

Successful development of the HPA axis is essential for adrenal glucocorticoid production, and therefore maturation of the fetus and parturition. However, the adrenal gland is the most sensitive level of the HPA and the neglected organ in endocrine disrupting research (Harvey et al., 2007). It contains a high

concentration of cytochrome P450 (CYP) enzymes, which are required for all steroid hormone synthesis from their precursor cholesterol (Figure 10). The CYP-subfamily of enzymes are monooxygenases, and are important targets for EDCs (Miller, 1988). Numerous organs within the body have the capacity to produce active steroid hormones; the gonads, placenta, brain, adipose tissue but most importantly, the adrenal gland (Sanderson, 2006).

In outline, the *de novo* steroidogenic pathway involves a series of enzymes that result in the bio-synthesis of steroid hormones from cholesterol including progestagens, oestrogens, androgens and glucocorticoids (Figure 10). A number of these are CYP enzymes, and the first step in steroid hormone biosynthesis involves the conversion of cholesterol to pregnenolone by mitochondrial CYP11A1. Pregnenolone is then converted to progesterone by 3-beta-hydroxysteroid dehydrogenase (3βHSD), which is found in both mitochondria and smooth endoplasmic reticula. Pregnenolone and progesterone are then the precursors for all further steroid hormones (Sanderson, 2006). Initially, external stimuli, including ACTH and angiotensin-II, activate steroidogenic acute regulatory protein (StAR) and steroidogenic factor-1 (SF-1), which induce the steroid synthesis pathway (Stocco, 2001).

Steroid 21 hydroxylase (CYP21), is unique to the adrenal cortex and is essential for the synthesis of the intermediates 11-deoxycorticosterone and 11-deoxycortisol for mineralocorticoid and glucocorticoid production, and is found in all three layers of the adrenal cortex. 11-deoxycorticosterone is converted to the mineralocorticoid aldosterone by the enzyme CYP11B2 and 11-deoxycortisol is converted to the glucocorticoid cortisol by the enzyme CYP11B1 (Sanderson, 2006).

In other steroidogenic organs, for example the testis, the weakly androgenic androstenedione, derived from progesterone by the action of CYP17A1, is converted to testosterone by 17-beta-hydroxysteroid dehydrogenase (17βHSD). CYP17A1 is bi-functional and has both 17, 20, lyase activity (required for androgen production) as well as 17, hydroxylase activity

(required for glucocorticoid intermediates and androgen synthesis) (Kitamura et al., 1991). In the ovaries, androstenedione and testosterone are converted to oestrone and oestradiol by aromatase (CYP19) and 17βHSD, 17βHSD types 1 and 7 favouring the conversion of oestrone to oestradiol (Sanderson, 2006).

Environmental effects on adrenal development and/or function may occur through alterations in CRH and ACTH secretion or at the level of the adrenal gland through altered steroidogenic enzyme activity (Kaludjerovic and Ward, 2012). Indeed, the gestational exposure to PCB congeners present in sewage sludge reduces ovine fetal cortisol levels and fetal adrenal size (Hatano et al., 1996). EDCs have also been linked to adrenocortical hyperplasia in seals (Bergman, 1999). In addition, PCBs have been found to alter growth, adrenal development and cortisol production in an ovine model of exposure (Zimmer et al., 2013). Zimmer and colleagues also found exposure to PCB congeners during gestation resulted in excessive and prolonged plasma cortisol levels when subjected to mild stress in offspring at nine months old, compared with controls (Zimmer et al., 2009). Human adrenocortical cell lines (H295R cells) showed increased cortisol production when exposed to an environmentally relevant EDC exposure, and multivariate analysis concluded this was due to PCB and, to a lesser extent, DDT mixtures. Additionally, increases in steroidogenic enzyme mRNA was also detected (Zimmer et al., 2011).

1.11i: The Steroidogenic Pathways

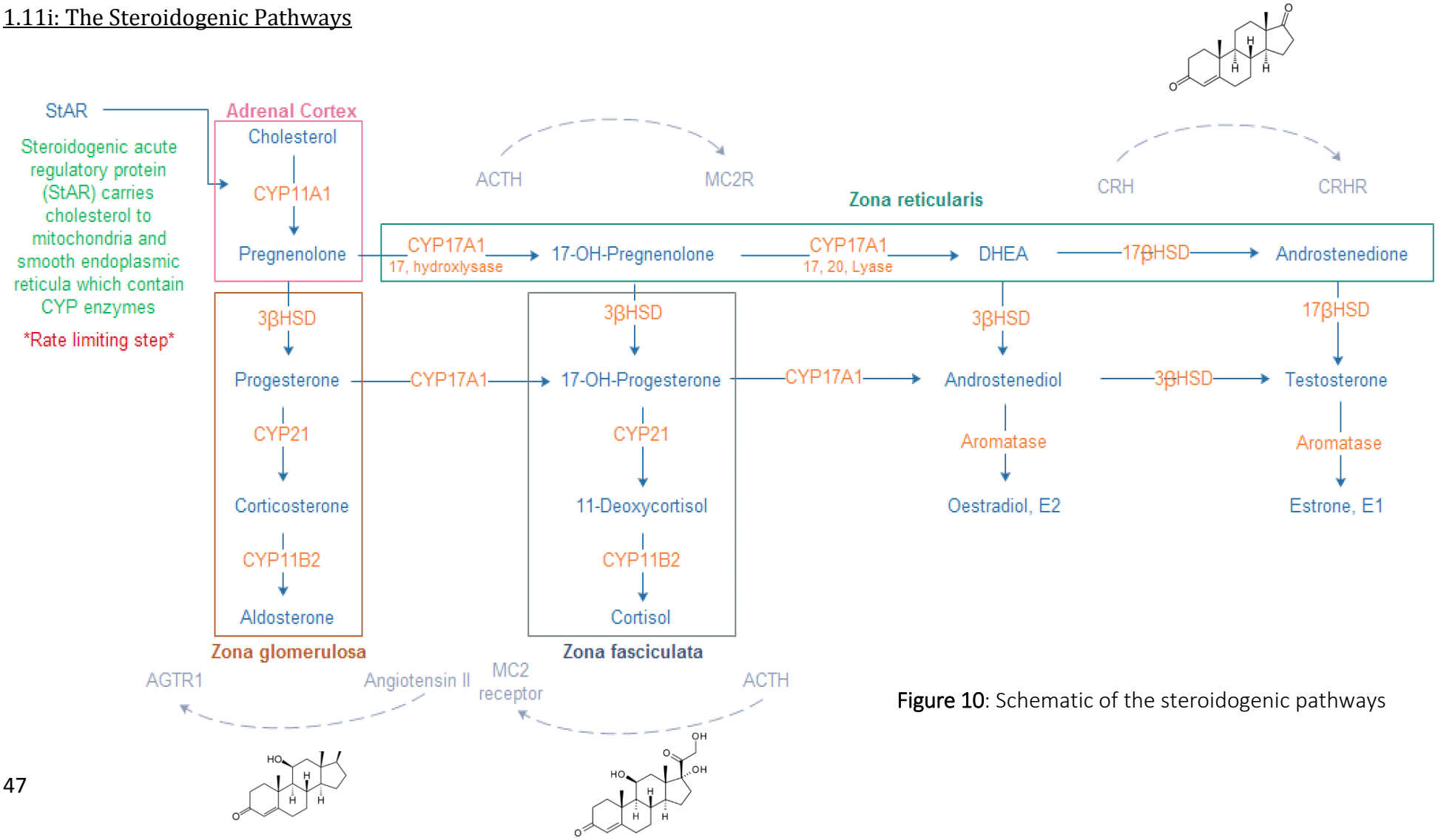

Figure 10: Schematic of the steroidogenic pathways

1.12: The Effects of Toxicological and Environmental Factors on the Adrenal Gland

1.12i: The Case of Pharmaceutically Induced Addisonian Crisis

Licensed pharmaceuticals have the ability to induce fatal adrenocortical toxicity, for example, etomidate, the anaesthetic induction agent, quickly induces fatal adrenocortical insufficiency (Addisonian crisis) by CYP11B1 inhibition, and the antidepressant and sedative aminoglutethiamide causes inhibition of CYPs 11A1 and 11B1, and MC2R receptor down-regulation (Harvey, 2014). Since both EDCs and pharmaceutical drugs inhibit the same target genes this raises concerns over the downstream clinical consequences of a more subtle perturbation.

1.12ii: Hyperadrenocorticism

Hyperadrenocorticism (HAC) or Cushing's disease is one of the most common endocrine diseases found in the companion dog (Behrend and Kemppainen, 2001). There are two main forms, the most common being pituitary dependent hyperadrenocorticism (PDH), where a benign pituitary microadenoma produces excessive levels of ACTH without being affected by the increasing levels of glucocorticoid being produced: there is no effective negative feedback. This results in adrenal cortical hyperplasia. The second, and less common cause of HAC results from high cortisol concentrations being produced by a malignant or benign adrenal tumour (adrenal dependent hyperadrenocorticism, ADH). It has been hypothesised that such adrenal pathology is likely to be caused by environmental toxins (Plechner, 2004).

Many have hypothesised that xenobiotic chemicals in the environment are having negative impacts on wildlife populations, in terms of reproductive health and endocrine related problems. A declining population of Baltic grey

seals and the ring seal population has been linked to high tissue concentrations of PCBs and DDT, which has resulted in reproductive pathology and adrenocortical hyperplasia, or Cushing's Disease (Bergman, 1999). Cushing's disease is thought to be a common problem in the Baltic Seal population. In seals, DDD metabolites showed marked binding to the adrenal cortex, via CYP interaction, which could be a route of adrenal dysfunction and hyperplasia reported in these animals (Bergman, 1999).

1.12iii: Metabolic Diseases

There is increasing evidence that fetal exposure to EDCs, especially steroid-mimicking EDCs, may lead to the development of chronic disease in adult life including metabolic diseases such as diabetes and obesity, both of which are on the increase. It has been documented since the 1970s that fetal exposure to chemicals including testosterone during early and mid-gestation induces masculinisation and virilisation in females in a dose-dependent manner, as reviewed by Padmanabham and colleagues (Padmanabhan and Veiga-Lopez, 2014). In addition to causing infertility, prenatal testosterone exposure (pregnant ewes were directly injected with testosterone proprionate twice weekly from day 30 of gestation) has been shown to cause intrauterine growth restriction including decreased fetal weight, reduced head and chest circumference, and reduced organ weights in female fetuses (Steckler et al., 2005). However, as testosterone proprionate was injected at toxicological concentrations, this is evidence of developmental origins of abnormalities rather than of EDCs causing diseases. However, certain EDCs have known androgenic properties and could therefore lead to similar abnormalities.

Obesity

In the Western World, obesity is increasing at an alarming rate, and even though most suggest this is due to a positive energy balance, there is increasing evidence of an additional environmental aetiology, including links to EDCs, as reviewed by Grun and Blumberg (Grun and Blumberg, 2009). Adipose tissue is not only the site for lipid storage, but functions as an endocrine organ and there is an ever-increasing list of obesogens, or chemicals that promote lipid accumulation. A number of EDCs have been found to act upon the PPAR receptors (peroxisome proliferator activated receptors, α, δ and γ). PPARs are nuclear receptors that have multi-functional activities including regulation of adipogenesis, involvement in glucose homeostasis and lipid metabolism. PPARγ regulates adipogenesis, and the diabetic treatments, the thiazolidinediones, cause the production of more adipocytes and therefore weight gain, via activation of PPARγ, as reviewed by Schug and colleagues (Schug et al., 2011). Schug and colleagues therefore hypothesise than any chemical with the capacity to activate PPARγ might have the same obesogenic effect. Indeed, the presence of a mutated version of PPARγ2 is significantly correlated with obesity (Ristow et al., 1998). However, a hypomorphic variant of PPARγ is associated with lower body mass and improved insulin sensitivity (Grun and Blumberg, 2009). Indeed, tributyltin, the persistent organic pollutant EDC, is an agonist of retinoid X receptors and PPARγ, and studies have shown this dual agonism by tributyltin leads to modulation of pro-adipogenic gene networks within liver, adipose tissue and bone marrow, as reviewed by Grun and colleagues (Grun and Blumberg, 2009). In addition, many other EDCs have been identified as ligands for PPARγ, including bisphenol A derivatives (Riu et al., 2011).

Metabolic Syndrome

Metabolic syndrome comprises obesity, type II diabetes, dyslipidaemia and hypertension, and is endemic in the Western World (Gluckman and Hanson, 2004). There are five hypotheses for the cause of metabolic syndrome: 1. Mitochondrial dysfunction; 2. Thrifty phenotype (able to gain weight quicker); 3. Epigenetic thrifty genotype; 4. Over-eating and little exercise; 5. EDCs (Kim and Lee, 2014). Kim and Lee reviewed EDCs as causes of mitochondrial dysfunction, and concluded that environmental chemicals, which are toxic to the mitochondria and therefore cause mitochondrial dysfunction, will predispose humans to metabolic disease development (Kim and Lee, 2014). Lee and colleagues reviewed correlations between POPs and obesity and type II diabetes, and concluded that background exposure to mixtures of POPs can increase the risk of type II diabetes in humans (Lee et al., 2014). A major economic study conducted in the EU by an expert panel evaluated exposure-outcome relationships between certain EDCs and obesity and diabetes reviewing numerous longitudinal epidemiological studies. Legler and the panel concluded DDE exposure cost €24.6 million in 2010 in obesity in 10-year-old old children and €835 million caused by adult diabetes. Phthalate exposure was predicted to cause €607 million in female diabetes associated costs, and prenatal BPA exposure was associated with a lifetime cost of €1.54 billion due to early childhood obesity. In total, EDC exposure associated obesity and diabetes is predicted to cost the EU €18-29 billion annually (Legler et al., 2015).

1.13: Hypothesis

Exposure to Endocrine Disrupting Compounds during fetal life perturbs the development of the adrenal gland, which could modify the functionality of the hypothalamic pituitary adrenal axis in adult life.

1.14: Aims

The current study is designed to use the ovine sewage sludge real-life model of exposure to a mixture of EDCs, to determine the following:

1. The effects of EDCs in sewage sludge on adrenal gland morphology and the expression of functional gene products (steroidogenic enzyme: CYP17A1 and receptor: angiotensin-II receptor 1, AGTR1) by immunohistochemistry.
2. The effects of exposure to sewage sludge on ovine fetal adrenal gland steroidogenic enzyme and receptor genes by qPCR. Specifically the expression of enzymes CYP11A1, CYP17A1 and 3βHSD and the receptors oestrogen receptor 1 (ER1), melanocortin receptor 2 (MC2R) and AGTR1.
3. To compare the effects of continuous exposure to EDCs throughout gestation to selected transient periods of exposure to the same EDCs. This will enable adrenal EDC sensitive windows to be identified in terms of the parameters listed in (1) and (2).

2. Materials and Methods

2.1: Sewage Sludge Model

Tissues used in these experiments were from animals used in a previous study performed at the James Hutton Institute, Aberdeenshire. All animals used in that study had been treated humanely according to the James Hutton Institute's Local Ethical Committee. Ewes were maintained at the Macaulay Land Use Research Institute research station, Lanarkshire, on pastures that had never previously been treated with sewage sludge. The pastures involved in this study were treated in the September of the service year with one single application of 2.25 tonnes of dry matter per hectare of thermally dried sewage sludge pellets. Control pastures were treated with 225kg per hectare per year of inorganic fertiliser to achieve similar nitrogen levels to the treated pastures. Experimental animals were maintained on the control pastures when they were not subjected to treatment exposure (see Figure 11). Grass sward in the control and treated pastures was maintained at the same height by the addition or removal of non-study sheep. After sewage treatment, the pastures were left for three weeks before grazing commenced, a legal requirement. No additional feed was given to the sheep throughout the study period.

Initially, 90 Texel ewes were included in the study, and these were weighed and their body condition score was measured prior to allocation into the five study groups, to enable nutritional standardisation throughout the groups. Ewes were euthanised at day 140 of the 147-day gestation period and fetuses collected. Four of these groups were maintained on sewage-fertilised pastures, and one group was maintained as the control on inorganic fertiliser treated pasture (Figure 11). Fifty soil samples of approximately 5cm in diameter and 5cm in depth were collected at the beginning of exposure and analysed for selected EDCs by the James Hutton Institute to provide an index

of animal exposure (data not shown, (Rhind et al., 2005a). Ewes underwent oestrous synchronisation with progestagen sponges (Chronolone, 30mg; Intervet, Cambridge, UK) before mating. Gestational age was based on conception occurring within 48 hours of sponge removal, as previously described by Fowler and colleagues (Fowler et al., 2008).

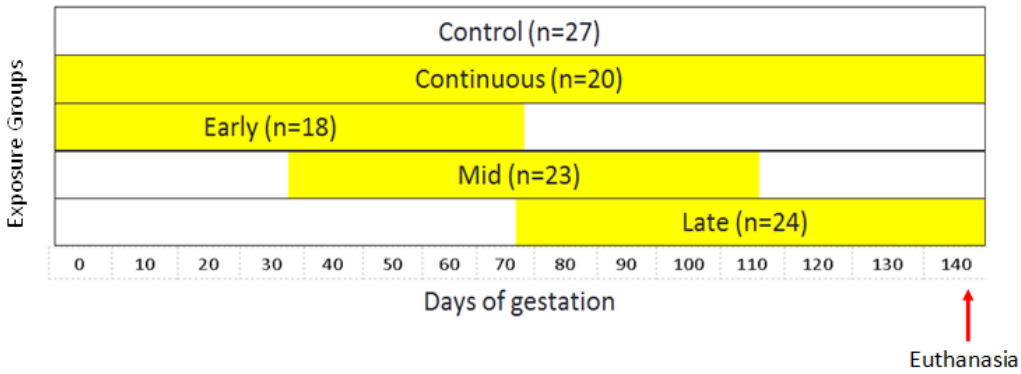

Figure 11: Exposure of pregnant ewes to sewage sludge fertiliser: experimental design. Ewes were randomly allocated to one of five treatment groups. Four of the treatment groups comprised ewes exposed to sewage sludge based fertiliser (indicated in yellow). One group of ewes was exposed continuously (0-140 days) and the remaining three groups were exposed for 80-day periods of gestation (early: 0-80 days, mid: 30-110 days and late: 60-140 days). Control ewes were maintained on inorganic fertiliser-treated pasture from 0 to 140 days. All ewes were maintained on inorganic fertiliser treated pastures when they were not in their treatment window (indicated in white). All ewes were euthanised at day 140 and fetal tissues were collected. "n" indicates number of fetuses collected from each treatment group.

2.2: Tissue Collection

All ewes from each treatment group were euthanised in 2008 at day 140 of gestation following United Kingdom Animals (Scientific Procedures) Act, 1986 Schedule 1 protocols. No animals in this study required Home Office Licencing. At euthanasia, maternal body condition score, fetal body weight and fetal anogenital distance were recorded. Blood and many tissues were collected from both dams and fetuses. Only fetal adrenal glands were used in this study. The left fetal adrenal glands were snap frozen in liquid nitrogen and stored at -80°C until simultaneous RNA, DNA and protein extraction was

carried out. The right fetal adrenal glands were fixed in Bouin's Solution (Sigma-Aldrich, Dorset, UK), and stored in 70% ethanol (Fisher-Scientific, Loughborough, UK) at 4°C until further processing was performed.

2.3: Tissue Processing

Processing for immunohistochemical and morphological analysis took place in 2014. By this time a number of specimens had dried out. Each adrenal gland was cut transversely approximately in half, placed in plastic cassettes and wax embedded into paraffin using a Leica TP1020 Tissue Processor (Leica Biosystems, Newcastle, UK). An established protocol was used during which samples were submerged consecutively in 70%, 80% and 95% ethanol, each for one hour and then finally in 100% ethanol for two hours. This was followed by three hours in Histoclear (Scientific Laboratory Supplies Ltd. Hessel, UK), followed by two-and-a-half hours in liquid paraffin. Once tissue processing was completed, the samples were embedded into wax blocks, ensuring the cut side of the adrenal gland was accessible for sectioning.

Sections of adrenal gland were cut to 5µm thick using a Leica Microtome (Leica Biosystems, Newcastle, UK) placed onto Polysine slides (ThermoScientific, Hamel Hepstead, UK), and dried on a 42°C hot plate.

2.4: Histological Analyses

Slides were stained following the standard haematoxylin and eosin staining method, and mounted with a mixture of distyrene, a plasticiser, and xylene (DPX) (VWR International Ltd. Lutterworth, UK) under a glass cover slip (VWR International Ltd. Lutterworth, UK).

2.4i: Immunohistochemistry: Manual Bench Method

Immunohistochemical staining was completed for the steroid enzyme CYP17A1 using polyclonal rabbit anti-CYP17 antibody (Biorbyt, Cambridge, UK), and the membrane-bound receptor AGTR1 using polyclonal rabbit anti-AGTR1 antibody (Abcam, Cambridge, UK). A manual bench method was initially used. Five-µm sections were submerged in histoclear for five minutes and, following wax removal sections were re-hydrated in 100%, 95% and 75% ethanol, consecutively for five minutes. Slides were then washed in distilled water for two five-minute time periods, followed by two five-minute washes in Phosphate Buffered Saline (PBS) buffer solution (Gibco by Life Technologies, Paisley, UK). The antigen retrieval step involved 15 minutes of microwave treatment at low-medium power in pH6 citrate buffer (Sigma-Aldrich, Dorset, UK). This treatment was split into three five-minute time blocks, with a one-minute rest period in between each. The slides were then rested in the hot citrate buffer for 20 minutes. Slides were washed in PBS buffer for five minutes, followed by five minutes in an endogenous peroxidase blocking solution (3% H_2O_2: Sigma-Aldrich, Dorset, UK) in distilled H_2O, followed by two five-minute buffer washes in PBS. Slides were transferred into a humidified box. Immunohistochemical staining was carried out using a Vectastain Universal Elite ABC kit (Vector Laboratories, Burlingame, USA). Sections were incubated with normal blocking serum for 20 minutes, ensuring the sample was completely covered. Primary antibody was added to the slide, a coverslip applied, and left to incubate at 4°C overnight. The coverslips were floated off using PBS with the addition of Tween20 (Merck, Germany) (100µl Tween20 per litre of PBS) (PBS/T). This was followed by two five-minute PBS/T washes. The secondary biotinylated antibody (Vector kit) was then added and incubated at room temperature for 30 minutes. After washing the slides in two five-minute PBS/T washes, an avidin biotinylated horseradish peroxidase macromolecular complex was added (Vector kit), and incubated at room temperature for 30 minutes. Following two five-minute PBS/T washes,

diaminobenzidine (DAB) chromogen (DAB Substrate Kit for Peroxidase, Vector Laboratories, Burlingame, USA) was added to each slide at 30-second intervals to ensure the time on each slide was exactly matched. When a colour change was observed (2-10 minutes), the reaction was stopped by immersion in in distilled water. Following two five-minute washes in distilled water, slides were counterstained with haematoxylin for 25 seconds. After a thorough wash in distilled water, sections were dehydrated through ethanol (two minutes each in 70%, 95% and 100% ethanol consecutively) submerged in xylene for five minutes and cover-slipped with DPX.

Optimisation: anti-AGTR1

To optimise the concentration of primary antibody, one concentration above and below the recommended concentration (taken from the antibody data sheet) was used. Dilutions used are shown in Table 1 below. Fifty-µL of diluted antibody was required per slide. Adult ovine liver tissue was used as a positive control.

Tissue	Anti-AGTR1 antibody Concentration
Liver (positive control)	20 µg/mL
	10 µg/mL
	5 µg/mL
Adrenal	20 µg/mL
	10 µg/mL
	5 µg/mL

Table 1: Working concentrations used to optimise anti-AGTR1 antibody for immunohistochemical staining

Optimisation Results

Optimal results were obtained at 10µg/mL with DAB staining taking approximately six minutes. Negative isotype controls comprised sections incubated with non-specific Rabbit IgG (Vector Laboratories, Burlingame, USA) at the same concentration as the primary antibody for each IgG concentration tested.

Optimisation: anti-CYP17A1

To optimise the concentration of CYP17A1, a range of concentrations was also tested (Table 2). Fetal ovine testis was used as the positive control.

Tissue	Anti-CYP17A1 antibody Concentrations
Testis (positive control)	5 µg/mL
	2.5 µg/mL
	1.25 µg/mL
	0.625 µg/mL
Adrenal	5 µg/mL
	2.5 µg/mL
	1.25 µg/mL
	0.625 µg/mL

Table 2: Working concentrations used to optimise anti-CYP17A1 antibody for immunohistochemical staining

Optimisation Results

The optimal concentration of anti-CYP17A1 was found to be 5µg/mL, with DAB applied for approximately nine minutes. Isotype controls (Rabbit IgG) were completed as for anti-AGTR1.

2.4ii: Leica-BOND Max

Since some variability in colour was observed between manual runs, an automated staining method was used for better standardisation. Polysine slides, mounted with paraffin embedded adrenal gland 5µm sections, were stained using a fully automated Leica-BOND Max machine (Leica BOND Max machine, Leica Biosystems, Milton Keynes, UK). The standard programme IHC procedure X was used comprising a bake and dewax step and heat-induced Epitope Retrieval (HIER) step with Bond ER (epitope retrieval) solution 1 (citrate buffer pH 6.0). The optimal concentration of antibody for immunostaining using the automated method was found to be 5µg/ml for both anti-AGTR1 and anti-CYP17A1 antibody. Corresponding isotype controls (Rabbit IgG) were used at the same concentrations as the primary antibodies, and were negative at this concentration. Each run included positive and

negative controls (Table 3). Two identical adrenal gland sections were incubated with primary antibody in each run as an intra-run control. Following completion of staining, slides were dehydrated through 70%, 90% and 100% ethanol for two minutes each, followed by mounting in DPX.

AGTR1	CYP17
Ovine liver: anti-AGTR1	Ovine testis: anti-CYP17
Adrenal: rabbit IgG x 3	Adrenal: Rabbit IgG x 3
Repeat adrenal x 2	Repeat adrenal x 2

Table 3: Controls for AGTR1 and CYP17A1 immunostaining.

2.4iii: Image analyses

Approximately eight images of each adrenal were taken with a Leica CTR5000 Light Microscope (Leica Microsystems (UK) Ltd, Milton Keynes, UK) at 50x optical zoom, and stitched together using Leica Application Software, version 3.8 (Leica Microsystems (UK) Ltd, Milton Keynes, UK) (Figure 12A). Stitched images were then analysed for percentage area stained using Image-ProPlus software (Media Cybernetics, Inc., MD, USA). Initially, the area of interest was highlighted by drawing a line around the capsule, then the DAB-stained area was superimposed with red, and the haematoxylin-stained area superimposed with yellow (Figure 12B). The percentage area stained by DAB was then calculated. This was completed for both CYP17A1 and AGTR1.

Percentage Area Stained

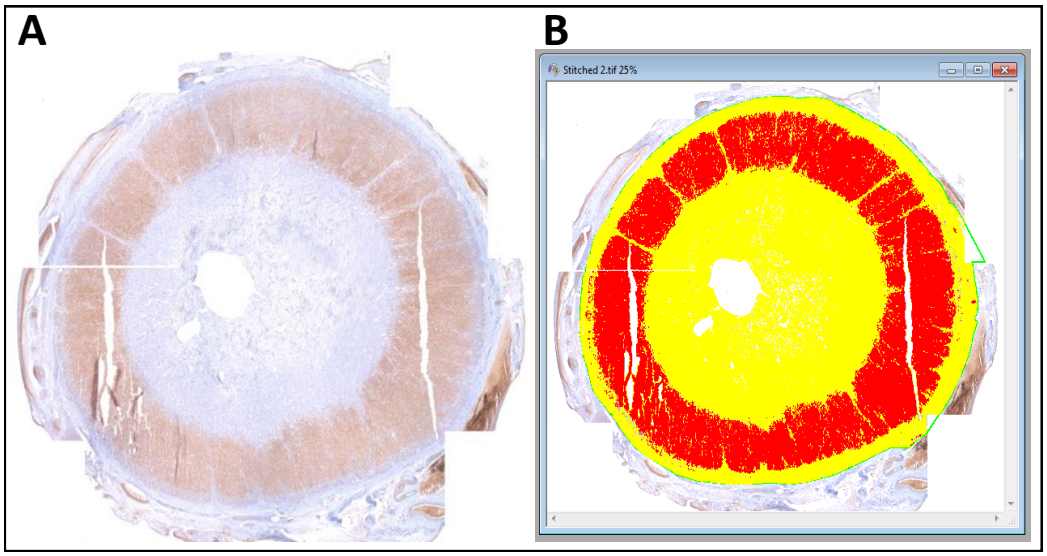

Figure 12: A: cross-section image of a typical adrenal gland stained for anti-CYP17A1. B: image analysis in which the DAB stained area has been highlighted in red and the haematoxylin stained area has been highlighted in yellow. Magnification: 50x.

The cortical thickness stained by both anti-AGTR1 and anti-CYP17A1 antibody was measured using calibrated images and Image-ProPlus software, as illustrated in Figure 13. Eight measurements were taken at regular intervals around the “clock-face” of the adrenal, and a mean value calculated.

Cortical Thickness

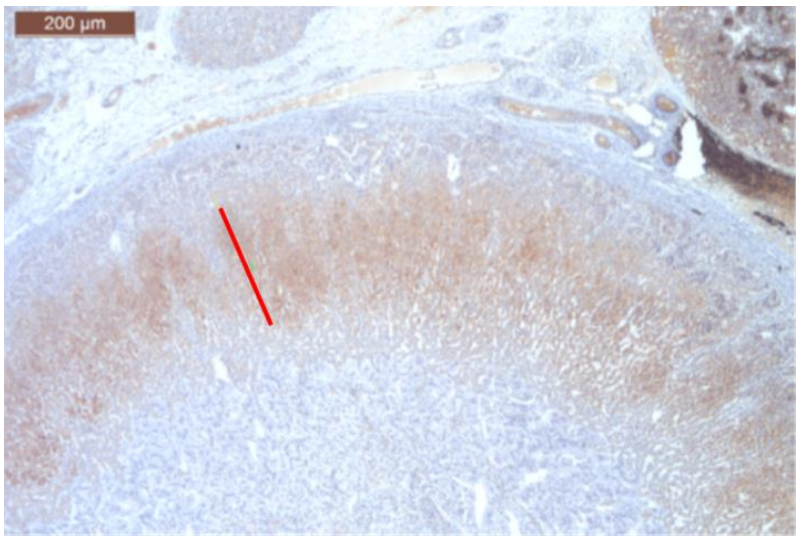

Figure 13: Representative image for AGTR1 immunostaining from which the thickness of the cortical stained region was measured using calibrated images and Image-ProPlus software. Red line shows an example measurement. Scale bar = 200µm.

2.5: Molecular Analyses

2.5i: Tissue Disruption and Homogenisation

Snap frozen left adrenal glands were weighed and homogenised in RLT lysis buffer (Qiagen, Crawley, UK.), a highly denaturing buffer containing isothiocyanate, which inactivates DNases, RNases and proteases. Twenty-µL of RLT buffer was used per 1mg of adrenal tissue. Ten-µL of β-mercaptoethanol (βME: Sigma-Aldrich, Dorset, UK) per mL of RLT was added to the lysis buffer; this was used to reduce R-S-S-R bonds in proteins, and also it acts as an antioxidant. After weighing the fetal adrenal glands, each was quartered to accommodate their relatively large size. Each quarter was placed into a 2mL RNase-free microcentrifuge tube with 600µL of the RLT/βME solution. Using a 2mm steel ball, the tissue was then homogenised using a bead-milling machine (frequency 30Hz: Scientific Laboratory Supplies Ltd,

Hessel, UK) for two minutes. The direction of the tubes was changed and homogenisation was then repeated for further two minutes. The lysates from all adrenal quarters were combined in a larger 5mL RNase-free Eppendorf tube, and the volume adjusted with RLT/βME solution required for the weight of the adrenal tissue (20µL/mg). The tubes were vortexed to ensure thorough mixing of the entire adrenal gland lysate. Six-hundred-µL aliquots of each lysate were placed into 2mL RNase-free Eppendorf tube and stored at -80°C.

2.5ii: RNA, DNA and Protein Extraction

RNA, DNA and protein were extracted from the lysate produced in section 2.5i using a Qiagen AllPrep DNA/RNA/Protein Mini kit (Qiagen, Crawley, UK); all reagents mentioned are from this kit, unless otherwise stated. The procedure was carried out following the standard kit protocol with the addition of an on-column DNase digestion step.

In brief, each 600µL aliquot of homogenised adrenal gland lysate was defrosted on ice before RNA, DNA and protein extraction. To maximise protein integrity, protease inhibitors were added: 1.5µL of Protease Inhibitor Cocktail, and 6µL of each Sigma Phosphatase Inhibitor Cocktail 2 and 3 (Sigma-Aldrich, Dorset, UK) per sample. The phosphatase inhibitors were added to allow for later analysis of phosphorylation, which was not included in this study.

RNA Extraction

The lysate was then centrifuged at >16,000*g* for three minutes, and the supernatant was transferred to a Qiagen AllPrep DNA column placed in a 2mL collection tube. This was then centrifuged at >8000*g* for 30 seconds and the column stored at 4°C for later DNA purification. Four hundred and thirty-µL of

100% ethanol was added to the remaining flow-through, and 700µL of this solution was transferred to an RNeasy spin column, placed within a 2mL collection tube. This was then centrifuged at >8000*g* for 15 seconds and the flow-through stored at 4°C for later protein purification. This step was repeated to ensure all the initial lysate was processed. Three hundred and fifty-µL of wash Buffer RW1 was added to the RNeasy spin column and centrifuged. DNase I incubation mix (10µL DNase I stock solution and 70µL RDD buffer: RNase-Free DNase Set, Sigma-Aldrich, Dorset, UK) was then added directly to the RNeasy column, and incubated at room temperature (20-30^{o}C) for 15 minutes. Once incubation was complete, a further 350µL of wash buffer RW1, followed by two 500µL applications of buffer RPE, a mild wash buffer to remove traces of salt, were added to further wash the column, with centrifugation at >8000*g* for 15 seconds following each addition. Finally to ensure no wash buffers remained, the RNeasy column was added to a new, dry collecting tube followed by a final centrifugation (>16,000*g* for one minute). The RNeasy column was placed in a 1.5mL collection tube and 50µL RNase-free water was directly added to the spin column membrane, centrifuged (8000*g* for one minute) and the RNA-containing flow-through was stored at -80°C for later cDNA synthesis.

DNA Extraction

The AllPrep DNA column was removed from the fridge and 500µL of wash buffers AW1 and AW2 were added successively to the column, with centrifugation at >8000*g* for 15 seconds after each addition, and the flow-through discarded. One-hundred µL of elution buffer EB (preheated to 70°C) was added directly to the column membrane, and incubated at room temperature for two minutes, followed by a final centrifugation at >8000*g* for one minute into a new 1.5mL collecting tube. The DNA-containing flow-through was stored at -80°C.

Protein Extraction

Nine hundred-µL of buffer APP, a protein purification buffer which contains zinc chloride, was vigorously mixed with the previously stored flow-through from the RNeasy column and incubated at room temperature for 10 minutes, followed by 10 minutes of centrifugation at >16,000*g*. The supernatant was then decanted and 500µL of 70% ethanol was added to the protein pellet, ensuring that the pellet was floating within the ethanol. The protein pellet was then centrifuged for one minute at >16,000*g*, the ethanol removed and the pellet was left to dryat room temperature for a maximum of four minutes. After drying, 50µL of Modified Reswell Solution (MRS; 7M urea, 2M thiourea, 4% (w/v) CHAPS, 0.3% (w/v) DTT) was added to enable protein solubilisation. The protein pellets were then stored on ice until full re-suspension had occurred, then stored at -80°C.

2.5iii: Complementary DNA Synthesis

RNA purity and concentrations were measured using a NanoDrop ® ND-1000 spectrophotometer and Labtech International Ltd software (Ringerm, UK). A cDNA copy of the RNA was then generated by reverse transcription using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Burgess Hill, UK) following kit procedure A using random hexamer primers. All reagents used were from the Transcriptor First Strand cDNA Synthesis Kit unless otherwise mentioned.

Eleven-µL of each RNA sample (diluted to matching concentrations using PCR grade water (Roche Diagnostics Ltd, UK)) was added to PCR tubes. Nine-µL of master mix containing transcriptor RT reaction buffer, protector RNase inhibitor, deoxynucleotide mix, transcriptor reverse transcriptase and random hexamer primers was premixed in bulk (volumes following manufacturer's

instructions) was also added. PCR tubes were centrifuged at <7000*g* for 15 seconds, and incubated in a Techne TC-512 thermal block cycler (Keison Products, Exeter, UK) for 10 minutes at 25°C, 30 minutes at 55°C, five minutes at 85°C and maintained at 4°C. The resulting cDNA was stored at -80°C. The same reaction was completed for pooled cDNA samples from each treatment group replacing the transcriptor reverse transcriptase with PCR-grade water to produce RT-negative controls.

2.5iv: Real-Time PCR

The expression of gene cDNA was quantified by quantitative real-time PCR (qRT-PCR) using a Roche LightCycler ® 480 machine (Roche, Burgess Hill, UK). The target gene was quantified using gene-specific primers for the following target genes: CYP11A1, CYP17A1, 3βHSD, ER1, MC2R, CRHR1 and AGTR1. The reference housekeeping genes were YWHAZ, GAPDH and HPRT1 as per previously published work on the ovine sewage sludge model (Lea et al., 2014a). The three reference genes and ER1 were detected using TaqMan® probes with a 5'-6'-carboxyfluoroscein (FAM) reporter and 3'-labelled 3'-6'-carboxyl-N,N,N',N'-tetramethtylrhodamine (TAMRA) quencher (Eurofins MWG Operon, London UK). All other genes were detected using Roche Universal Probe Library probes, 5'-labelled with FAM and 3'-labelled with a reporter (Roche Diagnostics Ltd., West Sussex, UK), designed using the Roche ProbeFinder™ software (http://lifescience.roche.com/ accessed on 15th and 23rd of July and 18th August 2014). All primers and probes were designed from the respective *Ovis aries* gene sequences. Details of primers and probes are summarised in Table 4.

Gene	NCBI Accession	Primer / Probe	Base Sequence
MC2R	NM_001009442.1	Forward	CGTCCTGACGGTCCTCTG
	Gene length: 1023bp	Reverse	ATGGGAGAAGGTCACAATG
	Amplicon length: 64bp		G
		Probe	CAGTGGCA
		Roche probe	#59
CRHR1	NM_001009727.1	Forward	GCCTGGGGTGTACACTGATT
	Gene length: 1248bp	Reverse	AATTGATCAGCAGGACCAAG
	Amplicon length: 62bp		AT
		Probe	CCAGGGCC
		Roche probe	#81
AGTR1	NM_001009744.1	Forward	CCACATTCTGCCATGTTTTG
	Gene ength: 2358bp	Reverse	TTCATCCAGTTTCTGACATCA
	Amplicon length: 71bp		GTT
		Probe	GATGCCCA
		Roche probe	#105
3BHSD1	NM_001135932.1	Forward	TGACCTCAATTACACTTTGAG
	Gene length: 1122bp	Reverse	CA
	Amplicon length: 61bp		AAGGCTCATCCGGGAATC
		Probe	CTTCTGCC
		Roche probe	#29
CYP11A1	NM_001093789.1	Forward	CCACTGCTGAATCCAGTGTC
	Gene length: 1826bp	Reverse	CTGCTTGATGCGCTTGTG
	Amplicon length: 60bp	Probe	CAGCCTCC
		Roche probe	#75
CYP17A1	NM_001009483.1	Forward	CCACCCCACACAATTAGAGG
	Gene length: 1728bp	Reverse	CGGGGAGGAAGAAGGAAT
	Amplicon length: 60bp	Probe	CTTCTCCC
		Roche probe	#7
ER1	Europhins	Forward	TTTCGAGGGTCACTACCAGT
	Gene length: 5779bp	Reverse	GTCAATTGCCTCATTGAACC
	Amplicon length: 66bp	Probe	CAGCTGGCCACCACTGGCTG
			C
YWHAZ	Europhins	Forward	GGAGCCCGTAGGTCATCTTG
	Gene length: 738bp	Reverse	CTCGAGCCATCTGCTGTTTTT
	Amplicon length: 85bp	Probe	CAGCACCTTCCGTCTTTTGCT
			CAATACTGGAGA
GAPDH	Europhins	Forward	ACTACCATGGAGAAGGCTG
	Gene length: 1285bp	Reverse	G
	Amplicon length: 103bp	Probe	GGTTCACGCCCATCACA
			AGAGGGTCATCATCTCTGCA
			CCTTCT
HPRT1	Europhins	Forward	GAACGGCTGGCTCGAG
	Gene length:1405bp	Reverse	CCAACAGGTCGGCAAAG
	Amplicon length: 100bp	Probe	AATGTGATGGCCACCCATCT
			CCT

Table 4: Primer and Probe Sequences for quantitive Real-Time PCR

2.5v: Optimisation

Primer optimisation

Primer optimisation to maximise the performance of qRT-PCR was completed by running cycles with varying concentrations of the forward and reverse primers using a 1-in-10 dilution of pooled cDNA for each gene. This involved a cocktail of 10µL of ProbeMaster master-mix (Roche Diagnostics Ltd., Burgess Hill, UK), varying volumes of forward and reverse primers and probes (see Tables 5 and 6), cDNA and PCR-grade water to make up to a final volume of 20µL. For TaqMan® Probes, 0.7µL of probe was required per reaction, whereas Roche Probes required 0.1µL of probe per reaction. Each optimisation was performed in duplicate. qRT-PCR 96-well plates were then centrifuged for two minutes at 1000*g*, before undergoing an automated Roche LightCycler® 480 qRT-PCR reaction following the parameters given in Table 7.

			Forward : Reverse Primer Ratio			
	Primer conc (pmol/µL)	Probe conc (pmol/µL)	300F/300R (µL)	300F/900R (µL)	900F/300R (µL)	900F/900R (µL)
Master Mix			10.0	10.0	10.0	10.0
Forward Primer	5		1.2	1.2	3.6	3.6
Reverse Primer	5		1.2	3.6	1.2	3.6
Probe		5	0.7	0.7	0.7	0.7
PCR-grade H_2O			4.9	2.5	2.5	0.1
cDNA			2.0	2.0	2.0	2.0

Table 5: Roche 20µL System Real Time PCR Optimisation for TaqMan® Probes

			Forward : Reverse Primer Ratio			
	Primer conc (pmol/µL)	Probe conc (pmol/µL)	300F/300R (µL)	300F/900R (µL)	900F/300R (µL)	900F/900R (µL)
Master Mix			10.0	10.0	10.0	10.0
Forward Primer	5		1.2	1.2	3.6	3.6
Reverse Primer	5		1.2	3.6	1.2	3.6
Probe		5	0.1	0.1	0.1	0.1
PCR-grade H_2O			5.5	3.1	3.1	0.7
cDNA			2.0	2.0	2.0	2.0

Table 6: Roche 20µL System Real Time PCR Optimisation for Roche Universal Probe Library Probes

Temperature (°C)	Time	Number of Cycles	Phase
95	10 Minutes	1	Pre-incubation
96	10 Seconds	45	Amplification
60	30 Seconds	45	
72	1 Second	45	
40	30 Seconds	1	Cooling

Table 7: Real-time PCR Run cycle

Standard Curve Protocol

Two-µL of each sample was combined to make a pooled cDNA stock solution. This was diluted in a 1 in 5 serial dilution: Neat, 1:5, 1:25, 1:125, 1:625 and 1:3125. A PCR run was completed for each gene at optimised primer concentrations, along with qPCR grade water as a negative control. The PCR was then completed following the protocol in Table 7, after a two-minute centrifugation at 2000rpm. All standard curves were repeated four times, and a mean of the efficiencies was used for later relative gene expression analysis.

Gel electrophoresis

As the amplicons were too small for sequencing (apart from YWHAZ, which was successfully and correctly sequenced by SourceBioScience, Nottingham, UK), electrophoresis was used to determine amplicon size. Ethidium bromide (7.5µL) fluorescence dye was added to a 1.5% agar gel, and PCR product was mixed with loading buffer and run with a 100-1000 base pair ladder at 100V for two hours in 1% Tris-Acetate-EDTA buffer. Images were taken using IQuant Capture 3000 (GE Health Care, Little Chafont, UK).

2.5vi: PCR Runs

All runs used the master mix cocktail (Tables 5 and 6) using 2µL of each sample cDNA, following the run protocol in Table 7 using a Roche LightCycler® 480. A pooled cDNA sample was run under the same conditions on each plate for each gene as a calibrator to control for plate-to-plate variation, as well as PCR grade water as a negative control (no template control). All samples were measured in duplicate. As for optimisation and standard curve protocols, each plate was sealed and centrifuged for two minutes at 2000rpm before running the PCR protocol (Table 7). RT-negative controls were run under the same conditions for each gene to determine the contribution of contaminating genomic DNA to the overall target sample.

Gene expression data were analysed relative to a stable reference gene using the Roche Applied Science E-method, using Roche software (Roche Diagnostics Ltd., Burgess Hill, UK). This normalises the genes of interest to the most stable reference gene, normalising sample-to-sample differences, and an inter-assay calibrator to normalise for run-to-run differences, using efficiencies produced by the standard curves.

2.5vii: geNorm Method

Gene expression levels need to be normalised using stable internal control (housekeeping) genes (Vandesompele et al., 2002), and a housekeeping gene is required to measure expression accurately. geNorm is an algorithm which determines the most stable housekeeping reference gene from a selection of tested reference gene Ct values. Ct values from three housekeeping genes (HPRT1, GAPDH and YWHAZ) from 53 different cDNA samples (samples from each treatment group and control) were entered into the geNorm excel macro. This macro determined variation of these housekeeping genes within

the different samples. The best housekeeping gene has the smallest variation in Ct value within the 53 samples across the treatment groups. In this case, YWHAZ was the most stable gene, and was therefore selected as the housekeeper gene. geNorm provides an M value (average expression stability value, which needs to be less than 1). The M value for YWHAZ was 0.76.

2.6: Statistical Analysis

Statistical analysis was completed using the GenStat® statistical software (VSN International Ltd, Hemel Hempstead, UK). Since data were generated from an unbalanced two-way design, they were analysed by the mixed linear restricted maximum likelihood (REML) algorithm in which treatment and gender formed the fixed effects, adrenal weights and fetal weights were covariates. The ewe formed the random effect and the model recognised the ewe as the replicated variable.

3. Results

3.1: Fetal Infomation

Fetal adrenals were collected from 112 ovine fetuses from 58 ewes allocated to each of the five treatment groups described in materials and methods and listed in Table 8. Significantly more male than female fetuses were included in this study ($p=0.05$, t-test).

Treatment Group	No. Ewes	No. Fetuses	Gender		Parity			Left adrenal (mg)	SEM	Right adrenal (mg)	SEM
			Male	Female	Singles	Twins	Triplets				
Control	14	27	18	9	1	26	0	298.23	12.05	327.69	15.23
Continuous	11	20	12	8	4	10	6	291.1	18.39	270.51	10.01
Early	10	18	9	9	2	16	0	256.28	14.02	238.84	10.12
Mid	11	23	12	11	1	16	6	270.7	10.47	292.03	13.42
Late	12	24	14	10	2	16	6	240.14	10.01	258.62	11.92
TOTAL	58	112	65	47	10	84	18	-	-	-	-

Table 8: Effects of sewage sludge chemicals on gender, litter size and fetal adrenal weight. Values represent mean ± SEM of average raw adrenal weights for the left and right adrenal glands. The left adrenal gland was snap frozen in liquid nitrogen, and then stored at -80°C for RNA, DNA and protein extraction, and further molecular analyses. The right adrenal was fixed in Bouin's solution, and then stored in 70% ethanol at 4°C for tissue processing and morphological analyses.

3.2: Histology Studies

3.2i: Adrenal Sample Set

Out of the 112 adrenals that were fixed in Bouin's solution and stored in 70% ethanol, only 50 could be processed for morphological analyses. A number of the stored adrenals had dried out due to the length of time between tissue harvesting and further processing and this was beyond the control of the study. Despite rehydrating all affected samples, it was not possible to obtain satisfactory 5µm sections from them all. Table 9 below shows the number of fetuses that were satisfactorily sectioned for morphological analyses across the treatment groups. As with the complete sample set, there were more male than female fetuses in this sample set (p=0.025, t-test).

Treatment Group	No. Ewes	No. fetuses	Gender		Parity		
			Male	Female	Singles	Twins	Triplets
Control	7	9	5	4	1	8	0
Continuous	8	8	6	2	3	4	1
Early	7	8	4	4	2	5	1
Mid	9	13	9	4	1	8	4
Late*	7	12	7	4	1	8	2
Total	38	50	31	18	8	33	8

Table 9: Fetal gender and total number of adrenals processed for immunohistochemical analysis for each treatment group. * Gender and parity of one fetus is unknown in this group.

3.2ii: Histological Appearance

Fetal adrenals were sectioned and stained with haematoxylin and eosin. Figure 14 below shows a representative section of a fetal adrenal gland (5µm) at 100x magnification. The layers of the cortex, the chromaffin cells of the medulla all encased within the fibrous capsule can clearly be seen. Figure 15

shows high power images of each region of the day 140 ovine fetal adrenal gland. Initial gross histological examination of these fetal adrenal glands showed no obvious differences between the different treatment groups.

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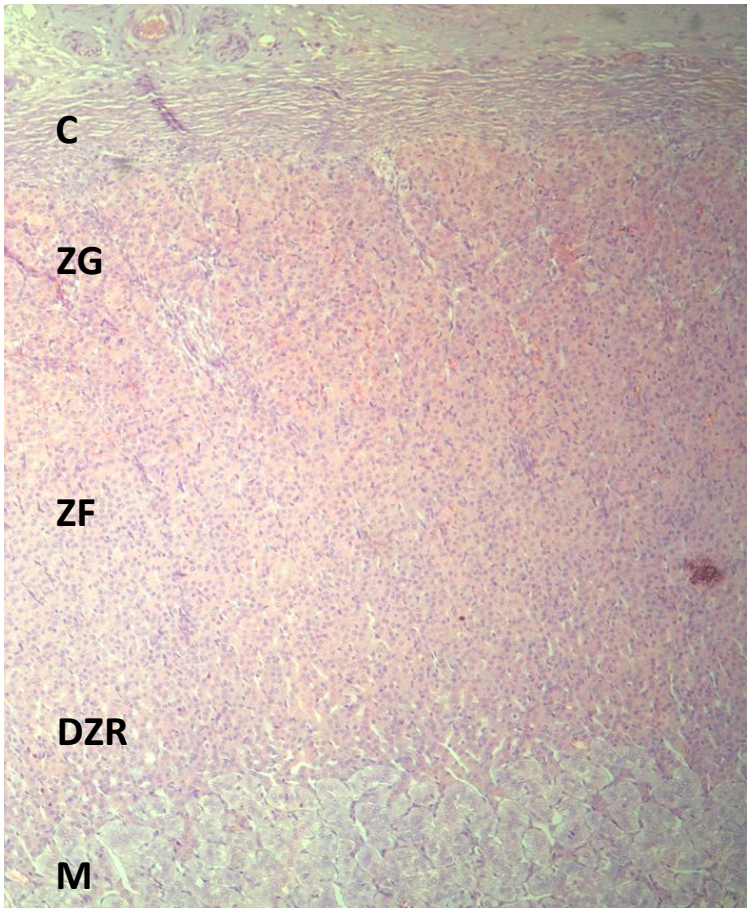

Figure 14: Morphological characterisation of a day 140 ovine fetal adrenal gland from the control group. ZG: zona glomerulosa: irregular ovoid clusters of cells surrounded by trabeculae. Continuous with fibrocollagenous capsule (C). Large number of capillaries. Large round nuclei and little cytoplasm. Produce mineralocorticoids. ZF: zona fasciculata: Narrow columns of cells with large capillaries. Cells contain pale staining cytoplasm (lipid droplets). Secrete glucocorticoids. DZR: developing zona reticularis: will develop post-natally to be an irregular network of branching clusters of small granular cells with darker staining cytoplasm, which secrete androgens and glucocorticoids. Not found in the fetal adrenal gland. M: medulla: clusters of granular, faintly basophilic chromaffin cells, supported by stroma, contains ample capillaries. The granules contain catecholamines: epinephrine and nor-epinephrine. Magnification = 100x.

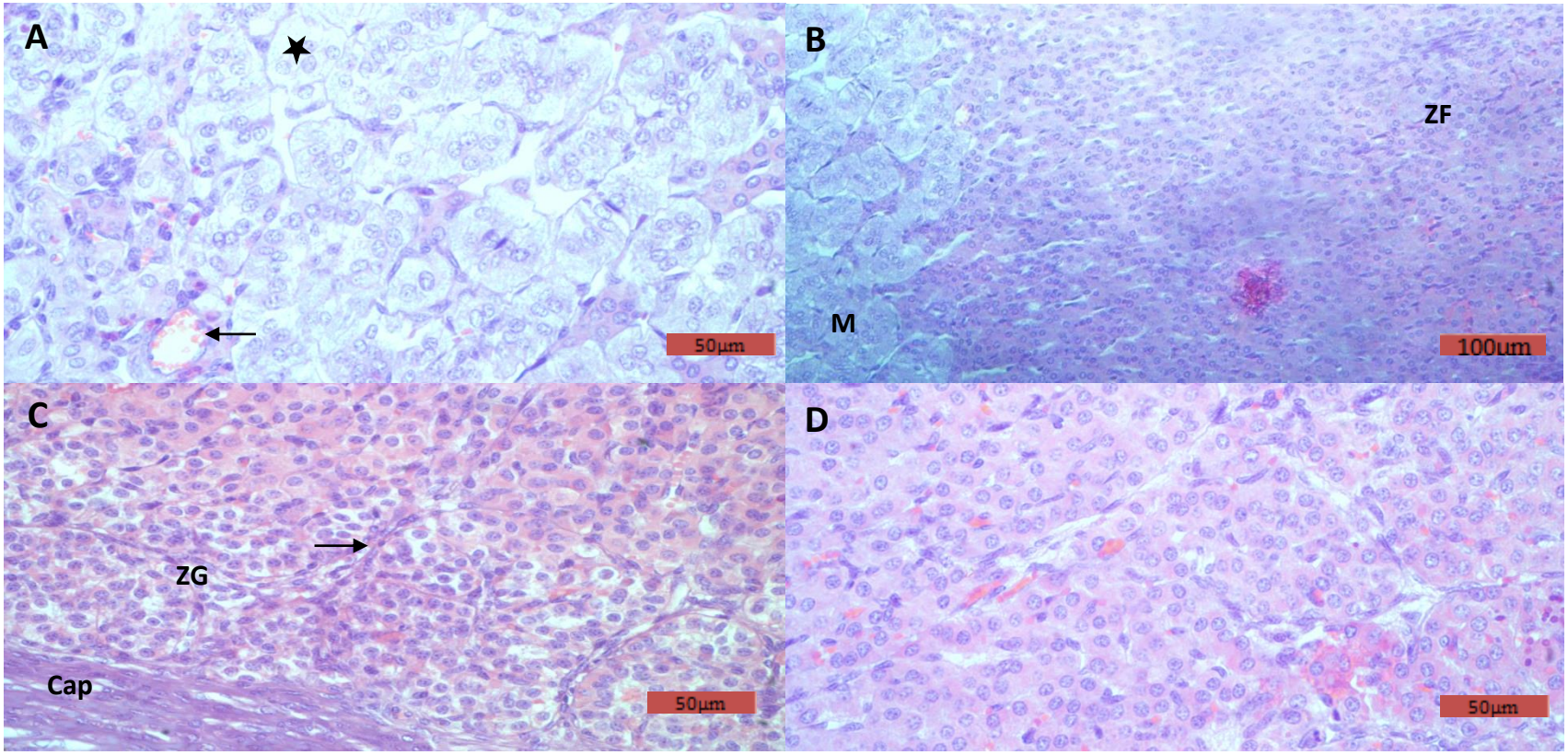
A
50µm
B
ZF
M
100um
C
ZG
Cap
50µm
D
50µm

Figure 15: Histological features of the day 140 ovine fetal adrenal gland from control group. A: Adrenal medulla. Predominantly chromaffin cells are present (*) with a capillary within the supporting stroma (arrow). B: Adrenal cortex: zona fasciculata (ZF) and adrenal medulla (M). C: Adrenal cortex: ovoid clusters of the zona glomerulosa (ZG), adrenal capsule (Cap) and fibrous trabeculae (arrow). D: Adrenal cortex: zona fasciculata. All sections were stained with haematoxylin and eosin.

3.3: Immunolocalisation of CYP17A1 and AGTR1

Sections (5µm) were incubated with one of two specific anti-ovine steroidogenic antibodies, specifically the enzyme CYP17A1 or the receptor AGTR1.

Figure 16 shows typical stained sections at 50x and 200x magnification for both CYP17A1 and AGTR1. The intensity of the staining for CYP17A1 varies throughout the cortex, with the outer most layer, the zona glomerulosa, staining relatively less than the rest of the developing cortex (mainly zona fasciulata, as the zona reticularis is not fully developed until after birth (Tangalakis et al., 1992)). The central medulla and capsule show minimal staining by the anti-CYP17A1 antibody, however, some steroidogenic cells were stained around the central medullary vein. Anti-AGTR1 also predominantly stains the adrenal cortex, including the outer-most zone, the zona glomerulosa and the zona fasciculata; however, some specific staining can also be seen in the medulla and around the central medullary vein. No staining was observed on negative isotype Rabbit IgG controls.

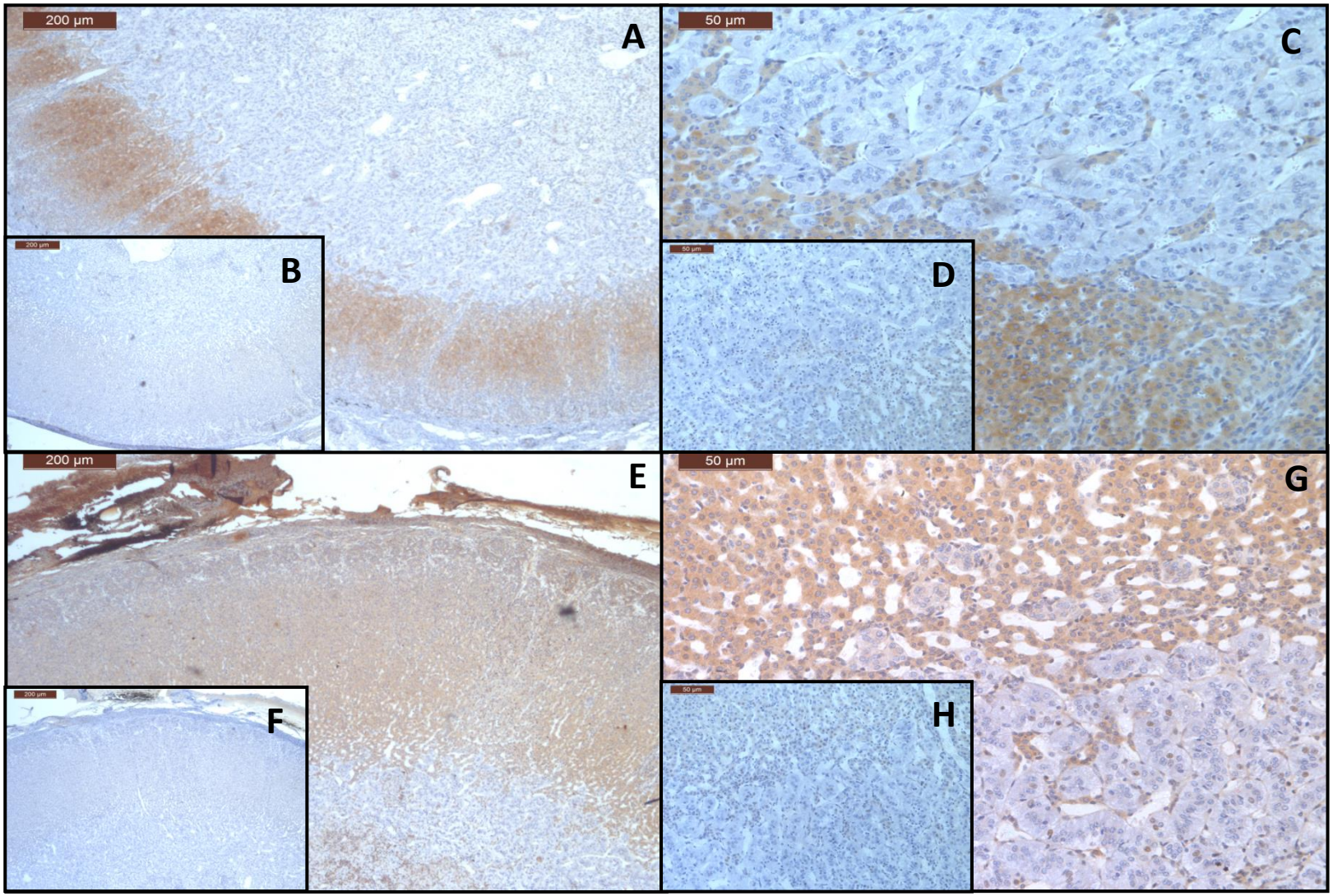
200 µm
A
B
50 µm
C
D
200 µm
E
F
50 µm
G
H

Figure 16: Localisation of CYP17A1 and AGTR1 protein in day 140 ovine fetal adrenal: A, C: CYP17A1 immunostaining of the adrenal cortex at low (A) and high (C) magnification. C shows the cortical-medullary boundary with some positive staining in the medulla. E, G: AGTR1 immunostaining at low (E) and high (G) magnification showing dark staining within the cortex and more limited staining in the medulla. Low and high magnification = 50x and 200x respectively. Inserts (B, D, F and H) are the corresponding negative controls incubated with non-specific Rabbit IgG. All are counterstained with haematoxylin.

3.3i: Cortical Thickness

Since CYP17A1 and AGTR1 antigen were primarily localised to the developing cortex (Figure 16), CYP17A1 primarily being found in the zona fasciculata, and AGTR1 being found in the zona glomerulosa and zona fasciculata, the thickness of the respective cortical regions were measured. A mean of eight measurements were taken at regular intervals across the whole adrenal section, for each sample (see materials and methods). Figure 17A and B show the scatter of the measurements for CYP17A1 and AGTR1 respectively. There was a significant treatment effect on cortical thickness as measured by CYP17A1 ($p=0.037$) and AGTR1 ($p=0.031$) (REML). This is further illustrated in Figure 18A and B showing the predicted means for CYP17A1 and AGTR1 generated by REML analysis. With both markers, the late gestation exposure groups showed the greatest difference from controls and had the lowest cortical thickness (Figure 17A, B, Figure 18A, B). AGTR1 also exhibited a significant gender effect ($p= 0.027$) and treatment by gender interaction ($p=0.048$) and Figure 18D illustrates that the greatest effects were seen in the females (REML). There was a suggestion of a treatment by gender interaction for CYP17A1 ($p=0.081$) and Figure 18C suggests that the greatest variation is between the females in the control group and the females in the mid and late gestation treatment groups.

3.3ii: Percentage Area Stained

No significant treatment effects were observed on percentage cellular area stained for CYP17A1 or AGTR1 (Figure 17C and D respectively). Percentage area stained by AGTR1 was consistently greater than that measured by CYP17A1 (Figure 17E). This is likely due to the staining observed in the zona glomerulosa and in the medulla. Despite the lack of a treatment effect, lowest levels were observed in the late gestation treatment group for both CYP17A1 and AGTR1. A significant CYP17A1 treatment by gender effect was observed ($p=0.04$, REML). Wide variation in staining was observed within the different treatment groups and this was particularly evident for AGTR1 (Figure 17D).

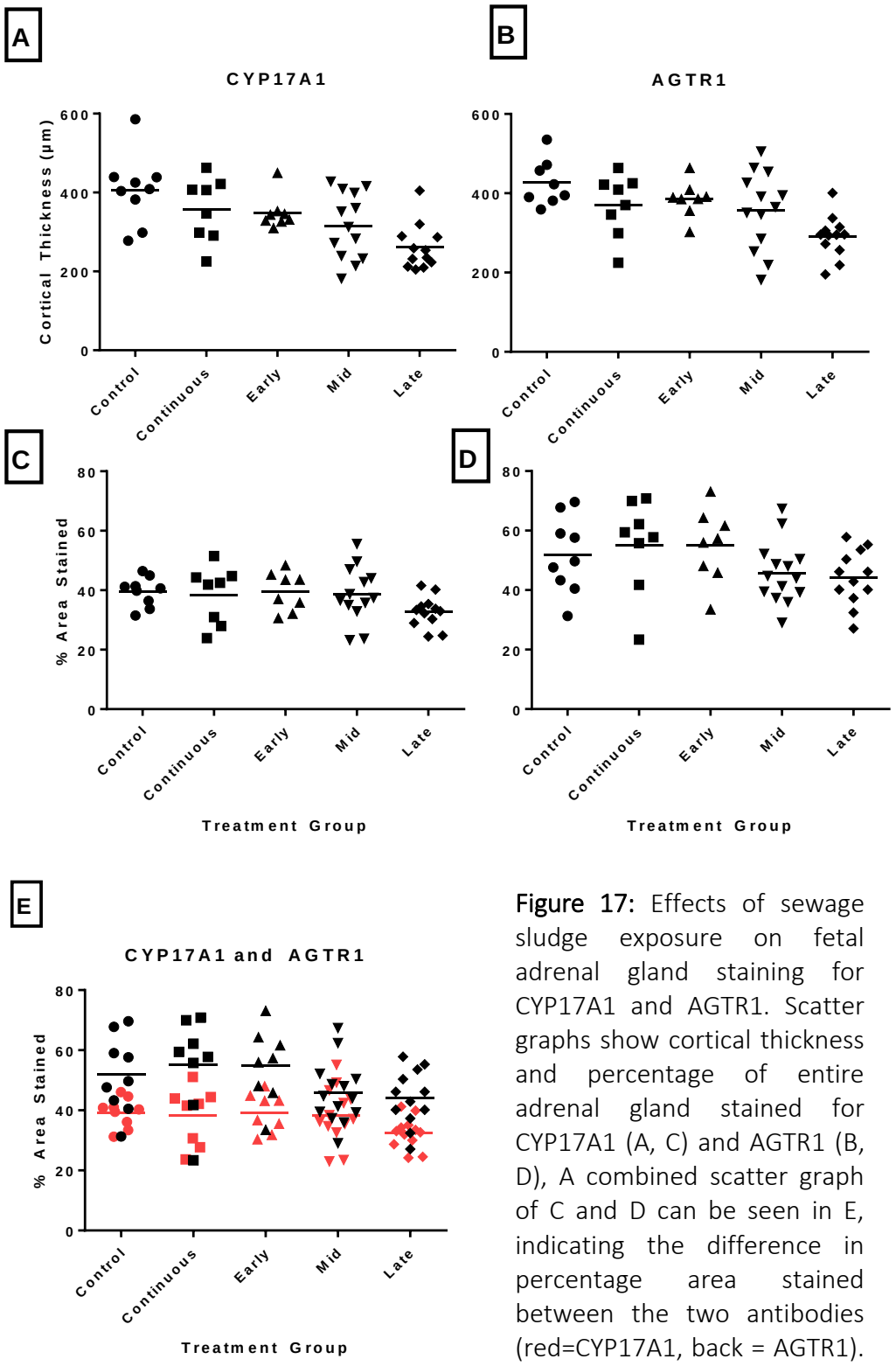

Figure 17: Effects of sewage sludge exposure on fetal adrenal gland staining for CYP17A1 and AGTR1. Scatter graphs show cortical thickness and percentage of entire adrenal gland stained for CYP17A1 (A, C) and AGTR1 (B, D), A combined scatter graph of C and D can be seen in E, indicating the difference in percentage area stained between the two antibodies (red=CYP17A1, back = AGTR1). Horizontal lines indicate the mean values. n=50 [Control (9), Continuous (8), Early (8), Mid (13), Late (12)].

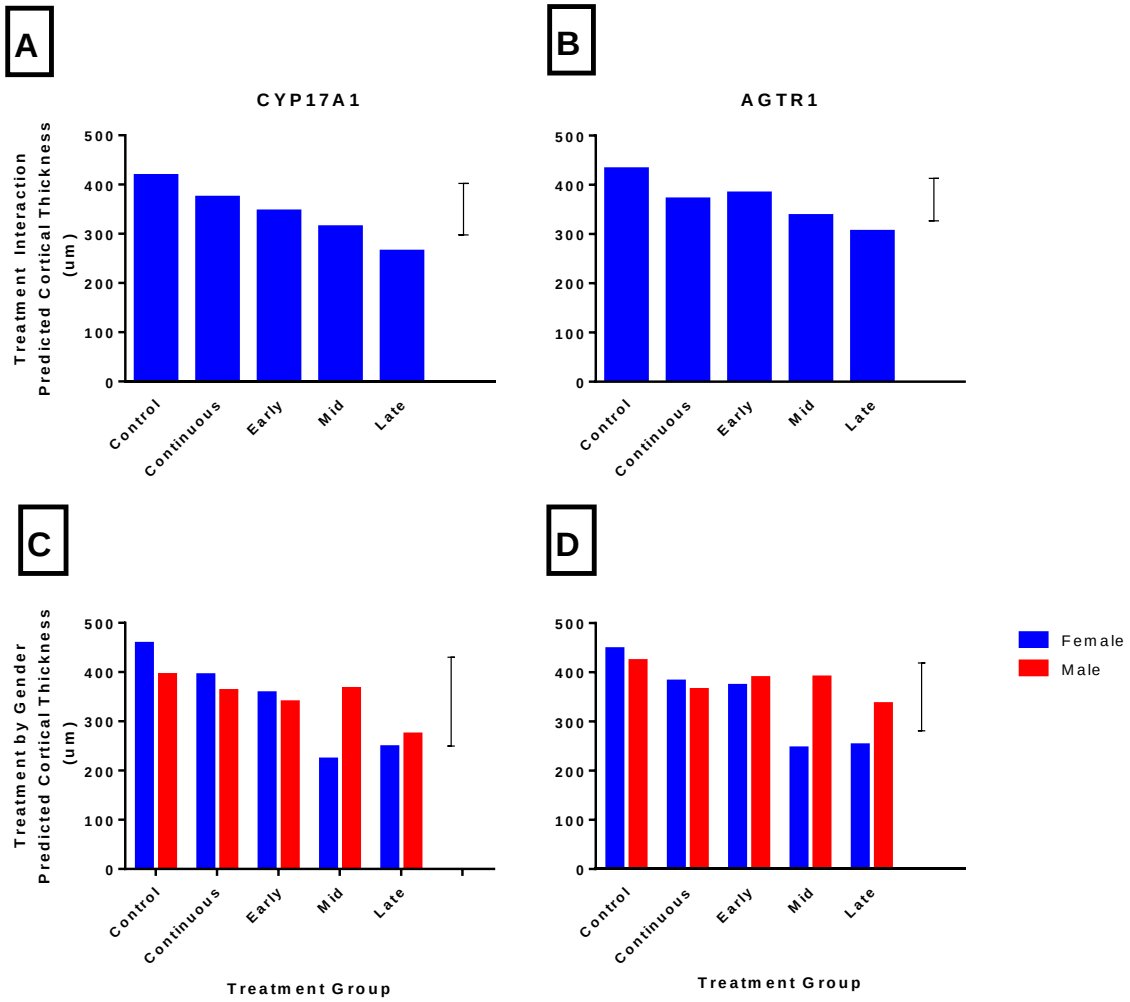

Figure 18: Effects of maternal sewage sludge exposure on day 140 fetal adrenal gland staining for CYP17A1 and AGTR1. Histograms showing predicted values for treatment interactions (A, B) and treatment by gender interactions (C, D) for cortical thickness staining for CYP17A1 (A, C) and AGTR1 (B, D). Line indicates SED.

3.4: Real-time PCR Optimisation

A number of steps were taken to optimise the real time PCR method in these experiments and to minimise non-specific effects, including investigating the presence of contaminating genomic DNA, optimising the concentration of the primers, checking the size (base pairs) of the PCR products as an indication of reaction specificity, and determination of the reaction efficiency.

3.4i: RT-Negative Controls

A reverse-transcriptase negative (RT-negative) control is a reaction containing all the reverse transcriptase PCR (RT-PCR) reagents but omitting the reverse transcriptase enzyme. Any subsequent amplification can therefore only be attributed to contaminating genomic DNA. During the extraction of RNA, a DNA digestion step using DNase was included to remove contaminating genomic DNA. However, since it is impossible to remove all contaminating genomic DNA, it is paramount to determine the residual level of contaminating genomic DNA within the samples. Figure 19 shows an example of RT-negative control results for 3βHSD using pooled cDNA from each exposure group. Amplification of cDNA occurring after 19 cycles represents 3βHSD whereas the amplification curves arising after 31 cycles represent contaminating genomic DNA. Figure 14 therefore shows that the Ct values for the contaminating genomic DNA are far higher than the Ct values of the cDNA. Since more cycles are required to amplify the genomic DNA, it is present at much lower levels than the cDNA of interest. An RT-negative control Ct value which is 10 cycles higher than that of the cDNA indicates that the contaminating genomic DNA is contributing 0.1% to the target signal and is therefore deemed negligible (Biosystems, 2015), and a difference of 6 cycles is deemed as acceptable (Dr M Rae, personal communication). Comparisons of the cDNA and the RT-negative controls (contaminating

genomic DNA) for all target genes (target and reference) are shown in Table 10 below.

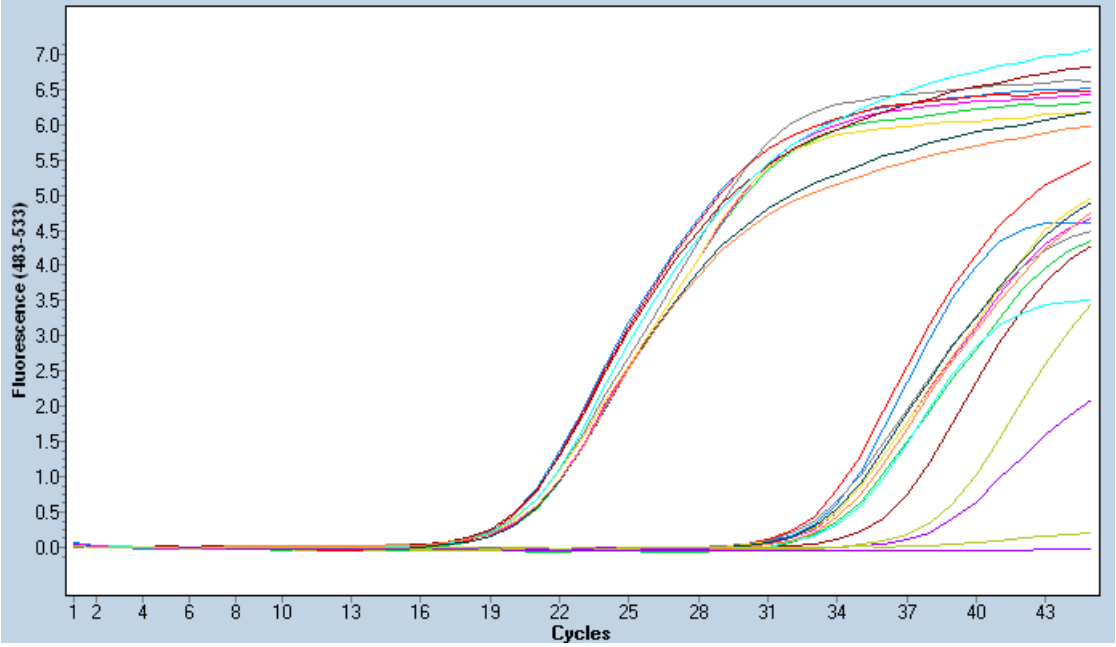

Figure 19: A representative graph for the target gene 3βHSD showing florescence of cDNA (left amplification curves) and contaminating genomic DNA (RT-negative controls; right amplification curves) for pooled treatment group samples.

Gene and Tx Group	RT Negative	cDNA	Difference
AGTR1			
Control	34.03840373	24.95536873	9.083035003
Continuous	34.555751	25.59812506	8.957625935
Early	34.66113711	25.1755701	9.485567005
Mid	34.82585518	24.08320749	10.74264769
Late	35.16197366	25.20807654	9.953897121
MC2R			
Control	33.81995755	25.67229426	8.147663289
Continuous	33.25877879	25.88866423	7.370114563
Early	34.89240839	25.70964675	9.182761645
Mid	34.62689545	25.22536167	9.401533774
Late	33.67649656	26.02063952	7.655857041
3βHSD			
Control	33.22814964	19.90563104	13.3225186
Continuous	33.64775655	20.56508657	13.08266997
Early	33.28737794	20.24032829	13.04704965
Mid	34.98590843	20.16419048	14.82171795

Late	33.49055115	20.54528345	12.9452677
CYP11A1			
Control	32.41179171	21.24861115	11.16318056
Continuous	32.87753198	21.93883129	10.93870069
Early	32.65564617	21.52271809	11.13292808
Mid	33.4741708	21.69021753	11.78395328
Late	33.18600191	22.4918259	10.69417601
CYP17A1			
Control	34.50495011	24.08810226	10.41684785
Continuous	34.19932009	24.64207021	9.557249884
Early	34.16133511	23.51026338	10.65107173
Mid	35.3485045	24.88495528	10.46354922
Late	34.8788779	25.42242003	9.456457873
YWHAZ			
Control	33.15157841	25.72101258	7.430565831
Continuous	32.98924913	26.08819492	6.901054207
Early	32.79128326	25.64137056	7.149912698
Mid	33.9268947	25.87597706	8.050917638
Late	33.32609568	25.80540707	7.520688609
CRHR			
Control	33.05441135	30.60428069	2.450130653
Continuous	32.327317	30.75694277	1.570374226
Early	32.20979277	30.58628746	1.62350531
Mid	33.8711995	30.07017954	3.80101996
Late	32.82194382	31.01364232	1.808301497

Table 10: Ct values for target genes and residual genomic DNA (RT-negative controls).

Table 10 illustrates that the amplification of contaminating genomic DNA is negligible for all genes other than CRHR1. Consequently, data from this gene were removed from further analysis.

3.4ii: Primer Optimisation

To obtain the most efficient amplification of target RNA, the primers used were tested over a range of concentrations. The concentration of forward and reverse primers that produced the most efficient amplification was then selected for use. Table 11 below shows the volumes of each reagent used in the optimised 20µL PCR reaction.

Genes	**AGTR1, MC2R and 3βHSD**	**CYP17A1 and CYP11A1**	**ER1 and YWHAZ**
Forward (F) and reverse (R) Primer Concentration (nM)	F: 300nM R: 900nM	F: 900nM R: 900nM	F: 300nM R: 900nM
Master mix	10.00	10.00	10.00
Forward Primer (pmol/µL)	1.20	3.60	1.20
Reverse Primer (pmol/µL)	3.60	3.60	3.60
Probe (pmol/µL)	0.10	0.10	0.70
cDNA	2.00	2.00	2.00
PCR-grade H_2O	3.10	0.70	2.50
Total Volume	20.00(µL)	20.00(µL)	20.00(µL)

Table 11: Optimised concentrations of forward and reverse primers for target and reference genes

Table 11 shows the optimised concentrations for each forward and reverse primer for all target and reference genes. The concentrations of reverse and forward primers used in the PCR reactions are highlighted in the table.

3.4iii: Gel Electrophoresis

In order to confirm that the size (base pair) of the PCR products (amplicons) were as predicted (an indication of reaction specificity), the amplification products were examined on agarose gel electrophoresis and compared to a

standard DNA ladder of 100-1000 base pairs. A representative electrophoresis gel of the PCR products, stained with ethidium bromide and viewed under ultra-violet light, is shown in Figure 20. The bands are indicative of the size of the amplicon produced in each PCR cycle. Only a single bright band is evident for each gene approximating to the anticipated amplicon size. This indicates that the gene of interest has been amplified rather than detectable amounts of higher molecular weight genomic DNA. The predicted amplicon size for each gene is shown in Table 12.

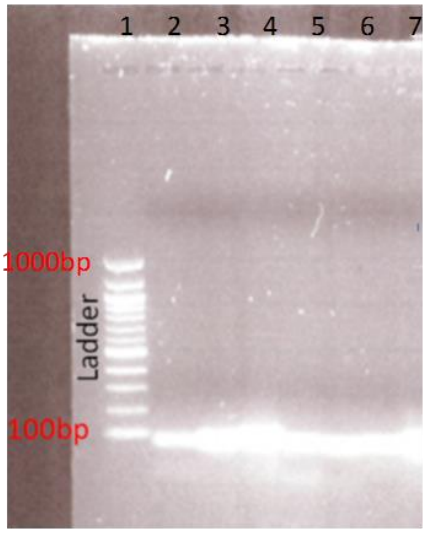

Figure 20: Electrophoresis of fetal adrenal gland nucleic acid amplified using the designed primers. 1: 1kb Ladder, 2: CYP17A1, 3: CYP11A1, 4: GAPDH, 5: MC2R, 6: 3βHSD, 7: AGTR1. Bands represent target genes and one housekeeping gene (GAPDH) (lane 4), compared with a 100-1000 base-pair ladder (1). All bands (except GAPDH) are less than 100 base pairs.

Gene of interest	Amplicon size (base pairs)
CYP17A1	60
CYP11A1	60
GAPDH	103
MC2R	64
3βHSD	61
AGTR1	71

Table 12: Amplicon size (in base pairs) of genes of interest and one reference gene (GAPDH).

3.4iv: PCR Efficacy

To determine the efficiency of the PCR reactions, a standard curve was completed for each target and reference gene using a range of dilutions of a pooled cDNA sample. A reaction which is 100% efficient (cDNA of interest doubles in each cycle), has an amplification factor of 2. The efficiency is calculated from the slope of a semi-logarithmic plot (Figure 21B) derived from the Ct values (Figure 21A).

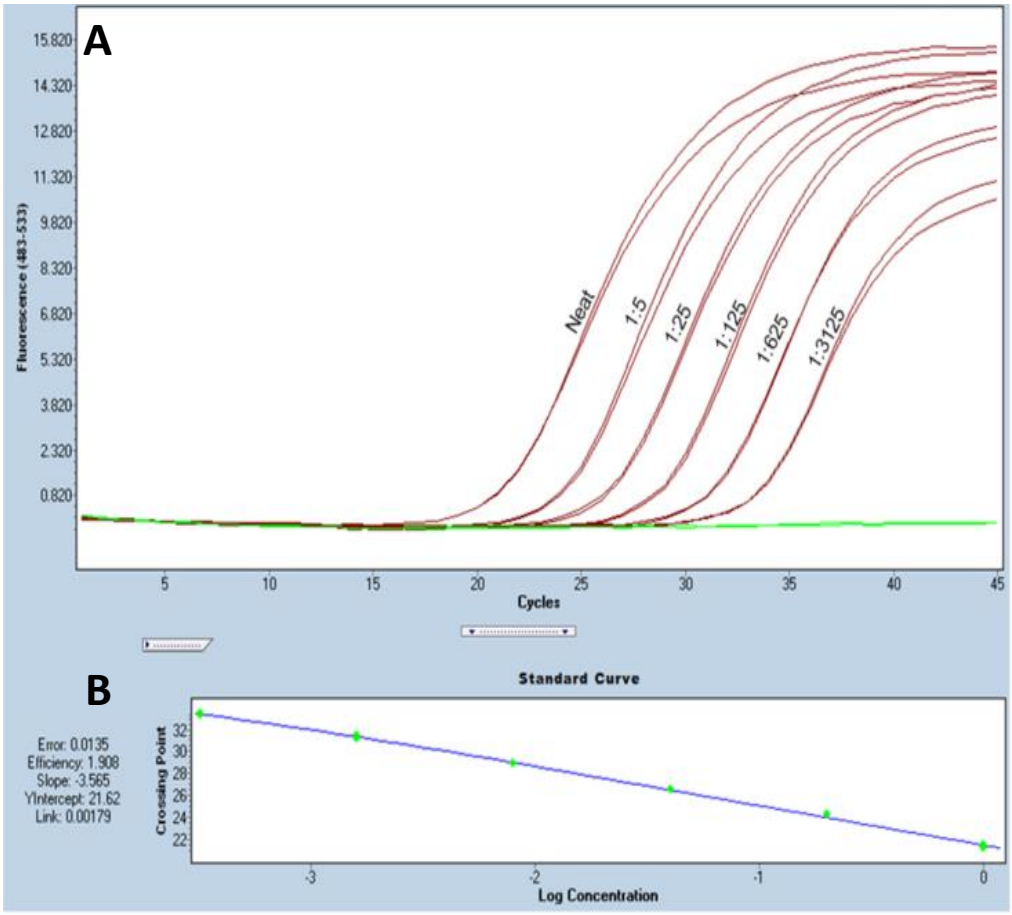

Figure 21: GAPDH amplification graph at different cDNA concentrations (A) and an example of a standard curve calculated from the crossing points and log concentration values (B).

The standard curves were repeated four times. The mean of the amplification numbers obtained are shown in Table 13.

Gene	Amplification Number
AGTR1	1.82
MC2R	1.98
CYP11A1	2.05
CYP17A1	1.91
3βHSD	2.01
ER1	1.95
YWHAZ	1.95
GAPDH	1.90
HPRT1	1.93

Table 13: Mean amplification number for each gene.

3.4v: Optimal Reference Gene Selection

Table 14 below shows the M values for the three tested reference genes (YWHAZ, GAPDH and HPRT1) from the geNorm algorithm. In tissue samples, reference genes are typically more variable than when they are in samples from a single cell type, and a value of M <1.0 is acceptable (Dahene, 2013). As can be seen, YWHAZ has the lowest M value (0.76) and is therefore the most stable reference gene.

Reference Gene	M-Value
YWHAZ	0.760
GAPDH	0.905
HPRT1	1.025

Table 14: Reference gene M-values

3.5: Real-Time PCR Results

To investigate the relative gene expression for the target genes across treatment groups, qRT-PCR was performed on samples from 105 fetuses. Following optimisation described in Section 3.1 above, and selection of the

most suitable reference gene (YWHAZ), qRT-PCR was carried out on all samples. Target genes amplified were AGTR1, MC2R, CYP17A1, CYP11A1, 3βHSD and ER1 (unfortunately, due to availability of ER1 primers, RT-negative controls and electrophoresis was not completed). In the control and each of the treatment groups, a proportion of fetal adrenals did not express CYP17A1. The largest proportion of CYP17A1 negative samples was found in the late gestation exposure group (54%) compared to 23% of the controls ($P<0.05$: Fishers Exact Test).

3.5i: Relative Gene Expression

The expression of all target genes was expressed relative to the reference gene YWHAZ using Roche software (see materials and methods). Initially, the residuals of the data were examined to ensure normal distribution (Figure 22).

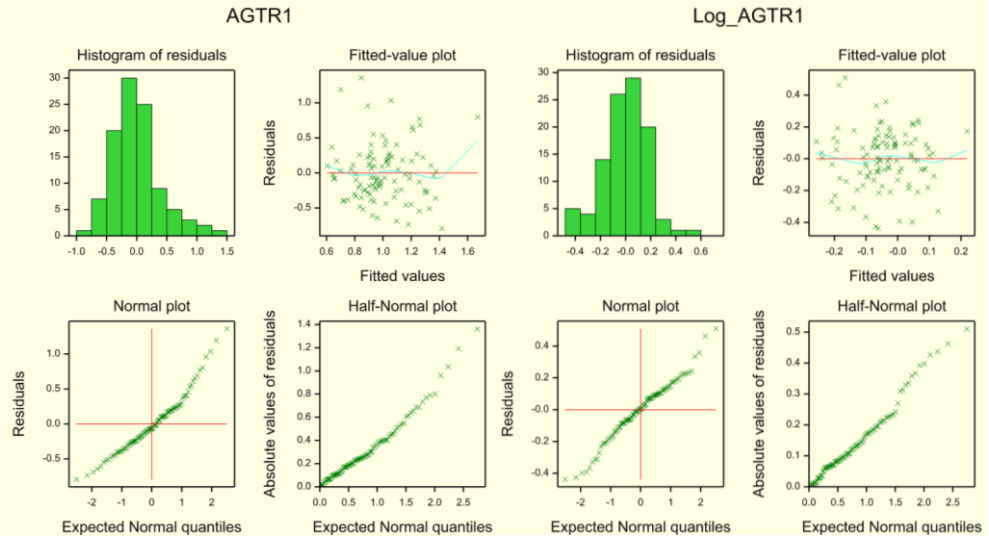

Figure 22: Example of testing for normality of real time PCR data. Residuals are shown for AGTR1 showing a positive skew in non-transformed data and a normal distribution following log transformation. Residual plots were generated by Genstat software (16th edition).

Examination of the residuals revealed abnormally distributed data for AGTR1; therefore data was log-transformed for analysis. All other data were normally distributed; and therefore original data sets were used.

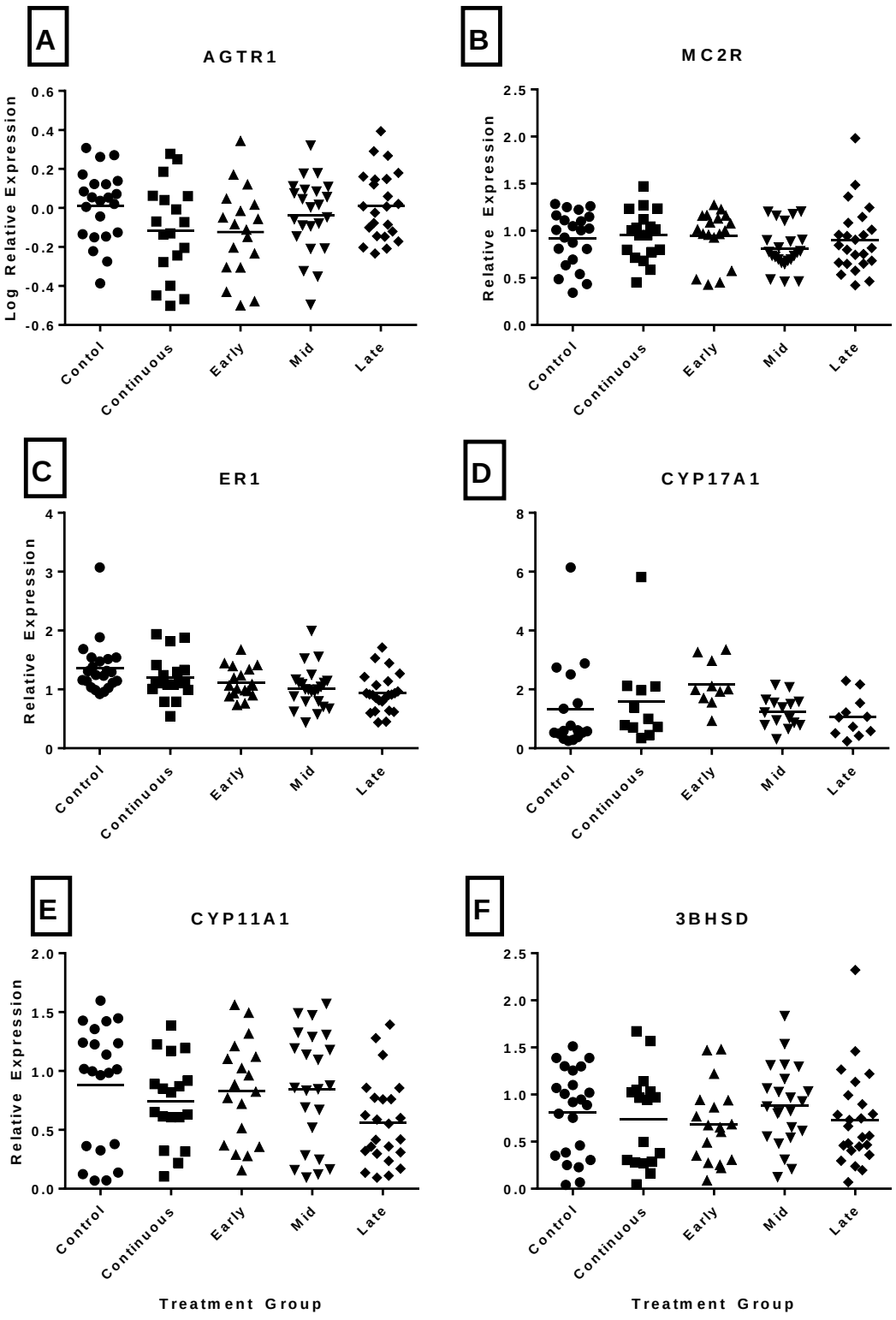

Figure 23: Effects of maternal sewage sludge exposure on fetal adrenal gland expression of target genes in day 140 fetal adrenals. Scatter graphs show gene expression data for AGTR1 (A), MC2R (B) and ER1 (C) and steroidogenic enzymes CYP17A1 (D), CYP11A1 (E) and 3βHSD (F). All genes are expressed relative to the YWHAZ housekeeping gene. Horizontal lines indicate means. n=105 [Control (22), Continuous (18), Early (18), Mid (23), Late (24)].

Due to the unbalanced experimental design, REML was used for statistical analysis of each of the target genes. No significant treatment effect was observed on AGTR1 (Figure 23A). However, there was a near significant effect of gender (p=0.053) but no treatment by gender interaction. No significant interaction between relative expression of MC2R and treatment, or any of the other covariates (adrenal size, fetal size) was observed (Figure 23B). In contrast, a significant treatment effect was observed on the expression of ER1 (p=0.042: Figure 23C). Relative to the control group, the lowest levels of ER1 expression were observed in the mid and late gestation treatment groups (Figure 23C: raw data; Figure 24A: predicted means). No significant treatment effect was observed on the expression of CYP17A1 (Figure 23D). However, a significant treatment by gender interaction was observed (p=0.032) and figure 24B indicates that this difference reflects measurements in the control and late gestation group with the females expressing significantly less CYP17A1. The expression of CYP17A1 also varied significantly with fetal weight (p=0.04: data not shown). There was no significant treatment effect on CYP11A1 or 3βHSD (Figure 23E,F) however there was a suggestion of a significant treatment by gender interaction for 3βHSD (p=0.067). Male fetuses within the continuous treatment group appear to express less 3βHSD gene than their female counterparts (Figure 24C).

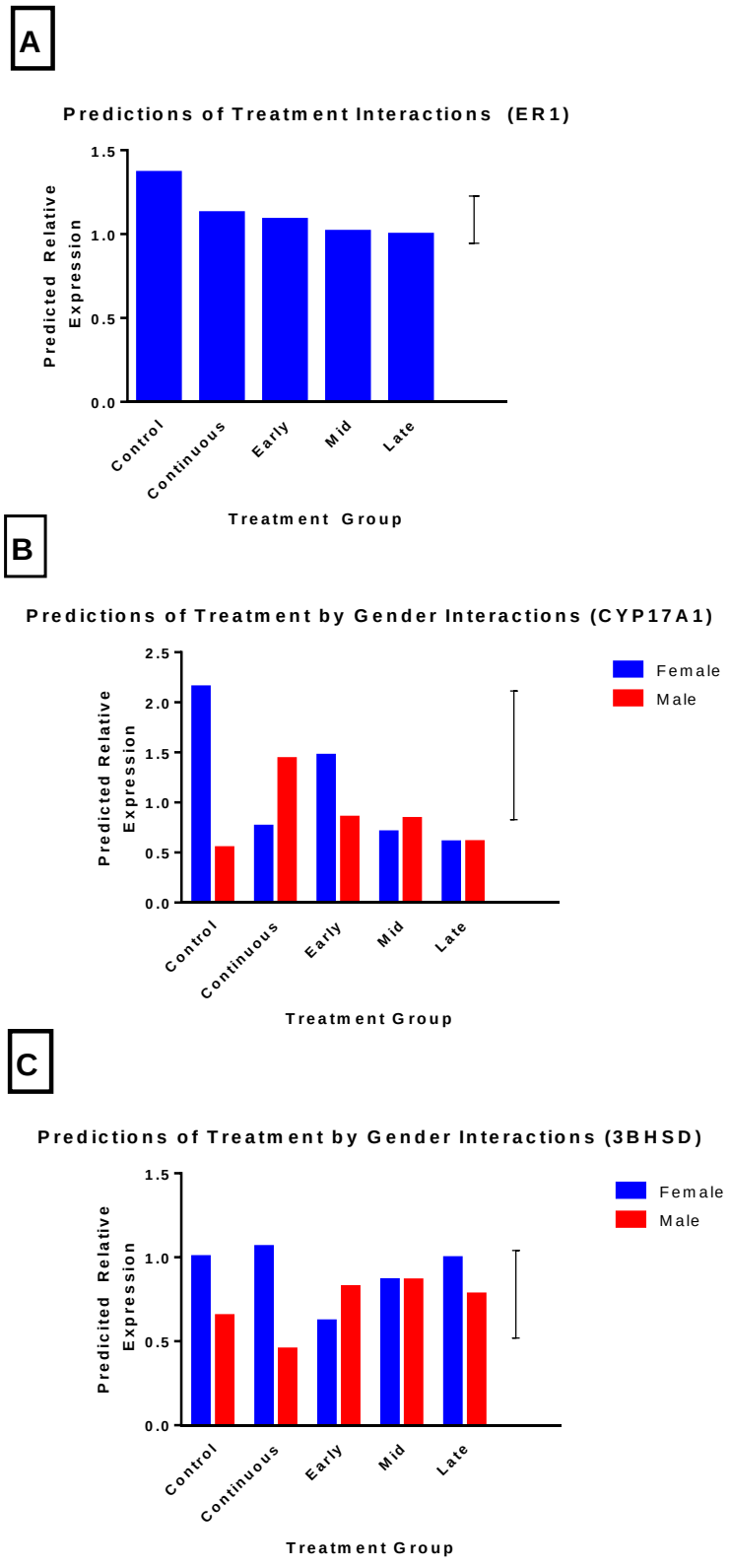

Figure 24: Effects of maternal sewage sludge exposure on fetal adrenal gland expression of target genes in day 140 fetal adrenals. Histograms show predicted gene expression levels from REML analysis showing a significant treatment effect on ER1 (A) and significant treatment by gender interactions for CYP17A1 (B) and 3βHSD (C). Error bars indicate SED.

4. Discussion

Many studies have investigated the effects of man-made environmental chemicals on various aspects of the endocrine system, particularly the reproductive system (hypothalamic pituitary gonadal (HPG) axis) and the thyroid hormones. However, the hypothalamic pituitary adrenal (HPA) axis and the adrenal gland have been rather neglected in this respect (Harvey et al., 2007). The adrenal gland is highly vascularised, and as many EDCs are lipophilic they are likely to accumulate within the cholesterol rich adrenal cortex (Harvey, 2014). In addition, the high concentration of CYP steroidogenic enzymes within the adrenal cortex have the potential not only to be the target of EDCs, but also have the ability to enzymatically convert the EDCs into possibly toxic metabolites and free radicals (Hinson and Raven, 2006). Although there have been a number of approaches to investigating the endocrine effects of EDCs, most have involved the administration of, or exposure to, a single defined EDC and at higher than environmentally relevant concentrations. However, in reality, the environment is polluted by a complex mixture of EDCs; it has been estimated that there are approaching 10,000 chemical entities implicated, including plasticisers, flame retardants, pesticides and pharmaceutical drug residues, each with the potential to interfere with the endocrine system (EPA, 2014). Furthermore, there are likely to be numerous interactions, both chemical and biological, between these compounds, resulting in the possibility of both enhancing and inhibiting their pathophysiological actions (Rhind et al., 2010a).

The aim of this project was to use an established sheep model of real-life human exposure to a mixture of environmental chemicals to determine the effects of maternal EDC exposure on the developing fetal adrenal gland at different windows of gestation. For the first time, it has been shown that the ovine fetal adrenal gland is sensitive to *in utero* exposure of an environmentally relevant cocktail of endocrine disrupting compounds at environmentally relevant concentrations. Furthermore, the results show that

the ovine fetal adrenal gland is more sensitive to a transient 80-day window of exposure compared to continuous exposure throughout gestation. This observation was facilitated by the unique study design, which allowed the effects of exposure during early, mid and late gestation to be determined. Data presented indicate that fetal adrenal glands are most sensitive to *in utero* exposure to EDCs within sewage sludge fertiliser during the mid and late gestation periods. This was manifested by reduced ER1 mRNA as well as reduced levels of the steroidogenic enzyme CYP17A1 and the angiotensin-II receptor 1, AGTR1 protein, as measured by thickness of staining within the cortex in fetal adrenals derived from these two exposure periods. Interestingly, with respect to CYP17A1 and AGTR1, the females were more affected than the males and there was no overall treatment effect at the gene expression level. Moreover, a treatment by gender interaction did not reflect differences in the mid and late gestation groups. The difference between changes in the adrenal gene expression (tanscriptome) and protein expression (proteome) is consistent with previous findings, and likely reflects additional post-translational processes (Klopfleisch and Gruber, 2012).

There are HPA gender differences which could result in this increased female sensitivity. In a cell-based ovine study, females were found to secrete more cortisol than their male counterpart in response to ACTH stimulation. However, basal cortisol production was found to be highest in adrenal cultures from intact male sheep (Canny et al., 1999) although this was an *in vitro* cell based study, and therefore had limitations. More importantly, in an *in vivo* ovine study across breeding and non-breeding seasons, again females showed significantly higher plasma cortisol levels post ACTH stimulation than their male counterparts (van Lier et al., 2003a). In addition to female sheep secreting more cortisol than their male counterparts, gonadectomy has been shown to reduce this difference, confirming the gonadal hormone roles in HPA activation (van Lier et al., 2003a, van Lier et al., 2003b).

4.1: Ovine Sewage Sludge Models

There has been limited research completed on the effects of EDCs on the intact adrenal gland. Instead, much work has been conducted on cell lines including human adrenocortical H296R cells (Ohlsson et al., 2010). Even more specifically, the effects of EDCs have been studied using isolated steroidogenic enzymes (purified bovine adrenal 3beta-hydroxysteroid dehydrogenase) (Wong and Keung, 1999). Such *in vitro* approaches obviously do not reflect the complexities of the whole organism. Indeed, it is important not only to take into account the whole of the HPA axis but all other complex physiological interactions to paint a realistic picture of EDC exposure at the entire organism level. Domestic sheep are ideal for use as human models of fetal development (McMillen and Robinson, 2005), are exceptional models to study the fetal origins of adult disease (Padmanabhan and Veiga-Lopez, 2014), and are recognised as a relevant human model of HPA toxicity, and therefore EDC exposure (Zimmer et al., 2013).

Previous work on the ovine sewage sludge model described in this study has shown that maternal tissue EDC levels are not generally affected by environmental EDC exposure; there was no difference in hepatic concentrations of specific EDCs in adult sheep comparing treated and control animals (Rhind et al., 2010b). However, fetuses of dams exposed to sewage treated pastures during pregnancy had significantly higher hepatic concentrations of EDCs (Rhind et al., 2010b) suggesting these chemicals accumulate in the fetus and that measuring maternal EDC tissue burden cannot predict fetal tissue levels. In addition, the ovine sewage sludge model has been used to demonstrate that low concentrations and complex mixtures of environmental EDCs impair fetal ovarian development (Fowler et al., 2008), fetal testis development, as shown by a reduction in gonocyte, Leydig and Sertoli cell numbers, and a reduced testicular weight (Paul et al., 2005), fetal thyroid development (Hombach-Klonisch et al., 2013) and fetal hypothalamic-pituitary axis development (Bellingham et al., 2010). Buckley and colleagues

showed that *in utero* exposure to sewage sludge before and after conception resulted in significantly reduced fetal adrenal subcapsular vasculisation, and separation of the adrenal cortex and medulla was significantly delayed in treated groups compared to controls. Fetal adrenal development was therefore delayed and the transcriptional regulation of adrenal proteins were negatively impacted (Buckley, 2012). Data presented here on ER1, CYP17A1 and AGTR1 provide further evidence for an EDC induced perturbation on the fetal adrenal.

A key observation in the current study is that transient periods of maternal exposure have a greater effect on fetal development than continual exposure throughout the entirety of gestation, particularly in the mid and late gestation treatment groups. This is not without precedent since transient effects of maternal EDC exposure were also demonstrated by Bellingham and colleagues. These authors found that fetuses exposed for transient periods (EDC exposure before or after conception) had reduced primordial follicles compared to continually exposed treatment groups (EDC exposure before and after conception). In addition, there was a greater number of gene transcript changes in the transient treatment groups compared with continual exposure groups, confirming transient maternal exposure is more detrimental to fetal development than continual exposure. This also highlights the susceptibility of the fetus to maternal exposure of EDCs before conception (Bellingham et al., 2013). Fetal development is therefore more sensitive to changing exposure than continual exposure. *In utero* exposure, followed by pre-weaning exposure via maternal milk followed by direct post-weaning exposure, caused major spermatogenic abnormalities in a subset of adult male sheep, confirming that EDC exposure in the developmental period prior to puberty is able to cause adult male disorders (Bellingham et al., 2012).

In the current study, the mid and late gestation treatment groups showed significant reductions in endocrine receptors (ER1 mRNA, AGTR1 protein) and the CYP17A1 steroidogenic enzyme protein within the fetal adrenal glands. Interestingly, the current findings are in agreement with results from the fetal

ovaries from the same study, which showed increased morphological atretic follicles and oocytes in the same transitory periods (mid and late gestation treatment groups) compared with controls (Lea et al., 2014a)(Lea et al., in preparation). Further evidence for a greater impact of a transient period of exposure compared to continuous exposure has come from unpublished studies of the same fetuses from the same mid and late gestation treatment groups. These fetuses were significantly smaller and had significantly reduced anogenital distances compared to the early and continuous gestation treatment groups and the controls (Lea et al., in preparation). Fetuses within this study also had significantly higher tissue concentrations of EDCs; this varies across different studies, suggesting an independent control of EDC concentrations (Rhind et al., 2010b).

4.2: Steroidogenic Enzymes

Toxicological studies have shown that steroidogenic enzyme gene targets are sensitive to pharmacological concentrations of specific EDCs. The data in this study have now shown that fetal adrenal glands are also sensitive to environmental concentrations and mixtures of EDCs. These anthropogenic chemicals have the capacity to interact within complex pathways, and therefore may exert their effects on several parts of the common steroidogenic pathway (Nielsen et al., 2012), which can precipitate dysfunction at lower exposure concentrations (Harvey, 2014).

The rate-limiting step for the production of steroidogenic hormones is the carriage of cholesterol to the cellular mitochondria and smooth endoplasmic reticulum by steroidogenic acute regulatory protein (StAR). Within the adrenal gland cortex, CYP11A1 (cytochrome p450, family 11, subfamily A, peptide 1) is the first steroidogenic enzyme in the system and converts cholesterol into pregnenolone, which is then further transformed to all other steroid hormones. Any inhibition of this enzyme could affect the whole of the

steroidogenic pathway (Figure 10) and would lead to a reduction in pregnenolone, the precursor for all steroid hormones (Nielsen et al., 2012). CYP17A1 (cytochrome p450, family 17, subfamily A, peptide 1) is found within the endoplasmic reticulum, and has both 17-alpha hydroxylase and 17, 20-lyase activity. It is required for the production of mineralocorticoids, glucocorticoids, androgens and oestrogens, by converting pregnenolone to 17-OH-pregnenolone (17-alpha hydroxylase activity), and this further to dehydroepiandrosterone (DHEA) (17, 20 lyase activity). 3βHSD (3-beta-hydroxysteroid dehydrogenase) is another essential enzyme in glucocorticoid, mineralocorticoid, androgen and oestrogen hormone production, and catalyses the conversion of pregnenolone to progesterone (and their OH-counterparts) (see Figure 10). Numerous studies have concluded that the weak oestrogenic chemicals, including phytoestrogens, inhibit the production of steroid hormones including cortisol (Ohno et al., 2002, Kaminska et al., 2013, Mesiano et al., 1999, Ohlsson et al., 2010), aldosterone and testosterone (Ohlsson et al., 2010). It can therefore be assumed that EDCs with mild oestrogenic activity will also have the capability to inhibit steroid production.

The presence of many EDCs have been confirmed within sewage sludge treated soil, these include DEHP, PCB congeners (as appropriate for PCB pollution), polybrominated diphenyl ethers (PBDEs) (including a range of brominated compounds) and a range of poly-aromatic hydrocarbons (PAHS) (Rhind et al., 2013). Many of these EDC examples have been found to have oestrogenic properties (Nishihara et al., 2000) as do the phytoestrogens found within many soy-containing food products. The phytoestrogens genistein and daidzein have been found to significantly suppress basal and ACTH-stimulated cortisol and corticosterone levels in porcine adrenocortical cells (Kaminska et al., 2013). Ohlsson and colleagues concluded that mixtures of phytochemical EDCs inhibit steroid production in an additive manner (Ohlsson et al., 2010). The two phytoestrogens genistein and daidzein, as well as additional flavenoid phytochemicals, were previously found not to interact

with oestrogen receptors (Kaminska et al., 2012, Ohno et al., 2002), and it was therefore hypothesised that instead they may interfere directly with steroidogenic enzymes (Kaminska et al., 2013). The mild oestrogenic activity of EDCs may therefore follow suit by interfereing with steroidogenic enzymes, inhibiting hormone synthesis. Indeed, maternal doses of PCB 118 and PCB 158 caused significantly reduced fetal plasma cortisol levels, a significantly thinner fetal adrenal cortex and significant down regulation of steroid hormones in a sheep model (Zimmer et al., 2013). Future studies investigating the plasma cortisol level of the fetuses within this study would confirm if the *in utero* exposure to a mixture of EDCs within sewage sludge can alter cortisol production.

4.2i: CYP17A1

CYP17A1 is expressed strongly in the ovine fetal adrenal gland in early and late gestation, with a quiescent phase in the mid-gestation period. CYP17A1 activity increases prior to the pre-term cortisol surge required for parturition (Han et al., 1997, Tangalakis et al., 1989). Coulter and colleagues propose that the supression of CYP17A1 during the mid-gestational quiescent phase may be mediated by AGTR1. This receptor is found in most abundance at mid gestation in the ovine fetal adrenal, and it is the fall in AGTR1 expression towards the end of gestation which allows for ACTH mediated activation of CYP17A1 (Coulter et al., 2000).

In the current study, CYP17A1 was found to be significantly affected by maternal exposure to sewage sludge fertiliser. Significantly less CYP17A1 protein was detected in the late gestation treatment group compared with control animals, and CYP17A1 gene expression in female fetuses was also signficantly reduced in the late gestation group. This is in general agreement with the findings of Ohno and colleagues who concluded that certain mildly oestogenic flavenoids inhibited CYP17A1 (Ohno et al., 2002). In addition,

CYP17A1 inhibition was investigated by quantifying CYP17A1 activity by measuring DHEA production in a porcine adrenal cortex microsome assay and its expression by real-time PCR in H295R human adrenocortical cells. Eight out of 20 EDCs tested were found to inhibit CYP17A1 activity (in either the enzyme activity assay or at the gene expression level) including DES, tetrabromo-bisphenol A, methylmercury, dioctyltin chloride, SU10603, para-methoxyamphetamine (PMA), BPA and conazole antifungal agents (Roelofs et al., 2013). In another study, the pesticide prochloraz was found to inhibit CYP17A1, shown by an increase in pregnenolone and progesterone (accumulated upstream of CYP17A1 in the steroidogenic pathway, see Figure 10), and a decrease in hormones downstream (Nielsen et al., 2012). Certain chemicals have the capacity to increase the activity of CYP17A1; in one cell-based study, forskolin and 8BrocAMP significantly increased the gene expression of CYP17A1, however, the EDC PMA significantly decreased CYP17A1 gene expression (Hilscherova et al., 2004).

CYP17A1 is necessary for the production of DHEA, which is the essential precursor for androgens and oestrogens. In humans, 40% of androgens in men, and 75% (increasing to 100% post-menopause) of oestrogens in women are produced intracrinologically by the adrenal gland (Labrie, 1991). Primate adrenal glands, including fetal adrenal glands produce large volumes of DHEA and DHEAS, whereas other mammals, including sheep, locally produce sex steroids within the gonads, therefore the sheep adrenal produces less DHEA than the primate counterpart (Labrie, 1991).

As DHEA production can be used to quantify CYP17A1 activity (Roelofs et al., 2013), a decrease in CYP17A1, as observed at the protein level in the current study, is likely to result in a decrease in DHEA. Logically it can be concluded that the outcome of this will be a reduction in testosterone. In support of this, testosterone concentrations in the fetuses used in the current study were reduced in males in the late gestation exposure group (Lea et al., 2010) and also, in a different study, testosterone was lower in male fetuses from sewage sludge exposed ewes euthanised at day 110 of gestation (Paul et al., 2005).

Interestingly, only the mid-gestation exposure group are reported to have reduced testicular CYP17A1 immuno-expression (Lea et al., 2010). This raises the possibility that the reduction in testosterone in the late gestation exposure group reported by Lea and colleagues may be a reflection of the reduced adrenal CYP17A1 protein levels seen in the current study. In addition, mid-gestational testicular CYP17A1 reduction could follow the quiescent CYP17A1 phase normally found in the adrenal gland during mid gestation. However, the caveat to this is that the reduced CYP17A1 protein reflects the staining within the adrenal cortex rather than a direct measure of CYP17A1 activity. Nevertheless, in a separate study, fetuses from ewes exposed continuously for the first 80 days of gestation also exhibit reduced numbers of testicular CYP17A1 immunopositive cells (Lea et al., 2010). Adrenal tissues from these animals have yet to be examined. Taken together, these data generated from the sewage sludge sheep model illustrate the sensitivity of CYP17A1 to EDC exposure, particularly at the protein level within both the fetal adrenal gland and testis.

Although the relationship between reduced fetal testosterone and adrenal function requires further study, there are important implications of these findings for population heath. If the low fetal CYP17A1 and testosterone levels continue into adulthood, adult phenotypes could change: fetal onset of adult disease (Gluckman and Hanson, 2004). Many epidemiological studies have shown that lower testosterone levels in men are associated with cardiovascular disease, and therefore higher mortality rates, as reviewed by Ikeda and colleagues (Ikeda et al., 2012). Furthermore, androgen deprivation therapy for prostate cancer is significantly correlated with increased coronary heart disease, acute myocardial infarction and heart failure, when used in combination with LH releasing homone receptor (LHRH) agonist (Martin-Merino et al., 2011). In addition, lower testosterone levels in men is associated with many chronic metabolic disease states including metabolic syndrome, hypertension, increase body mass index and type 2 diabetes, as reviewed by Moulana and colleagues, and Grossmann (Moulana et al., 2011,

Grossmann, 2011). The global increasing incidence of cardiovascular and metabolic diseases may therefore be in part atributed to EDCs, via fetal CYP17A1 inhibition influencing the adult phenotype.

Fetal onset of low female adult DHEA could by hypothesised to be even more detrimental than in males due to the large proportion of oestrogen derived from this adrenal precursor. DHEA decreases with age, and therefore a decrease may be linked with post-menopausal problems including osteoporosis, muscle atrophy, vaginal atrophy, fat accumulation, hot flushes, type 2 diabetes and memory loss, as reviewed by Labrie (Labrie, 1991). Therefore a reduction in CYP17A1, and thus DHEA, from birth could exacerbate post-menopausal conditions, and indeed reduce the age of menopause, a reported effect of many EDCs (Eskenazi et al., 2005). In further support of this concept, smoking is a source of the EDC PAH (Fowler et al., 2014), and women smokers tend to experience earlier menopause (Tawfik et al., 2015). However, a review of published and non-published randomised control trials comparing any dose and any form of DHEA administration concluded that there was no benefit of DHEA to reduce post-menopausal symptoms, other than possibly improving sexual function (Scheffers et al., 2015).

Lower fetal concentrations of circulating DHEA/DHEAS due to CYP17A1 reduction could also have major consequences on pregnancy maintenance. The majority of pregnancy-maintaining oestrogen precursors (DHEA/DHEAS) are of fetal adrenal origin (Kaludjerovic and Ward, 2012), therefore it can be assumed that a fall in adrenal DHEA/DHEAS, either fetal or maternal, could result in a fall in pregnancy oestrogens. During pregnancy this can result in miscarriage (Albrecht et al., 2000), which was the original idea behind the use of the synthetic oestrogen DES to prevent miscarriage in the 1940-1970s (Smith, 1949). Maternal serum concentrations of conjugated BPA is significantly correlated with increased miscarriage rates (Lathi et al., 2014) and BPA has been shown to inhibit ovarian steroidogenesis in mouse follicle cultures including a significant decrease in DHEAS from the treated follicles

(Peretz et al., 2011). It could therefore be hypothesised that BPA could negatively affect fetal and maternal DHEA/DHEAS production, leading to a fall in oestrogens, resulting in the increase in miscarriages seen following BPA ingestion.

Maternal DHEA and DHEAS levels are also important in conception. In a double-blind, randomised, placebo-controlled trial of ageing infertile women, DHEA administered prior to *in vitro* fertilisation (IVF) therapy caused a significantly higher live birth rate than placebo controls (Tartagni et al., 2015), suggesting the importance of maternal, in addition to fetal, adrenal DHEA and DHEAS. The result of fetal exposure to EDCs may not be seen until adulthood, highlighting the concept of fetal origin of adult disease (Gluckman and Hanson, 2004). A fetal reduction in CYP17A1 could result in a reduction in DHEA and DHEAS in the adult, leading to reduced fertility. In a study of 1950 Chinese women undergoing IVF, low basal testosterone levels were correlated with a significant decrease in mature oocytes, lower fertilisation, and lower pregnancy rate when measured on day four of the menstrual cycle (Guo et al., 2014). Indeed androgens, including testosterone, act synergistically with FSH during folliculogenesis, and are therefore important in the early stages of follicle maturation (Gleicher et al., 2011). Mitotaine, a drug used to combat the glucocorticoid excess caused by hyperadrenocortisism, is an isomer of dichlorodiphenyldichloroethane (DDD), and is a derivative of the well researched EDC DDT. Mitotaine treatment in pre-menopausal women was associated with a significant decrease in androgen levels including androstendione and testosterone, contributing to the development of ovarian macrocysts, menstrual abnormalities and pelvic pain (Salenave et al., 2015). This illustrates that EDCs may induce androgen and oestrogen deficiency via inhibitory effects on CYP17A1 and DHEA, and this inhibition can lead to female reproductive problems.

It has been reported that exposure to EDCs can result in hypertension (Arrebola et al., 2015). A proposed mechanism for this observation is that a general reduction in physiological adrenal CYP17A1 activity, resulting from

adult or fetal EDC exposure as seen in the current study, causes an androgen and oestrogen deficiency and a mineralocorticoid excess. As previously mentioned, decreased DHEA leads to a subsequent decrease in oestrogens and androgens. Due to the reduction in 17-hydroxylase activity of CYP17A1, there are also reduced 17-OH-progesterone levels, resulting in lowered cortisol levels. All these lowered hormone concentrations reduce the negative feeback on the hypothalamus and pituitary gland, resulting in an increase in ACTH production. ACTH activates the zona glomerulosa resulting in the production of pregnenolone and progesterone. Lack of CYP17A1 results in more precursor for CYP21 to convert to corticosterone (see Figure 10). High circulating concentrations of corticosterone cause supression of renin and expand plasma volume leading to hypertension, as shown by Marsh and colleague's work on congenital CYP17A1 defficiency (Marsh and Auchus, 2014). Due to high corticosterone, CYP11B2 expression and aldosterone are very low, or absent in patients with congenital CYP17A1 deficiency (Marsh and Auchus, 2014).

4.2ii: CYP11A1

In the current study, no treatment effect was found for CYP11A1 gene expression across the five treatment groups. In agreement with the current results, CYP11A1 was found not to be the mechanism by which oestrogenic genistein and daidzein affect human corticoadrenal H295R cells (Kaminska et al., 2013). Within the steroidogenic pathway, CYP11A1 converts cholesterol to pregnenolone, which, via a number of enzymatic conversions, results in the end product cortisol (see Figure 10). Phytoestrogens inhibited cortisol production from H295R cells, however, the addition of pregnenolone to the cell media did not remove this inhibition, therefore reduced pregnenolone due to reduced CYP11A1 activity was not the reason for the inhibition. This suggestes that phytoestrogens inhibited cortisol production further down the steroidogenesis pathway (Kaminska et al., 2013). Mesiano and collagues also

found that there was no interaction between phytoestrogen exposure and CYP11A1 gene expression (Mesiano et al., 1999). In contrast to the current study, in a sheep model of maternal exposure to PCB 118 (49µg/bw/day) and PCB 153 (98µg/bw/day) CYP11A1 gene expression was reduced (Zimmer et al., 2013). This could suggest that chemicals within EDC mixtures interact and therefore have the capability to affect adrenal glands differently when combined than when individually tested. This highlights the importance of the sewage sludge model in the current study, which exposes ewes to an environmentally relevant cocktail of EDCs.

PAHs are EDCs found within cigarette smoke. In a study of human fetal ovaries of 11-21 weeks of gestation from social abortions, Fowler and colleagues demonstrated that CYP11A1 was dysregulated *in utero* by maternal smoke exposure (Fowler et al., 2014) and concluded, as maternal hepatic PAH levels were increased nearly 6-fold and female fetal hepatic PAH concentrations were significantly increased by maternal smoking, that chemicals in the cigarette smoke were responsible for this effect. However, of relevance to the current study, liver concentrations of PAHs in the ovine sewage sludge model were not significantly affected by sewage sludge exposure compared with controls in either maternal or fetal liver samples (Rhind et al., 2010b). Therefore the lack of PAH accumulation within the fetuses observed here could be one reason for a lack of any observed effect of maternal exposure to contaminated pasture and fetal CYP11A1 gene expression. This underlies the strengths of the real-life ovine model, as the effects seen reflect the EDC composition of the model.

4.2iii: 3βHSD

Using porcine adrenocortical cells, Kaminska and colleagues concluded that phytoestrogens inhibit steroidogenesis at the level of 3βHSD. 3βHSD converts pregnenolone to progesterone, which is then further converted to cortisol

within the steroidogenic pathway. Cortisol production was inhibited by the addition of the EDCs, however, on the addition of progesterone, this inhibition was reversed, concluding that the inhibition was at the level of 3βHSD (Kaminska et al., 2013) (see Figure 10). In contrast, the current study showed no treatment effect on 3βHSD gene expression, although there was a gender difference. Male fetuses expressed significantly more 3βHSD than female fetuses in the mid-gestation treatment group. Interestingly the Kaminska study described above used adrenocortical cells from only female adult pigs, thus effects may be gender specific.

Isoflavones have also been found to inhibit the activity of 3βHSD in bovine adrenocortical cells (Wong and Keung, 1999) and human H295R adrenocortical cells (Ohno et al., 2004). However, it is important to note that these two studies, as well as that of Kaminska, were all *in vitro* cell-based studies which do not take into account whole animal physiology. Nielson and colleagues investigated the inhibiting effects of the three EDCs prochloraz (a pesticide), ketoconazole (an anti-fungal) and genistein (a phytoestrogen) on human H295 adrenocortical cells, concluding that only genistein out of the three compounds tested inhibited 3βHSD. This was highlighted by an increase in pregnenolone and dehydroepiandrosterone (upstream of 3βHSD) and a decrease in androstendione and estrone (downstream) (Nielsen et al., 2012). This highlights the variablility of EDC activity and the importance of investigating an everyday cocktail found within the current study, as well as an in *in vitro* versus *in vivo* models.

4.3: Steroidogenic Receptors

4.3i: Angiotensin Receptors

In addition to chronic low levels of glucocorticoids and androgens caused by EDC interactions within the adrenal, EDCs have also been found to reduce

mineralocorticoid levels (Martinez-Arguelles et al., 2014). This could be at receptor level, including AGTR1, or again within the steroidogenic pathway. Data generated in this study showing reduced adrenal AGTR1 protein and the effects on adrenal CYP17A1 may therefore reflect a sensitivity of the zona glomerulosa to EDCs. Additionally, cortical staining for AGTR1 was significantly reduced in the late exposure group with the females more affected than the males.

AGTR1 is activated by the vasoconstrictor angiotensin-II which, initiates aldosterone production within the zona glomerulosa of the adrenal cortex. In sheep, by mid-gestation half the aldosterone in fetal blood is of fetal adrenal origin, however, increased plasma potassium or angiotensin-II do not increase aldosterone secretion until near term (Coulter et al., 2000). Therefore the reduction of AGTR1 protein in the late gestation exposure group in the current study could lead to reduced aldosterone in late pregnancy. There is a high fluid volume demand during pregnancy, and this increase is partially induced by aldosterone (Chapman et al., 1998). Indeed, failure of this volume expansion can result in the development of preeclampsia and growth retardation (Hays et al., 1985). As aldosterone concentrations correlate with plasma volume expansion (Salas et al., 2006) and placental size (Gennari-Moser et al., 2011) during pregnancy, it has been hypothesised that low aldosterone, leading to lower blood volume and resulting vasoconstriction, is a contributing factor to preeclampsia and reduced placental perfusion (Shojaati et al., 2004) in primates with initial placental pathology. In aldosterone synthase knock out mice, dams were found to be hypotensive before pregnancy, and even more so during pregnancy, leading to a reduced litter size and reduced placental perfusion (Todkar et al., 2012). In addition, aldosterone has been suggested to be part of an adaptive pathway in conditioning pregnancies to the changing environment by modulating trophoblast growth and placental size (Gennari-Moser et al., 2011). Since 80% of mid-gestation fetal blood aldosterone is of fetal adrenal origin, environmental factors that affect aldosterone levels may indirectly affect

blood pressure, placental development and function and thus theoretically negatively impact on pregnancy maintenance via a number of different mechanisms including the reduced AGTR1 found in the current study. The reason for the apparent increased sensitivity of the female adrenal to EDCs is uncertain, however, there are gender differences within the normal physiology of the HPA, whereby the female HPA is more active than in the male.

In terms of the localisation of adrenal AGTR1, the current study showed staining of both the zona glomerulosa and zona fasciculata of the developing adrenal cortex. In a separate published study, Coulter and colleagues also reported intense zona glomerulosa staining but in contrast to the current study, they found less intense and more limited staining in the zona fasciculata (Coulter et al., 2000). Both studies found intense staining at the cortico-medullary boundary and around the central medullary vein, however, in the current study localised, sporadic medullary staining was also present. In agreement with data presented in this thesis, Wintour and colleagues found immunostaining for AGTR1 in the developing zona glomerulosa, within the steroidogenic cells of the developing zona fasciculata, and scattered throughout the medulla at 60, 100 and 130 days of gestation (Wintour et al., 1999). Subtle differences in localisation between studies probably reflect different antibody sources and specificities (polyclonal from Abcam (current study), polyclonal from Santa Cruz Biotechnology (Coulter) and three antibodies in Wintour).

In utero exposure to phthalates, in particularly DEHP, has been shown to have anti-androgenic effects. A DEHP induced reduction in testicular testosterone production in rat adult male offspring which were exposed *in utero* throughout the second half of gestation, was found to be due to a decrease in mineralocorticoid receptors (MRs) within Leydig cells (Martinez-Arguelles et al., 2014). A novel hypothesis for further testosterone reduction, in addition to reductions in CYP17A1 and testicular MRs, is a reduction of adrenal aldosterone. Indeed, a reduction in aldosterone was found in 60-day-old male

offspring after *in utero* DEHP exposure (Martinez-Arguelles et al., 2014) and aldosterone is known to stimulate testosterone and LH production in the testis as an exogenous ligand of MRs (Ge et al., 2005). This suggests that the adrenal-testis-testosterone-producing axis is sensitive to DEHP (Martinez-Arguelles et al., 2014), again highlighting the importance of the adrenal in producing gonadal steroids (intracrinology). Martinez-Arguelles and colleague's rat study of *in utero* exposure to DEHP also concluded that DEHP affected cholesterol bioavailability and steroidogenesis in the adrenal gland of offspring. Although aldosterone levels were decreased, neither angiotensin-II, potassium, corticosterone nor ACTH levels were affected, indicative of a DEHP affect on the zona glomerulosa. In addition, angiotensinogen synthesised in the liver, and renin synthesized in the kidney, were normal, again suggesting an adrenal aetiology of aldosterone reduction (Martinez-Arguelles et al., 2014).

Further studies by Martinez-Arguelles and colleagues showed that, after *in utero* exposure to DEHP, adult rat aldosterone concentrations remained similar to those from pubescent rats (approximately half the normal adult concentration), suggesting that DEHP targets maturation of the zona glomerulosa (Martinez-Arguelles et al., 2014). In the same rat study, expression of angiotensin-II receptors, including AGTR1, were also reduced by DEHP at very high concentrations (more than 300mg/kg/day), further highlighting the importance of the current study, in which AGTR1 was decreased by an environmentally relevant concentration and mixture of EDCs. Indeed, these authors hypothesised that the reduction in AGTR1 is likely linked to the reduced aldosterone observed in the same animals (Martinez-Arguelles and Papadopoulos, 2014, Martinez-Arguelles et al., 2014).

In the DEHP rat study described above, Martinez-Arguelles and colleagues also found a gender specific action of DEHP on male offspring. In the current study, however, a treatment by gender interaction was observed with the females being more affected than the males in the mid and late exposure treatment groups. Since their study only focused on DEHP, the current

investigation is more representative of real life exposure to a mixture of environmental contaminants. In addition, within this mixture there will inevitably be both synergistic and additive interactions also representative of real life exposure.

The mechanism by which reduced AGTR1 may cause reduced testosterone levels has been discussed. However, since aldosterone is responsible for maintaining blood pressure, EDC induced changes in blood pressure could also work via this mechanism. In the Martinez-Arguelles rat model, DEHP exposure administered by maternal gavage from day 14 of gestation until parturition decreased the night time systolic blood pressure of male offspring when they reached 200-days of age ("old"). These male offspring maintained on a low sodium diet (0.01% NaCl) demonstrated decreased diastolic blood pressure and there was also a significant decrease in their night activity. This suggests DEHP alters basal aldosterone mechanisms (Martinez-Arguelles et al., 2014). This also suggests that EDCs within sewage sludge, which reduce AGTR1, have the potential to reduce aldosterone concentrations and to reduce blood pressure. They also have the potential to cause behavioural changes via reduced testosterone (Martinez-Arguelles et al., 2014). In an ovine model of *in utero* exposure to sewage sludge throughout the entirety of maternal life and gestation has also been shown to affect behaviour in 5-month-old offspring (Erhard and Rhind, 2004). Since the ovine model described in this thesis investigated actual environmental concentrations and cocktails of ubiquitous EDCs at different windows of gestation, it follows that hormone concentrations, blood pressure and behaviour may be useful indices for further studies using this established ovine paradigm.

4.3ii: Oestrogen Receptors

In the current study, fetal oestrogen receptor 1 (ER1) gene expression was significantly affected *in utero* following maternal exposure to EDCs; with ER1

being expressed significantly less in the mid and late gestation treatment groups compared to controls. Many EDCs are mildly oestrogenic (Nishihara et al., 2000) and therefore have been found to act upon oestrogen receptors (ERs) (Hamlin and Guillette, 2011). There is little known about ERs within the ovine adrenal glands; however, ERs are found within all three adrenocortical zones, and within the adrenal medulla in the adult sheep (van Lier et al., 2003a). van Lier and colleagues showed that there was no difference in ER1 distribution or levels in male and female sheep or gonadectomised sheep (van Lier et al., 2003a). Similarly, in the current study, no gender differences were found in ER1 gene expression. The presence of ERs within the adrenal glands indicate the importance of oestrogen activation of the adrenal gland. That oestrogen stimulates the adrenal gland was shown by Lo and colleagues, who demonstrated that administration of oestrogen benzoate significantly increased plasma corticosterone levels from the rat zona fasciculata and zona reticualis in a dose dependent manner (Lo et al., 2000). Therefore any disruption of ERs within the adrenal gland could affect the ability of the HPA axis to fully respond to stressors. The decrease in ER1 gene expression demonstrated in the current study could therefore result in reduced activation of the HPA axis by endogenous oestrogens at the adrenal level.

Oestrogen modulators also have the capacity to alter catecholamine secretion from the adrenal medulla. In bovine adrenal medullary cells, low concentrations of raloxifene and tamoxifen modulate ERs (via their allosteric site), and at high concentrations inhibit catecholamine synthesis and secretion by inhibiting sodium and calcium influx via acetylcholine (Inagaki et al., 2014). The isoflavenone daidzein stimulates catecholamine synthesis via ERs in bovine adrenal medullary cells; however, high concentrations inhibit catecholamine synthesis and secretion (Liu et al., 2007). In a mouse model, *in utero* exposure to arsenic and post-natal exposure to DES did not separately affect ER1 gene expression; however, *in utero* exposure to arsenic enhanced ER1 modulation by post-natal DES exposure; this combined exposure increased ER1 gene expression (Liu et al., 2009). The current study is the first

to investigate and conclude that *in utero* exposure to EDCs alter the fetal expression of ER1 within the adrenal gland when exposed from 30 to 100, or 60 to 140 days of gestation to a mixture of chemicals.

4.3iii: Melanocortin Receptors

Alterations in the melanocortin receptor MC2R can result in adrenopathology including clinical signs associated with hyperadrenocortisism (Cushing's disease) or hypoadrenocorticism (Addison's disease) (Yang et al., 2015). The current yet limited literature on the interaction of EDCs and MC2R suggest adrenal dysregulation caused by EDCs occurs at a different receptor level than MC2R or within the steroidogenic pathways. The current real life study also shows a good indication that an everyday cocktail of EDCs does not affect fetal MC2R development; no differences in gene expression of MC2R was found across the gestation treatment groups within this study. In a rodent study, chronic *in utero* exposure to the EDC tetrabromobiphenol A was found to significantly modulate the transcription of MC4R within fetal hypothalamic PVN samples, however, organotin tributylin (TBT) exposure caused no such effect (Decherf et al., 2010). This suggests different MCRs may be sensitive to the effects of specific EDCs; however, the study by Decherf and colleagues did not include MC2R, and only investigated two EDC out of possibly thousands. Similarly to the current study, the EDC cadmium was shown not to alter mRNA levels of basal MC2R levels in a cell-based study of adrenal cells in rainbow trout kidneys (Sandhu and Vijayan, 2011), Teleosts do not have separate adrenal glands instead adrenal tissue is located within the cranial pole of the kidney.

4.4: Future work

In order to understand more fully the effects of exposure of EDCs on the developing fetus, and the long term implications of such exposure, there are a number of lines of enquiry that it would be profitable to pursue. It would be interesting to investigate the rate limiting step of steroiodogenesis in the sewage sludge model by investigating gene expression of StAR in addition to the other steroidogenic enzymes within the hormone pathway which were not included in the current study including CYP12A1 (aldosterone synthase). Serum hormone concentrations of cortisol, aldosterone, androgens and oestrogens from dams and fetuses would also be of interest. In addition to fetal studies, investigating possible disease progression in offspring including behaviour, mating behaviour, stress responses, and fertility, as well as the molecular makers included in this study would be of great importance in F1, F2 and F3 generations. Measuring systolic blood pressure of offspring across the different treatment groups, and changes during growth especially during pregnancy would be beneficial. In addition, it would be advantageous to complete a study on the impacts of direct depots of sex hormones, investigating a purely androgenic or oestrogenic insult on ovine fetal development. Future work on environmentally relevant mixtures of EDCs could then be investigated and referred to the “oestrogen” or “androgen” pattern to decipher the hormonal activity of the EDC cocktail as a whole, to investigate linkages or common effects. Larger screening studies including micro-arrays, next generation sequencing and proteomics could be used to find novel EDC targets.

4.5: Limitations

4.5i: Immunostaining

The sections processed for immunohistochemistry analyses were harvested and immediately fixed in Bouin's solution in 2008 and stored in 70% ethanol at 4^{o}C; however, some tissues had dried out during this time period, and these could not be included in analyses. It is unknown if this time period may have affected the expression of protein in the tissue samples that were investigated. However, due to the specificity of the staining, and the negativity of the IgG controls, it is likely that these results are valid and not compromised by non-specific binding. Presumably as a consequence of the age of the tissue, a number of sections did not remain intact during the immunohistochemical procedure; the edges of the tissue curled over, or areas of the adrenal tissue dislodged completely from the slide. In these situations, the eight measurements of cortical staining for each of the antibodies was completed at even distances across the available tissue to get as full a representation as possible of the entire cortex. Percentage area stained was completed for the section of tissue that remained on the slide. All results therefore have to be considered with this in mind.

4.5ii: Real Time PCR

Ideally primers could have been designed to span intron boundaries to obviate any possibility of amplifying contaminating genomic DNA. However, as an alternative approach to minimise the chance of genomic amplification, a DNase digestion step was included during the RNA extraction step. In addition, reverse transcriptase negative controls were run, and results from these controls clearly showed the negligible contribution of contaminating genomic DNA to the amplification. Sequencing of the PCR products was not

possible for any gene other than YWHAZ due to the small amplicon size. However, further optimisation by electrophoresis of the qRT-PCR products confirmed that the size of the amplicons was as expected for each gene. Unfortunately, due to limited availability of one of the ER1 primers that had been designed prior to the start of this project, gel electrophoresis and RT-negative controls were not completed for this gene of interest.

4.6: Broader Implications

4.6i: Wildlife

Adrenal dysfunction caused by EDCs has been found in wildlife, including marine mammals, arctic mammals and birds. High body concentrations of PCBs and DDT compounds within Baltic seal populations caused a massive population decline in the 1970s. The seals exhibited characteristics of Cushing's disease and adrenal cortex hyperplasia (Bergman, 1999). Dolphins from the oil-spill area of Baratana Bay have significantly lower adrenal hormone blood levels than dolphins in clean waters. This was found to be the case for cortisol eve after taking into account circadian variations of this hormone, and aldosterone, which were both significantly lower than blood concentrations in the control animals (Schwacke et al., 2014). High blood plasma concentrations of brominated flame-retardants and organochlorides in glaucous gulls (*Larus hyperboreus*) has been associated with high corticosterone concentration in both genders, and an altered stress response in males (Verboven et al., 2010). EDCs have the potential to alter and decrease the natural stress response, by dampening down glucocorticoid production, which therefore has the ability to impair an animal's survival fitness (Harvey, 2014).

4.6ii: Humans

It is hypothesised that in domestic mammals, there is a low-grade cortisol insufficiency, and this is likely to have an environmental aetiology (Plechner, 2004), and, in addition, such a low-grade cortisol deficiency has been suggested in humans (Jefferies, 1994). As previously described, most investigations have been completed on the reproductive aspects of EDC perturbations, focusing on the HPG axis, therefore this work on the fetal adrenal is novel and of great importance. Indeed, the adrenal gland is the most commonly targeted endocrine organ in toxic insults, more specifically, the adrenal cortex, and more specifically still, the zona fasciculata and zona reticularis are the most commonly affected regions of the adrenal gland by toxins (Rosol et al., 2001). Inhibition of normal adrenal cortex physiology and function has been noted to cause detrimental and fatal consequences in the human. For example, to causes of fatal adrenal dysfunction include the anaesthetic agent etomidate, and the anti-convulsant medication aminogluthiamide, both of which caused death via Addisonian crises. These are examples where single doses, at reasonably low concentrations, can still have fatal consequences via adrenal inhibition (Harvey, 2014, Harvey et al., 2007).

Pharmaceuticals can fatally inhibit the adrenal gland, and low level adrenal deficiency is common in domestic mammals (Plechner, 2004). The current study shows EDCs perturb fetal adrenal gland development, and therefore it is hypothesised that EDCs may be linked to low-level adrenal pathology. Altered adrenal function, however mild, can have very wide pathological responses in mammalian physiology, including effects on the stress response, the thyroid system, the pancreas and the gonads (Harvey, 2014). The HPA axis is activated at times of stress (physical, infection, injury, toxic insult), when the body is most vulnerable to toxic insults, and therefore any suppression of the full HPA axis response can be detrimental (Harvey, 2014).

In humans, there are a number of conditions that are caused by abnormalities of the adrenal cortex and adrenal steroidogenesis, and as EDCs are already known to interfere with steroid synthesis, it is plausible that such conditions could at least in part be chemically induced, leading to adrenal insufficiency (Harvey, 2014). Some examples of altered or mutated adrenal steroidogeneisis causing human disease are: adrenogenital syndrome (Hakki and Bernhardt, 2006), an increase in aldosterone production leading to hypertension and cardiovascular disease (Arrebola et al., 2015), an increase in certain CYP17A1 genotypes known to correlate with increased prostate cancer (Madigan et al., 2003), and defects in aldosterone synthesis leading to reduced aldosterone and salt-wasting syndrome in babies, which can be fatal unless treated (White, 2004).

4.7: Conclusion

This study, for the first time has demonstrated that *in utero* exposure to an environmentally relevant concentration and mixture of EDCs found within processed human sewage sludge dysregulates the developing ovine fetal adrenal gland. More specifically, *in utero* exposure to EDCs within sewage sludge during mid and late gestation reduces ER1 gene expression and, particularly in female offspring, reduces levels of AGTR1 and CYP17A1 protein. It is hypothesised that this fetal dysregulation, leading to altered fetal and possible adult steroidogenesis, could contribute to increasing human chronic dieases including infertility, hypertension and metabolic diseases, and to the declining biodiversity in wildlife. More importantly, this study highlights the need for further EDC research into delining fertility to be broadened to include the perturbed reproductive activities of the HPA axis.

In terms of the agricultural practice of using sewage sludge based fertiliser, data presented in this study add to the weight of evidence that this practice may require regulation at national and international levels. Indeed, over 10

million tonnes of dry matter sewage sludge fertiliser containing a mixture of EDCs are used annually in the EU (Laturnus et al., 2007). Therefore these new data could be used to argue for a reduction in the treatment of agricultural land with such material for use in animal management, and thus reduce exposure to these harmful chemicals. Indeed, Switzerland has already banned sewage sludge fertiliser usage for animal management practices (Wager, 2007). Adding to the previous work completed on the ovine sewage sludge model, these results further strengthen the augment that the EU should follow in Switzerland's footsteps to ban or at least reduce the use of processed human sewage sludge as a fertiliser in animal management systems.

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