The impact of grazing oestrogenic clover on the reproductive tract of ewes in Western Australia: a histomorphometric and immunohistochemical analysis

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THESIS DECLARATION

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The research involving animal data reported in this thesis was assessed and approved by The University of Western Australia Animal Ethics Committee. Approval #RA/100/1657. The research involving animals reported in this thesis followed The University of Western Australia and national standards for the care and use of laboratory animals.

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ABSTRACT

Reproductive wastage and neonatal lamb mortality occur annually in Australian sheep farming systems—with significant implications for animal welfare and profit—from causes that are not always clear. A significant historical contributor to ewe reproductive wastage was clover disease, seen in ewes grazing cultivars of subterranean clover (subclover). Clover-diseased ewes were frequently infertile with high rates of uterine prolapse, dystocia and neonatal lamb loss, and masculinised behaviours and external genitalia. The source of this dysfunction was the structural similarity of subclover phytoestrogens, in particular formononetin, to mammalian sex steroid 17-β-oestradiol, causing permanent morphological damage to the ovine reproductive tract. Resultant changes in agricultural practice reduced pasture phytoestrogen availability, and clover disease has become rare in recent decades. However, recent reports outline concerns that permanent phytoestrogen infertility (PPI) may occur without the observable symptoms traditionally associated with clover disease.

This thesis examined ewe reproductive tract histology for evidence of markers associated with clover disease. Treatment groups included phytoestrogen-exposed ewes that failed to conceive over two subsequent joining seasons [double-dry (EX-DD)], ewes that conceived, lambed then lost their lamb around the time of birth (EX-LL), healthy ewes that most recently lambed and raised the lamb to weaning age (EX-LW) and non-exposed controls. Samples from mid and uterine cervix regions underwent histopathology analysis, including cervical fold morphology, muscularis dimensions and cervical gland numbers. Uterus samples were examined for morphological parameters, quantitative stereological classification of tissue types, and oestrogen receptor (ERα and ERβ) and androgen receptor (AR) expression.

The number of mucus glands markedly increased in mid and uterine cervix, and uterus in EX-DD and EX-LL but not EX-LW compared to the control. All ewes exposed to phytoestrogens had increased cervical fold length and muscularis width in the mid cervix compared to the control. Only EX-LW increased the cervical fold area. Stereological quantification of uterine tissue types revealed increased smooth muscle volume density in EX-DD ewes and increased extracellular matrix (ECM) in EX-LW ewes compared to the control ewes. All exposed groups had reduced endometrial volume and increased volume density of uterine blood vessels compared to the control. All groups had similar ERα, ERβ and AR expression in uterine epithelia and glands. All exposed groups had significantly reduced cervix and organ size compared to the control group.

This thesis links the proliferation of cervix glands and aberrant reproductive outcomes in ewes exposed to phytoestrogens, indicating that current pasture phytoestrogen levels are sufficient for permanent infertility in a subset of ewes. Further research is required to establish updated diagnostic guidelines for veterinarians, farmers and researchers examining PPI relevant to the current occurrence of phytoestrogens in pastures.

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nIteb Qob qaD jup 'e' chaw'be' SuvwI'."

DEDICATION

This thesis is dedicated posthumously to Dr NR Adams; without his detailed and methodical analysis over many years of contribution to the study of clover disease, I would have had much less to write about.

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ABBREVIATIONS

AU = arbitrary units

BPA = bisphenol-A

DM = dry matter

DDT = dichlorodiphenyltrichloroethane

EDC = endocrine-disrupting chemical

EE = environmental oestrogen

ERα = estrogen receptor alpha

ERβ = estrogen receptor beta

FSH = follicle-stimulating hormone

H&E = hematoxylin and eosin

HPG = hypothalamic-gonadal-pituitary

LH = luteinising hormone

PPI = permanent phytoestrogen infertility

CHAPTER 1. INTRODUCTION

Management of ewe fertility and neonatal lamb mortality present ongoing challenges in terms of sustainable profitability and animal welfare management (for a review, see Bruce et al. 2021). A significant historical contributor to reproductive wastage in the southern half of Australia has been fertility disturbances caused by endocrine-disrupting phytoestrogens present in pasture forage legumes, particularly subterranean clover (*Trifolium subterraneum L.*), commonly known as subclover (Bennetts et al. 1946). Subclover is ubiquitous in pasture forage systems within the higher rainfall areas of southern Australia (Walker et al. 2003). Older cultivars of subclover often contain very high amounts of the phytoestrogen formononetin that, once consumed, interferes with reproductive function in ewes in a syndrome so connected to subclover that it was named 'clover disease' (Smith et al. 1971).

Historically, ewes with clover disease displayed distinct physical symptoms after exposure to high levels of formononetin. These included low conception rates (as low as 15%) despite repeated exposure to fertile rams, frequent uterine and vaginal prolapse, low lambing percentages, and high dystocia rates with resultant increases in neonatal lamb and ewe mortality (Beck and Gardiner 1965). The cause of this syndrome was determined to be the structural similarity of subclover phytoestrogens to the endogenous hormone oestrogen and the consequent disruption of oestrogen's physiological effects in the ewe (Tapiero et al. 2002). Oestrogen adversely affects the organisational structure and development of reproductive organs and is the main driver of female reproductive tract organogenesis during embryonic life (Bondesson et al. 2015). In adulthood, oestrogen triggers activational effects, whereby time-critical fluctuations of oestrogen mediate functional changes in glandular activity and the epithelial and muscular layers of the reproductive tract to prepare the ewe for insemination and pregnancy (Murray 1995). As such, consuming oestrogen-mimicking phytoestrogens can have devastating downstream consequences for ewe fertility and fecundity (Adams 1990).

It was discovered that two forms of subclover phytoestrogen infertility occurred in exposed ewes (Adams 1990; Adams 1995). First, similar to other female mammal species, temporary infertility in ewes can occur when exposed to phytoestrogens during adult life, with similar effects to administering high doses of oestrogen (Jefferson 2010). Temporary phytoestrogen infertility acts at the ovary level and results in compromised follicular function, embryo mortality, suppressed gonadotrophin production and sequentially depressed flock fertility and lambing percentage (Smith et al. 1979). The effects can be short-term if the phytoestrogen source is removed, and fertility recovery is possible within weeks (Adams 1990). However, ewes become permanently infertile after months or years of consistent grazing of oestrogenic clovers, even when examined several years after removal from oestrogenic pastures (Schinckel 1948). This second, permanent form of infertility acts primarily at the cervix level and results in pathological proliferation of cervical glands, epithelia and muscularis, similar to the organisational

rearrangement caused by high doses of exogenous oestrogen administered during fetal life (McLachlan et al. 1980).

Studies on clover-exposed ewes identified the cervix as the primary site of clover disease infertility, with affected ewes often exhibiting regular ovarian function and ovulation but significant differences in the number of sperm recovered from the cervix and fallopian tubes after insemination (Lightfoot et al. 1967). The ovine cervix is critical for ewe fertility, as a facilitator of spermatozoa transportation post-copulation, due to its anatomical structure and mucosal secretions (Naqvi et al. 2005). The tortuous, convoluted inner cervical structure acts as a reservoir for ram semen, with the glandular mucosal layers guiding spermatozoa towards the ovum via cervical mucus that changes composition, volume and viscosity in response to oestrogens immediately prior to oestrus (Schumacher 1970). After months or years of subclover phytoestrogen exposure, the ovine cervix exhibits flattening of cervical folds, hypertrophy of underlying muscularis, and reduced luminal surface area (Adams 1995). In addition, cervical glands atrophy and multiply in number, preventing the production of mucus necessary to facilitate insemination, resulting in high rates of infertility and bacteriological infestation (Adams 1995). Ovine species are the only experimental animal studied to demonstrate permanent, organisational changes in reproductive tract morphology in response to phytoestrogen exposure in adult life (Adams 1990). Temporary infertility from subclover phytoestrogens poses little economic threat to most of southern Australia, as subclover, an annual legume, grows only in autumn, winter and spring while sheep in this region are joined for mating in summer; however, permanent clover disease could cause substantial losses in terms of profit and animal life (Adams 1995).

Despite key insights linking cervical malfunction and infertility, many questions around clover disease remain unanswered, including the mechanism by which clover disease results in dystocia and neonatal lamb loss. Many ewes have conceived successfully after exposure to phytoestrogens, only for the lamb to perish at birth or shortly after; uterine contributions to clover disease may have been overlooked (Adams 1990). Structural alterations to uterine tissues, particularly volume ratios of smooth muscle and extracellular matrix (ECM) linked to dystocia in other species, may be mediated by high phytoestrogen doses (Wu et al. 2012). Further, masculinisation of ewe external genitalia and mating behaviour has been attributed to consumption of high phytoestrogen pastures, although the mechanism underlying this is currently unclear (Adams 1979). Unanswered questions about the phytoestrogen mechanism of action are complicated by the natural resistance of some ewes to clover disease. Commonly, a subset of ewes show no symptoms of either permanent or temporary infertility and have normal fertility and fecundity (Adams and Croker 1987).

More recently, the selection and release of low-formononetin subclover cultivars and modifications to agricultural practices reducing oestrogenic subclovers in pastures reduced the frequency of clover disease symptoms, with the disease presumed eliminated (Nichols et al. 1994). However, a subsequent publication warned against the common presumption that clover disease had disappeared (Adams

1995). Adams wrote of a ‘subclinical’ presentation of clover disease, which included significant rates of infertility but lacked observable physical symptoms in ewes grazing reduced volumes of subclover phytoestrogens (Adams 1995): he named this syndrome permanent phytoestrogen infertility (PPI). Five million ewes Australia-wide were predicted to be afflicted with PPI in 1995 (Adams 1995). However, with no readily accessible evidence of its presence or absence, research interest in PPI was lost.

Recently, renewed concerns of a knowledge gap arose as research into phytoestrogen infertility syndromes had not been updated since the 1990s (Martin et al. 2021). Lamb losses in Australia are highly variable, ranging from 10–77% in a given year (Refshauge et al. 2015); it is unknown how many of these deaths occur on pastures containing persistent oestrogenic subclovers that can persist for decades without encouragement. Additionally, dystocia and ewe infertility continue to cause significant economic losses and concerns for animal welfare, and the causes are not always obvious (Bruce et al. 2021). Further research is required to assess any risk to ewes grazing southern Australian pastures, clarify the mechanism of action of subclover phytoestrogens, and examine their effects in presently available volumes.

CHAPTER 2. REVIEW OF LITERATURE

2.1. Endocrine-disrupting chemicals

Endocrine function is critical for biological processes that maintain homeostasis, allowing the animal to respond to environmental cues that govern physiological processes, including those that relate to fertility and pregnancy. Endocrine-disrupting chemicals (EDCs) refer to a diverse class of synthetic and environmentally derived chemicals that, once ingested or absorbed by animals, are capable of disruptive sequelae on aspects of endocrine function. An EDC is defined by the United States Environmental Protection Agency as "an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action" (Gore et al. 2015). While the effects of EDCs are numerous and diverse, they most commonly suppress reproductive function by interfering with oestrogen action (Chen et al. 2010).

While studies have focused on environmental oestrogens (EEs) derived from synthetic chemicals from industrial contaminants (e.g. dioxins,), plastics (e.g. bisphenol-A [BPA]) and pesticides (e.g. dichlorodiphenyltrichloroethane [DDT]) (Boverhof et al. 2006; Lemaire et al. 2006; Yang 2011). Less research has been undertaken in recent decades on the effects of EEs of plant-based origin, termed phytoestrogens. Of principal interest for female livestock reproductive capacity are the naturally produced phytoestrogens present in many pasture forage legumes (Saloniemi et al. 1995). Phytoestrogens, once ingested, interfere with endogenous oestrogen biosynthesis and cellular metabolism in mammals primarily via competition for oestrogen receptors, resulting in downstream consequences for reproductive function (Morito et al. 2001). All phytoestrogens share a relatively low affinity for oestrogen receptors, including the isoflavone formononetin present in a small number of older cultivars of subclover (Schoo and Rains 1971). However, despite low affinity for oestrogen receptors, high phytoestrogen clovers can significantly interrupt various oestrogen roles in female livestock when consumed in large quantities (Marshall et al. 1971).

2.2. *Trifolium subterraneum* and sheep farming

Since its introduction to Australian agriculture in the early 1900s, subclover has been the most widely sown pasture legume in the agricultural zone of temperate southern Australia and is estimated to be present on over 29 million hectares (Hill and Donald 1998). Subclover and other legumes are prized for their ability to enrich soils with nitrogen sequestered from the atmosphere, a trait particularly important in the southern half of Australia, where soils are naturally low in nutrients (Dolling 2017). Subclover has a high protein content and a favourable nutritional profile and is ths a good resource to feed sheep (Robertson and Friend 2019). While initially introduced to Australia from Europe accidentally, cultivars have been developed over the last hundred years to further adapt subclover for southern Australian pasture agriculture conditions (for review, see Nichols et al. 2013). Subclovers have a hard seed which allows a percentage of subterranean seed banks to remain dormant each season, preventing the need for

farmer maintenance such as frequent re-sowing (Smith 1988). Other advantageous subclover traits include tolerance to the wide range of annual rainfall volumes and growing season lengths across temperate southern Australia and resistance to pests and pathogens such as red-legged earth mite (Foster et al. 2017; Nichols et al. 2019). Subclover pastures were widely sown in southern Australia's early agricultural history when the harsh infertile soil conditions necessitated rapid enrichment (Summerfield and Bunting 1980). Subclover was commonly sown in monoculture swards, comprising one or two cultivars of clover as 100% of the pasture biomass, and contributed to flourishing early sheep enterprises (Summerfield and Bunting 1980). However, some decades later, naturally occurring phytoestrogens in subclover were linked to deleterious reproductive outcomes in grazing livestock (Bennetts et al. 1946).

2.3. Phytoestrogens in subclover

Phytoestrogens are a class of oestrogen-mimicking plant compounds comprising isoflavones, coumestans, lignans and stilbenes (Setchell 1998). Subclover contains the isoflavones genistein, its methylated derivative biochanin A and formononetin (Tava et al. 2016). Other species of legumes such as alfalfa (*Medicago sativum*) contain coumestans that are demethylated intra-ruminally into coumestrol, which is also oestrogenic (Lookhart 1980; Bora and Sharma 2011). Biochanin A and genistein are harmless in ewes due to their rapid intra-ruminal reduction into non-oestrogenic metabolites excreted without effect (Beck 1964). However, formononetin is metabolised in ovine rumen to the isoflavandiol equol (Figure 1), which is rapidly absorbed into the bloodstream to compete with endogenous oestrogens for oestrogen receptors within hours of initial consumption (Dewi et al. 2012) Formononetin is only produced in oestrogenic subclover when the plant is green and growing from autumn to spring, with the concentration varying significantly depending on available nutrients and environmental stresses such as waterlogging and pathogens (Booth et al. 2006). It is debated why subclover produces phytoestrogens, but they appear to enhance clover immune function and growth, perhaps explaining why the presence of certain plant viruses amplifies phytoestrogen production in legumes (Kellock 1971).

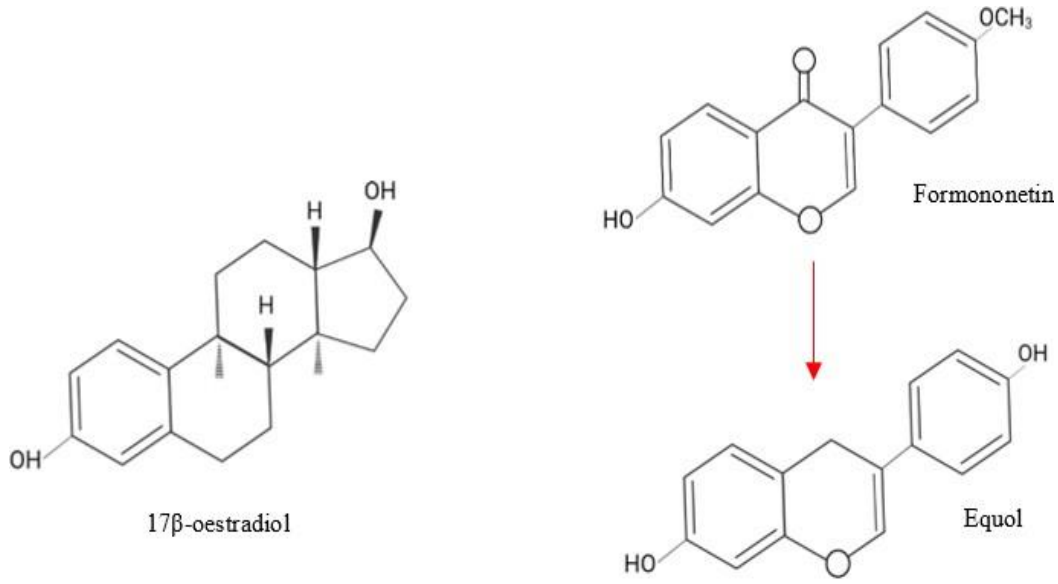


Figure 1. Conformational similarity of 17β-oestradiol, formononetin and equol. Mammalian sex steroid 17β-oestradiol (left), critical to reproductive processes in female sheep, bears a structural and functional similarity to phytoestrogens such as the isoflavone formononetin (top right), which are naturally occurring in many plant species, including certain subclover cultivars. Formononetin is harmless to ewes until reduced intra-ruminally (red arrow) to its oestrogenic metabolite equol (bottom right). Created with BioRender.com

2.4. Clover disease in Western Australia

Severe reproductive anomalies in sheep grazing subclover were reported in Western Australia in the early 1920s and first linked to subclover in 1942 (Bennetts et al. 1946). On oestrogenic subclover pasture, ewe fertility markedly declined, and the number of stillborn lambs increased, with high rates of uterine prolapse and dystocia—usually fatal to the lamb and often to the ewe due to infection or exhaustion (George 1975). Uterine prolapse was observed even in virgin, unmated ewes, which is unusual for ovine species (Adams 1977). Masculinised, swollen external genitalia, often alongside infected and swollen mammary glands, and masculinised sexual behaviour were also observed (Adams 1979). Some studies also reported 'high tail', where dysfunctional pelvic ligaments cause pelvic rotation, elevating the ewe tail unnaturally high (Beck and Gardiner 1965). It was not long before the aberrant reproductive outcomes of clover disease were linked to specific subclover cultivars (Guggolz et al. 1961). With a significant proportion of the highly prevalent subclover pastures across southern Australia 100% pure subclover swards, it was critical to identify offending cultivars and the mechanism involved. Of the 45 cultivars registered in Australia today, only five are oestrogenic; four of them—Dinninup, Dwalganup, Geraldton and Yarloop—were heavily relied upon in the early days of agricultural expansion in the south-west of Western Australia and thus disproportionately prevalent in this region (Collins et al. 1996).

After the discovery of isoflavones and identification of formononetin as the culprit, subclover's persistence—a perceived benefit for its inclusion in pasture forage systems—was recognised as a disadvantage (Marshall et al. 1971). Oestrogenic subclover cultivars continued to cause clover disease symptoms for years and even decades after re-sowing ceased (Taylor and Rossiter 1974). Farmers were advised to adequately fertilise pastures, as phosphate-deficient soils can increase formononetin concentration (Neil and Marshall 1970), avoid mating ewes on oestrogenic pastures, and dilute pasture phytoestrogens by planting more grasses, broadleaf and non-oestrogenic legumes (Adams and Croker 1987).

Changes in pasture management, aided by subclover breeding programs in the 1960s and 1970s supplying novel low-formononetin cultivars, saw a steady decline in pasture phytoestrogens in the following decades (White et al. 2010). Pure subclover swards are no longer seen across southern Australia, and subclover cultivars bred since the 1970s contain negligible formononetin content (Nichols et al. 1994). Consistent with pasture improvement has been the steady decline in reports of severe clover disease (Adams and Croker 1987). The observable external symptoms—masculinisation of external genitalia, high rates of neonatal lamb loss, extremely low lambing percentages and high rates of dystocia and prolapse afflicting unmated ewes (Adams 1995)—are no longer seen. However, as research into this condition has ceased, dietary xenoestrogens in other species—including soy products in human diets, commercial laboratory and pet feed in rodents and non-human primates, and mycoestrogens such as zearalenone in commercial livestock feeds—have accrued some research interest (Faber and Hughes 1991). Often these animals present as outwardly healthy, which can obfuscate the relationship between dietary intake of these compounds and reduced reproductive rate (Stopa et al. 2014). Generally, pasture containing more than 20% oestrogenic subclover is potentially dangerous to ewe fertility, but this has been understudied. Calls for updated information on the current volumes of subclover phytoestrogens on livestock have gained traction across southern Australia (Martin et al. 2021).

The study of clover disease in ewes has been fraught with contradictory findings, chief among these, not all ewes on oestrogenic clover pastures suffer the disease (Marshall 1973). While clover disease is considered cumulative and permanent, it is considerably more advanced in some ewes than others within the same flock grazing the same pastures (Adams and Croker 1987). Even more puzzlingly, some ewes appear immune from phytoestrogen effects, able to graze oestrogenic subclover pastures, conceive, give birth to live lambs and remain fertile in following joining seasons (Adams and Croker 1987). Several theories were postulated for the mechanism of immunity, including differences in the rumen microbiome responsible for breaking down formononetin, with differential microbial populations linked to phytoestrogen infertility resistance in other species (Williams et al. 2019). A significant proportion of humans who consume dietary soy products also lack adequate intestinal bacteria to reduce isoflavones into equol, with the compounds excreted without effect (Liu et al. 2010). Similar variations in individual microbiomes may be true for ewes, considering the sheep rumen rather

than human intestine as the site of isoflavone reduction. Other theories include a genetic selection bias from repeated culling of infertile ewes, resulting in a phytoestrogen-resistant flock (Davenport 1967). Sheep often show grazing preferences that vary among individual animals, perhaps detecting the taste of phytoestrogens and avoiding those clovers (Penning et al. 1997). However, none of these theories has been tested comprehensively. Prior to the present investigation, clarification of the pathology of PPI and its testing methods were lacking for sheep in current systems.

2.5. Oestrogen, phytoestrogens and sources of endocrine function in ewes

Dietary phytoestrogen exposure has been linked to endocrine disruption in rodents (Glover and Assinder 2006), avian species (Rochester 2009), fish (Clotfelter and Rodriguez 2006) and captive wildlife such as the cheetah (Setchell et al. 1987) and southern white rhinoceros (Tubbs et al. 2016). The degree of severity varies depending on dosage and duration of exposure, species studied, and environmental conditions (Branham et al. 2002). The cause of this disruption is the conformational and functional similarity of phytoestrogens to endogenous oestrogens, especially 17β-oestradiol, which is the primary oestrogen produced by mammals (Amenyogbe et al. 2020). The effects of oestradiol throughout the life cycle of female mammals can be classified as either organisational or activational (Abbott et al. 2006). Organisational effects of oestradiol occur in early fetal life when the female phenotype develops from an undifferentiated embryo, and organs in the reproductive tract and associated brain regions gain complexity and maturity (Hannema and Hughes 2007). Activational effects of oestrogen begin after puberty when cyclical steroidal release mediates the timing of reproductive tract processes critical for fertility (Figure 2). This is known as the oestrus cycle in ewes, does, sows and domestic pets like the cat and dog, and the menstrual cycle in humans, some ape species, and the spiny mouse (Bellofiore et al. 2017). These processes, while variable among species, share a common end goal of successful ovum maturation and release, and preparation of the reproductive tract to support conception and pregnancy (Ortavant et al. 1988).

While considerable variation exists between and within breeds, sheep in Western Australia are generally ‘short-day breeders’, entering a period of fertility during late summer when the increasing night length (reduced photoperiod) triggers a photosensitive neuronal system to increase the duration of nightly secretion of the pineal hormone, melatonin (Figure 2) (Chemineau et al. 1992). This occurs from December to January in Australia, with resulting lambs born about five months later onto peak pasture density, ensuring that maximal nutrition minimises the risk of lamb starvation (Réale and Bousses 1999). While Merinos are considered seasonal breeders, they are not strongly so. Adherence to reproductive seasonality varies significantly among individual Merinos and is less predictable than for other sheep breeds (Ortavant et al. 1988). Fertile periods can be managed with nutrition, implants carrying melatonin or progesterone, and exposure to the ram effect, all of which trigger the start of cyclicity in preparation for fertilisation and conception (Martin and Scaramuzzi 1983; Mutiga and Baker 1984; Jorre de St Jorre et al. 2014). Non-pregnant ewes enter a period of diestrus, or seasonal infertility, when decreasing night length reduces the duration of melatonin secretion (Pérez et al. 1998). The

photoneuroendocrine circuit is the catalyst for subsequent reproductive sequelae across the hypothalamic–pituitary–gonadal (HPG) axis (Chemineau et al. 1992).

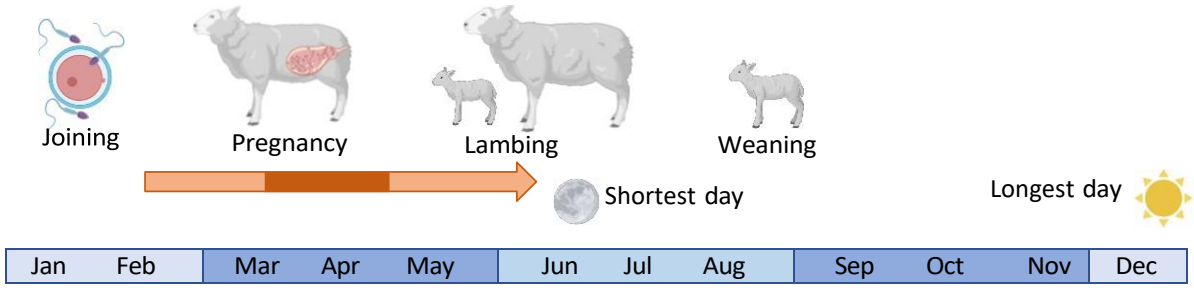


Figure 2. Breeding cycle of a Merino ewe and interaction of pregnancy stage typical in the southern half of Australia. Ewes begin oestrus cycles as day length begins to shorten, and joining with rams begins early in the year. Ewes are pregnant for ~147 days, with lambing in Western Australia between May and July and weaning ~12 weeks later (Curnow and Conte 2019). Individual farm management, weather variability, geographical differences and other factors can influence the precise timing of this process by several weeks.

The HPG axis in mammals comprises the hypothalamus region of the brain, pituitary gland and gonads (ovaries in females) (Figure 3) (Chappel and Howles 1991). Regulation of oestradiol and other sex steroids occurs through positive and negative feedback processes (Acevedo-Rodriguez et al. 2018). When environmental conditions are appropriate, the reproductive control centre in the brain produces gonadotrophin-releasing hormone gonadotrophin-releasing hormone (GnRH), triggering the pituitary gland to release the gonadotrophin's luteinising hormone (LH) and follicle-stimulating hormone (FSH) (Dwyer and Quinton 2019). This sequence triggers the aromatisation of androgens to oestrogens, most notably oestradiol, primarily from the granulosa cells of the ovary (Kirilovas et al. 1999). Biologically significant amounts of oestradiol are also synthesised by the adrenal glands, adipose tissue and brain (Setchell et al. 1998). A homeostatic process involving negative feedback to the brain-pituitary system (Figure 3), establishes an equilibrium where there is sufficient circulating oestradiol to maintain reproductive tissues. This equilibrium shifts when the brain detects appropriate environmental conditions, timing ovulation to result in birth at what is generally optimal time of the year for the survival of the newborn.. All stages of the reproductive cycle are dependent on appropriate oestrogen signals and HPG feedback, and all levels of the HPG axis are susceptible to disruption through the consumption of endocrine-disrupting compounds (Adams 1976).

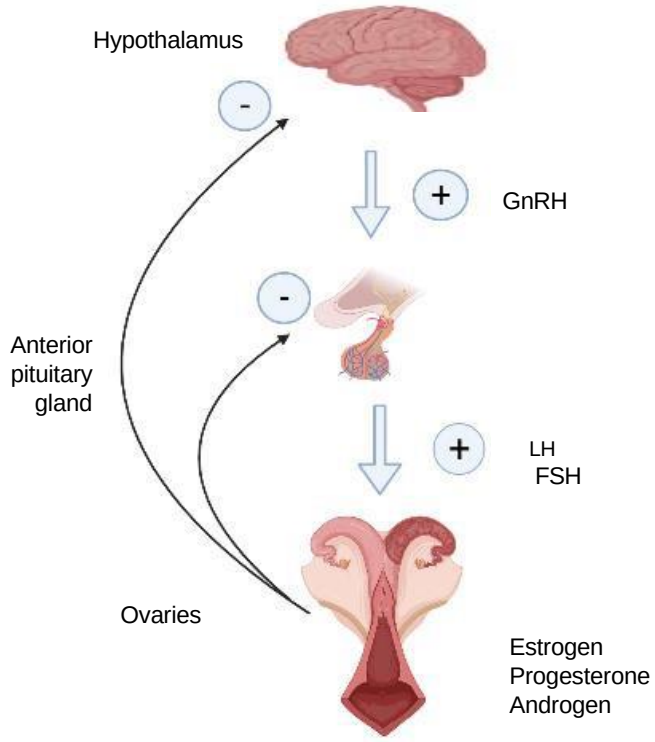


Figure 3. Negative feedback loop (*arrows*) of the hypothalamic–pituitary–gonadal (HPG) axis in the mature ewe. The hypothalamus, located at the base of the brain, releases GnRH, which stimulates the anterior pituitary gland to release luteinising hormone (LH) and follicle-stimulating hormone (FSH), triggering the production of sex hormones from the gonads—specifically, the ovaries in the female. The sex hormones in blood plasma supply negative feedback to the hypothalamus and anterior pituitary gland to establish an equilibrium with relatively constant concentrations of LH, FSH and sex steroids. Created with BioRender.com

2.6. Mechanisms of oestrogen action

17β-oestradiol influences oestrogen-responsive organs and tissues primarily via steroid receptors located in the nucleus of cells (Aronica et al. 1994). Oestradiol and other steroid hormones are small and lipid-soluble, allowing them to pass through the phospholipid cell membrane bilayer and form a hormone-receptor complex with their receptor (Klinge 2001). This 'ligand-hormone receptor complex' activates target genes, triggering changes in cellular processes that influence downstream tissue and organ morphology and functional sequelae (Kushner et al. 2000). Oestradiol less frequently exerts effects via signalling molecules in the cell membrane (Xu et al. 2019). Signalling pathways such as g-coupled protein receptors and tyrosine kinases act outside of DNA amplification and protein synthesis, which can activate change in minutes rather than hours or days (Xu et al. 2019).

While the classical oestrogen receptors (ERα and Erβ) share common functions, they are produced by different genes located on separate chromosomes and have distinct distributions and roles (Gosden et al. 1986). ERα and ERβ are expressed in the cardiovascular and central nervous systems; however, ERα is mainly expressed in the female in ovarian thecal cells, mammary gland, bone, liver and adipose tissue,

while ERβ is expressed in ovarian granulosa cells, colon, adipose tissue and immune system (Paterni et al. 2014). Both ERα and ERβ are expressed in the cervix and uterus and regulate tissue-trophic action, although the exact contribution of each is uncertain (Wang et al. 2000). ERα knockout mice show marked abnormalities in all organs of the reproductive tract and are functionally sterile (Antonson et al. 2012). ERβ knockout mice are capable of pregnancy but at a reduced frequency and fecundity (Couse and Korach 2001). While ERα has a more prominent role in the reproductive tract, the HPG axis and predominantly the uterus, phytoestrogens have a more profound affinity for ERβ (Pike et al. 2000).

Progesterone and its associated receptor (PR) are important for regulating the luteal stage of the oestrus cycle and opposing the effects of oestrogens and critical for gestation (Mesiano et al. 2011). The role of androgen receptors in the ewe reproductive tract are not fully understood but appear to play a key function in endometrial decidualisation and myometrial proliferation (Duan et al. 2019). Androgens may facilitate the role of oestradiol in trophic activity in the reproductive tract through cell signalling, gene expression and cellular metabolism (Kowalski et al. 2004).

Oestrogen receptor expression and localisation mapping for diagnostic examination of disease phenotypes have been heavily studied in mammals, and historically used in human cancer pathogenesis (Burstein 2020). Detection and quantification of ligand binding to ERs is a reliable indicator of tissue-trophic activity in the reproductive tract (Shanle and Xu 2011). Isoflavones can activate oestrogen receptors, preferentially ERβ, in a similar manner to oestradiol but at a fraction of the potency (Markiewicz et al. 1993). However, this appears sufficient for severe reproductive damage if large amounts of oestrogenic clover are consumed continuously, especially as all or most of the total diet (Marshall et al. 1971).

In non-ovine mammals, dietary isoflavones appear to cause mildly anti-oestrogenic effects due to their competitive binding with oestradiol for oestrogen receptors but comparatively weak biological effect (Morito et al. 2001). While phytoestrogens have variable relative binding affinities that differ from other environmental oestrogens, such as plastics, the low levels of isoflavones in human diets combined with their low potency makes them harmless or mildly beneficial (Cederroth et al. 2012). Traditional Asian diets contribute roughly 15–50 mg of soy isoflavones per day, with western countries reporting less than 2 mg (Eisenbrand 2007). The therapeutic dosage of isoflavones for oestrogen supplementation for a 60 kg postmenopausal woman is 1500 mg of red clover (Chen et al. 2021). A ewe consuming several kilograms of clover daily as their only food source could easily surpass any estimated safe dosage for humans by orders of magnitude (Chen et al. 2021). The immediate effect of such high volumes of dietary isoflavones on oestrogen receptors in clover-diseased ewes has not been studied. However, shorter-term studies of high-dose phytoestrogens on rats yield many of the same symptoms seen in clover-diseased ewes, including vaginal keratinisation, cervical squamous hyperplasia and a marked increase in uterine weight (Chen et al. 2021). This increase in proliferative activity mimics the effects of chronic oestradiol treatment, similar in human, rodent and ovine species (Martin et al. 1973).

Therefore, it is reasonable to assume that, with shorter-term exposure, oestrogen receptor activity in the reproductive tract in ovine species is similar to rodents and humans, although more research is needed to support these assumptions (Kuiper et al. 1998).

Merino ewes in the southern half of Australia are the only experimental animal studied that is vulnerable to long-term effects from phytoestrogen ingestion in adult life. In other examined species, phytoestrogens have similar effects on reproductive tract architecture, steroid metabolism and gametogenesis to temporary infertility studied in ewes—the primary symptom of concern is ovulation suppression, in addition to altered steroid hormone production and increased uterine weight, with effects reversed upon withdrawal of the phytoestrogen source (McGarvey et al. 2001). Exogenous oestrogens and phytoestrogens can induce similar and permanent effects in humans, sheep and rodents if administered in large quantities during fetal and early neonatal life, with affected animals showing permanently increased reproductive organ size, glandular proliferation, suppressed gametogenesis, altered puberty commencement, and reduced or absent offspring (Awoniyi et al. 1998). The precise timing of sex steroid exposure and withdrawal is critical for reproductive tract development (Tzschentke and Plagemann 2006). Developmental processes do not recover if interrupted, and permanent alterations to reproductive tract morphology and subsequent function can persist in adult life (Hayashi et al. 2004), even across subsequent generations, after the phytoestrogen source is removed (National Toxicology Program 2008).

The unique susceptibility of ewes to temporary and permanent infertility from phytoestrogen exposure in adulthood makes it challenging to elucidate the direct mechanism of action of permanent clover disease based on research in other species (Adams 1995). Ewes with clover disease show permanent ‘oestrus-like’ appearance in their reproductive tract histopathology, including increased uterine wet weight, vaginal keratinisation and cervical squamous metaplasia, even after removal from phytoestrogenic pasture and subsequent ovariectomy (Adams 1990). Only two studies have investigated oestrogen receptor activity in ewes with permanent clover disease (Tang and Adams 1981, Tang and Adams 1986) with both showing an increase in oestrogen receptors in response to exogenous oestradiol, similar to that in unexposed controls, despite marked abnormal histopathology. Clover-diseased ewes had increased metabolic activity, as measured by DNA, RNA and glycoprotein quantity, after oestradiol treatment, but no change in oestrogen receptor proteins in either study (Tang and Adams 1981). Tang and Adams (1986) noted that this differed from the expression patterns observed in rats and mice exposed to chronic exogenous oestrogen in neonatal life, which had permanent reductions in oestrogen receptor expression. This suggests that permanent clover disease in ewes operates independently of the quantity of steroid receptors, which cannot be deduced easily from studies on other species. Further steroid receptor research may yield more clues since recent technological advances in laboratory procedures offer more accurate methods for measuring steroid receptors in tissues and mapping their distribution (Janardhan et al. 2018).

2.7. Endocrine control of fertility in the life cycle of ewes

Lambs are born with immature, underdeveloped reproductive organs (Hayashi et al. 2008). In early postnatal life, ewe lambs grow rapidly in the size and complexity of their reproductive organs, followed by a period of dormancy until puberty (Hayashi et al. 2008). Sexual maturation is precipitated by increasing activity in the HPG axis, as evidenced by the first oestrus at 5–12 months of age (Fitzgerald et al. 1982). Photoperiod, adequate adiposity and minimum threshold body weight stimulate the preoptic area to begin GnRH production, increasing the frequency of pulsatile LH release in the months leading up to the first oestrus (Wood and Foster 1998). The anti-Mullerian hormone, controlling sexual differentiation in fetal life and ovarian follicular recruitment in adulthood, also increases in the lead up to puberty (Baruselli et al. 2018). As follicles increase in size and mature, more oestrogen is produced, increasing the plasma concentration (Pellicer 1997). Oestradiol levels are highest immediately prior to ovulation, responsible for behavioural receptivity to copulation and preparation of the uterus for pregnancy (Young 2013). Heightened oestrogen availability peri-oestrus correlates with peak ERα and ERβ expression in the uterus of ewes (Duan et al. 2019).

Pulsatile secretion of LH also increases after exposure to pheromones from a novel, sexually active ram, a phenomenon known as the 'ram effect', used by producers to optimise oestrus timing (Jorre de St Jore et al. 2015). Ovulation is initiated by a surge of GnRH and LH, with an approximate 30-hour window where the ewe is in oestrus and amenable to mating (Quirke et al. 1981). Following oestrus, circulating oestradiol levels decrease rapidly, and the corpora lutea formed from the postovulatory follicle remnants produces progesterone for potential trophic support of a fertilised oocyte (Wathes and Hamon 1993). In the absence of a viable oocyte, progesterone drives endometrial decidualisation in preparation for the following cycles (Ng et al. 2020). In the absence of a pregnancy, ewes experience this cycle every 13–19 days until seasonal anoestrus is triggered by the above-mentioned change in day length (Chemineau et al. 1992).

2.8. Oestrogen in pregnancy, parturition and birth

In sheep, progesterone levels critical for fetal development and pregnancy retention are maintained by the corpora lutea during early pregnancy, with the placenta taking over full production by mid-pregnancy. Progesterone is necessary to prevent early labour, with a gradual decline at approximately 19 weeks (130–135 days) into ewe pregnancy and a sharp decline in the days leading up to parturition (Hamon and Heap 1990). Concomitant with a spike in oestradiol concentration, corticosteroids produced by the fetal adrenal glands trigger parturition in ewes (Thorburn et al. 1991). Oestradiol feedback regulation of sex steroids, and signalling molecules such as eicosanoids and neuropeptides, is critical for parturition progression (Weiss 2000). Placental and endometrial glands supply prostaglandins for parturition initiatian, fetal membrane rupture, cervical contractility, and uterine involution (Olson 2003). Oxytocin is released by the medial preoptic region of the maternal brain to

advance labour contractions and stimulate maternal bonding and milk release (Kendrick et al. 1992). The development of mammary glands—immature until the end of the first pregnancy—is triggered by decreasing progesterone concentrations and increasing oestrogen concentrations at birth, resulting in prolactin release from the anterior pituitary gland and onset of milk production (Curlewis 1992). Studies on phytoestrogen-afflicted ewes have not determined whether these processes are responsible for infertility and neonatal lamb mortality or where interventions should occur (for review, see Adams 1976; Marshall 1973).

2.9. Ultrastructure of the ovine cervix and response to oestrogen and phytoestrogens

The cervix is critical for ewe fertility as a facilitator of spermatozoa transportation post-copulation and is the organ responsible for phytoestrogenic infertility (Adams and Sanders 1993). The cervix comprises an ectocervical portion accessible via the vagina and a fibromuscular inner canal (ectocervix) that is continuous with the uterus (Naqvi et al. 2005). The endocervix comprises a luminal epithelium, glandular-underlying stroma, fibromuscular or 'muscularis' layer, and outer serosa (Naqvi et al. 2005). The proportions of ECM to smooth muscle in the muscularis layer, length of folds and luminal epithelia vary according to the level of the cervix examined and fluctuate in response to hormones—particularly oestradiol—over the oestrus cycle (Restall 1966; Breeveld-Dwarkasing 2000). The inner luminal folds of the ovine cervix are uniquely convoluted and ridged compared to other species (Wulster-Radcliffe et al. 2004).

The biochemical composition and hydroelastic properties of cervical mucus are highly responsive to changes in circulating oestradiol concentration, owing to their varied functions throughout oestrus and the reproductive cycle (Suarez and Pacey 2006). Adequate production of cervical mucus is critical for protection against microbial infection throughout the ewe's life, becoming vastly more hydrated and elastic to assist sperm transit during oestrus (Maddison et al. 2014). The composition of cervical mucus is consistently more than 90% water, although hydration varies throughout the oestrus cycle, as do the proportions of carbohydrates, lipids, proteins and immune cells such as immunoglobulins, leukocytes, alkaline phosphatase and lactate dehydrogenase (Schumacher 1970). Cervical mucus is produced in relatively low volumes during non-fertile periods. However, an increase in measurable elasticity (termed spinbarkheit) accompanies an increase in volume immediately prior to oestrus in response to high circulating concentrations of oestradiol (Abril-Parreño et al. 2021). The viscosity, chemical composition, pH and spinbarkheit of cervical mucus are critical for protecting cervical tissues, spermatozoa transit after copulation, and retaining semen within cervical folds (Adams 1977).

Cervical mucus also contributes to cervical relaxation and remodelling throughout the oestrus cycle via the production of glycosaminoglycans in mucus that contribute to the hydration of ECM (Akgul et al. 2012). Glycosaminoglycans are polysaccharides produced in all regions of the reproductive tract, and secreted via cervical and uterine mucus that contribute to ECM remodelling, protecting vaginal and cervical tissues from bacterial invasion, and acrosome reactions during spermatozoa capacitation prior

to oocyte fertilisation (Meizel and Turner 1986; Ruscheinsky et al. 2008; Akgul et al. 2012). Rising oestradiol concentrations during the first half of the oestrus cycle act on cervical ECM via multiple pathways, including triggering an increase in oxytocin receptor activity that mediates the separation of collagen bundles (Kershaw et al. 2007). Therefore, the tensile properties of the ovine cervix rapidly respond to changing oestrogenic stimulation and ECM is in greatest proportion, most hydrated and most relaxed when oestrogen levels are highest (Leethongdee et al. 2007). The cervix generally displays the highest ratio of ECM to smooth muscle immediately prior to the preovulatory LH surge when the circulating plasma oestradiol concentration is greatest, but the percentage of each type of tissue varies slightly depending on the region of the cervix examined (Kershaw et al. 2007).

In clover-diseased ewes, the cervix exhibits a permanent reduction in the number and height of cervical folds (Figure 4; Adams and Sanders 1993). As cervical folds decline under a continuously strong oestrogenic stimulus, marked hypertrophy of the muscularis layer widens individual folds as they reduce in length, increasing the individual fold area but reducing the total luminal surface area (Adams and Sanders 1993). As the number of cervical glands increases, the glands atrophy and take on the distorted, elongated shape of uterine glands (Adams and Sanders 1993). Staining clover-diseased cervices with acid and alkaline phosphatase, NADH-diaphorase, amino acid radicals and gallocyaninchromalum reveals very little mucin in clover-diseased glands and an intermediate morphological presentation more similar to uterine glands than cervical glands in healthy controls (Heydon and Adams 1977). As a result, clover-diseased ewes produce watery cervical mucus of poor spinbarkheit, irrespective of oestrus cycle stage, which continues after ovariectomy (Adams and Tang 1986).

Abnormal cervical morphology from clover disease was understood to reduce ewe fertility via two mechanisms: first, through failure of cervical mucus to adequately support spermatozoa transit (Adams 1986) and; second, through reduced complexity of folds preventing retention of semen in the reproductive tract. Together, these two mechanisms can prevent or heavily reduce rates of conception (Adams 1986). In a study of cervices excised post-copulation, clover disease ewes retained only 350 spermatazoa per Fallopian tube, compared to 17,160 in healthy controls (Lightfoot et al. 1967). The clover- exposed groups also exhibited fewer ova with sperm attached to the zona pellucida compared to the control (Lightfoot et al. 1967). Cervices of clover disease ewes also exhibited profound change in immunological function, with increased rates of cervicitis and bacterial invasion (Adams 1976). Cervical glandular epithelia is most responsive to the proliferative effects of oestradiol, with previous research indicating that the more caudal segments most markedly demonstrate changes due to high oestrogen environments such as accumulations of stromal cells, squamous metaplasia, and marked inflammation when compared to healthy controls (Adams 1986). Clover disease histopathology displays similar morphological abnormalities to long-term treated ewes with large oestrogen implants, indicating that high doses of phytoestrogens have a strongly oestrogenic effect on reproductive tract morphology (Adams and Sanders 1993).

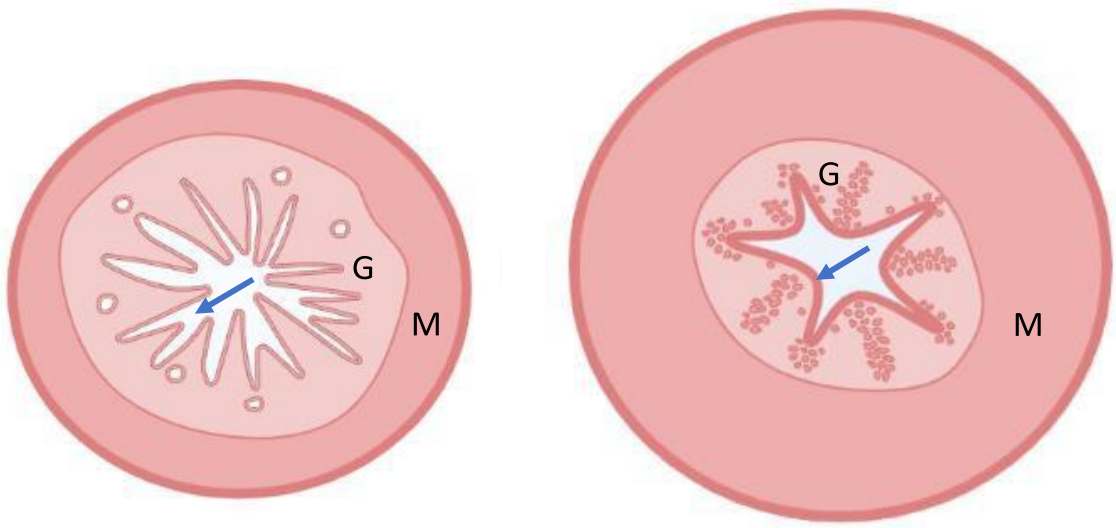


Figure 4. Phytoestrogen ingestion in ewes damages the cervix in a similar manner to treatment with high doses of 17β-oestradiol administered by implants. A healthy ovine cervix, shown here as a transverse cross-section through endocervix (*left*), exhibits a complex series of finger-like folds (*indicated by arrow*) that extend into the lumen and increase the surface area available to both ram semen and secreted mucus. After prolonged ingestion of large amounts of phytoestrogens (*right*), the muscularis mucosa layer exhibits marked hypertrophy (*muscularis mucosa layer marked M*), and the cervical glands atrophy and multiply exponentially (*glandular layer indicated by G*), while the cervical folds widen and fuse, and the crypts between cervical folds shallow. Thus, the surface area in the cervical lumen markedly declines. Epithelial tissue can also show inflammation, numerous cysts, large numbers of macrophage cells and squamous metaplasia. Created using BioRender.com, adapted from historical images (Adams 1986).

2.10. Effect of phytoestrogens on the ovine uterus

The uterus shares some similar morphological features to the cervix, with an inner glandular epithelium, smooth muscle muscularis layer and outer stroma (Adams and Sanders 1993). However, the myometrial layers are more numerous, thicker and more prominent than the cervix and contain less ECM (Arens et al. 2000). Uterine glands are long and tubular-shaped, rather than circular, and the glandular epithelial layer is smooth without folds (Adams and Sanders 1993). The ovine uterus is also bicornate, with two elongated horns ascending laterally from a common uterine corpus (Cooke et al. 2013).

The architecture of the uterus can be separated functionally into two zones, the endometrium and myometrium (Allison Gray et al. 2000). The endometrium comprises tightly connected fibroblasts adjacent to the luminal space, above hundreds of densely packed endometrial glands that radiate from the lumen towards the myometrium (Cooke et al. 2013). Endometrial gland secretions are critical during the initial stages of pregnancy for recognising the conceptus, implantation, adequate placentation and fetal nourishment (Goryszewska-Szczurek et al. 2021). Increasing availability of oestradiol from blood plasma during the luteal phase of the oestrus cycle directs the proliferation of luminal and glandular

epithelium and increases glandular activity in direct correlation with uterine thickness (Bertolin and Murphy 2014). Progesterone opposes these effects, resulting in dose-dependent atrophy of glands and stroma (Bertolin and Murphy 2014). In the absence of the opposing progesterone, high plasma concentrations of oestradiol can result in continued endometrial proliferation, hyperplasia, polyps and/or other lesions, similar to those in clover-diseased ewes (Lacey et al. 2005).

The uterine myometrium comprises multiple layers of smooth muscle cell bundles running in multiple directions. It is highly vascular, supplied by the uterine artery via branches of the ovarian and vaginal arteries (Elmetwally 2016). ECM volumes are relatively low in the myometrium but maintain critical roles in the vasculature and tensile support of uterine contractility during labour (Tiemann et al. 2020).

During periods of high phytoestrogen ingestion, as in other hyper-oestrogen syndromes, the uterus exhibits increased weight, attributed to both oedema and hypertrophy (Adams 1990). Cystic endometritis is also commonly seen, although not necessarily linked with reproductive outcomes (Adams 1975). The contribution of uterine abnormalities to the clover disease phenotype is difficult to elucidate. Clover disease has been considered a failure to conceive caused by damage to the cervix (Adams 1986). Axiomatically, ewes that overcome clover disease infertility can conceive successfully but fail to deliver a live lamb, perhaps not falling under the classically accepted phenotype, indicating greater uterine involvement. Curiously, the cervix and uterus in clover disease appear under oestrogenic stimulation at all times of the year, even after removing the ewes from oestrogenic pastures for several years or after ovariectomy (Tang and Adams 1982). The mechanism by which this ‘permanent oestrus’ phenotype persists, in the absence of any oestrogenic or phytoestrogen stimulation, is unknown.

2.11. Complexity of phytoestrogen effects

Outside of the clear connection between the morphological change in the cervix and infertility, the mechanisms leading to other clover disease symptoms remains mysterious. A lack of folds and increase in glands have been noted, with the cervix reverting to a more ‘uterus-like’ appearance (Adams 1979). It has been suggested that high levels of phytoestrogens have an androgenising effect on the cervix, reversing the feminisation of the cervix from uterine tissue during fetal development (Adams 1979). This process was attributed to the gene pathway for sexual differentiation, usually inactivated by birth in mammals, remaining transcriptionally active after birth and through adulthood (She and Yang 2017). Clover-diseased ewes have historically shown masculinisation of sexual behaviour (Adams 1983) and external genitalia, evidenced as redness and marked swelling of the clitoris (Adams 1979). However, masculinisation of ewes is uncommon, usually resulting from prenatal exogenous testosterone treatment (Lamm et al. 2012) or the virilising effects of adrenal hyperplasia, usually caused by a rare genetic mutation (Hall et al. 2004). The mechanism linking phytoestrogen exposure to masculinised reproductive organs, and the concentration and duration of oestrogenic exposure at which this occurs, is unknown. Androgenised play behaviour and genitalia are more commonly seen in ewes administered

with exogenous androgens and may indicate androgen receptor activation from high phytoestrogen doses (Pihlajamaa et al. 2011). Exogenous oestradiol treatment in humans can have opposing effects on endogenous oestrogen if administered doses are sufficiently high, but whether this phenomenon can activate androgenic effects in ewes has not been studied (Bayer et al. 2018).

The mechanism of action whereby exposure to high levels of phytoestrogens causes dystocia and prolapse is also unknown but likely due to inadequate functionality of support structures of the reproductive tract. Failure of adequate cervical effacement, a process of cervical softening prior to parturition, can lead to dystocia (Balamurugan et al. 2012). The lamb, if it survives the protracted birthing process, is more likely to suffer facial injuries that can be life-threatening due to an inability to nurse (Ismail 2017). Dystocia in humans has been linked to abnormalities in the biomechanical properties of the cervix and uterus (Buhimschi et al. 2006). Excessive ECM in humans is associated with uterine stiffness, increasing labour difficulty and often necessitating medical intervention (Buhimschi et al. 2006).

The relationship of oestradiol to tendon, ligament and ECM structures remains poorly understood, although strongly oestrogenic environments have been linked to several connective tissue malformations (Leblanc et al. 2017). Oestrogenic activity directly promotes the proliferation and activity of myofibroblasts, the matrix-producing cells that generate ECM proteins such as collagen (Muñoz-de-Toro et al. 2017). Increased oestrogen levels are also correlated with a pathological increased in ECM, seen as fibrosis (Ewies et al. 2003). The direct relationship between increased oestrogen and fibrotic phenotypes has been implicated in systemic sclerosis (Aida-Yasuoka et al. 2013) and scleroderma (Frost et al. 2019) in humans.

If the biomechanical properties of the reproductive tract are affected similarly by the ingestion of high quantities of subclover phytoestrogens and high oestradiol concentrations, this may explain the birthing difficulties associated with clover disease (Purohit 2006). The cervix undergoes a period of softening and relaxation known as ripening throughout pregnancy that accelerates immediately prior to parturition (Muñoz-de-Toro et al. 2017). Cervical ripening involves localised regulation of oestradiol and progesterone metabolism through a complex relationship between the cervical epithelium and stroma, although the exact process differs between species (Bornstein and Sage 2002). Generally, the process is controlled by the effects of oestradiol and progesterone on prostaglandins and oxytocin-induced positive feedback, causing an inflammatory cascade that produces excess collagenase, elastase, metalloproteinases and cytokine that rapidly alter cervical tissue types (Andersson et al. 2008). Cervical ECM becomes less structured and organised, permitting cervical opening and fetal expulsion (House et al. 2009). Incomplete cervical opening is a common cause of dystocia in livestock, often co-occurring with increased rates of prolapse (Prasad et al. 2017).

Cervix and uterus ECM comprises types I and III collagen (in stroma) and type IV collagen (in glands), the ratios of which facilitate uterine function generally and uterine contractility during parturition (Bornstein and Sage 2002). Abnormal collagen type III production in other animals has been linked to uterine prolapse (Ewies et al. 2003) and cystic glandular hyperplasia (Iwahashi and Muragaki 2011), both observed in clover-diseased ewes. Pathological increases in collagen type III, especially when measured in relation to collagen type I, are also seen in mice with cystic glandular hyperplasia, adenomatous hyperplasia and uterine prolapse, all of which are symptoms of clover disease (Diao et al. 2011). Aberrations in ECM proteins other than collagen have been linked to incomplete cervical ripening and/or uterine 'inertia', and a mixed double biglycin/decorin knockout mouse is often used as an experimental model for dystocia (Wu et al. 2012). Further research is needed to determine whether impaired volume density of ECM in response to high dietary phytoestrogen levels is relevant to clover disease dystocia and prolapse.

2.12. Barriers and confounding variables to clover disease and PPI research in ewes

Latency of effect

Estimating how much exposure a ewe has received to oestrogenic clover is difficult; even methods for assessing current intake are limited. Formononetin in clover is metabolised rapidly by ruminal microbiota and reduced quickly to equol (Lundh 1990). Within minutes or hours of grazing oestrogenic clover, the compounds of interest are circulating in the ewe's bloodstream and starting to be excreted (Dickinson et al. 1988). Tissue damage from months or years on oestrogenic clover persists long after the offending equol is gone (Shackell et al. 1993). Ewes can sometimes graze oestrogenic pastures for a year or more without evidence of ill effects and show evidence of infertility in one breeding season yet fall pregnant in a subsequent season (Adams and Sanders 1988). With continued exposure, damage to reproductive tissues is presumed to accumulate, however this is not yet possible to quantify with any certainty (Adams 1995). The only diagnosis for clover disease requires cervical tissue samples excised from autopsy, which is impractical for a breeding ewe flock (Adams 1986). Additionally, if ewes are sold or moved between pastures, the first evidence of clover disease may manifest months after removing the phytoestrogen source (Adams 1995).

Lack of agreed diagnosis criteria for PPI ewes

Affected ewes can only be diagnosed with clover disease via autopsy and pathology examination of the reproductive tract, which is impractical and costly in breeding flocks (Adams and Tang 1986). No readily accessible diagnostic tool exists for layperson use when examining subclover pastures for phytoestrogens or ewes for tissue damage. While the most commonly cited paper for clover disease assessment in ewes is comprehensive and robust, it was published in 1986 and compared high doses of phytoestrogens to supraphysiological doses of 17β-oestradiol administered via implants (Adams 1986). This level of exposure does not reflect the average pasture in southern Australia in recent decades (Adams 1998). It is unknown how the histopathology for milder phytoestrogen exposure—and thus the

average PPI phenotype—will present in comparison with previous studies of clover-diseased ewes. PPI is likely a spectrum of observable histological changes, reflecting individual degrees of susceptibility, with many ewes potentially resistant to the effects (Adams 1998). No specific criteria have been established for differences in histological presentation of PPI and clover disease, and little is known of the duration of exposure or dosage of isoflavones that induce the transition of PPI to clover disease.

Specificity of geographical limitations in clover disease epidemiology

Hundreds of edible plant species produce phytoestrogens, of which many can be used as pasture fodder; however, the geographic distribution of oestrogenic subclover cultivars in sheep production systems occupies a very small region of southern Australia (Cederroth et al. 2012). Permanent clover disease predominantly affects ewes on subclover pastures in high rainfall regions of the south-west of Western Australia, with less common incidents in isolated eastern regions of southern Australia, such as Kangaroo Island (Obst et al. 1971).

Ewes likely to be affected are not exposed to constant low-dose isoflavones year-round. Subclovers are oestrogenic when green and growing in mid-winter, resulting in high intake for several months, followed by a rapid reduction in dosage as spring approaches, and no exposure when the plants are senescent for six months of the year (Figure 5). The normal breeding season coincides with the senescent period, so ewes in southern Australia are not likely to experience exposure during mating or early pregnancy when temporary suppression of gametogenesis might be expected, as reported in many rodent studies (Brown and Setchell 2001; Cederroth et al. 2010; Lehraiki et al. 2011). Ewes are pregnant for ~147 days, with lambing in south-western Australia between May and July, followed by weaning ~12 weeks later (Figure 5; Curnow and Conte 2019). Different cultivars will flower at varying intervals in mid–late winter with the most intense exposure to phytoestrogens concentrated in the third trimester of pregnancy and the first months of neonatal life (Nichols et al. 2013). While other regions may rely on silage collected during winter for consumption during the summer months, resulting in year-round subclover availability, this is not common practice in southern Australia.

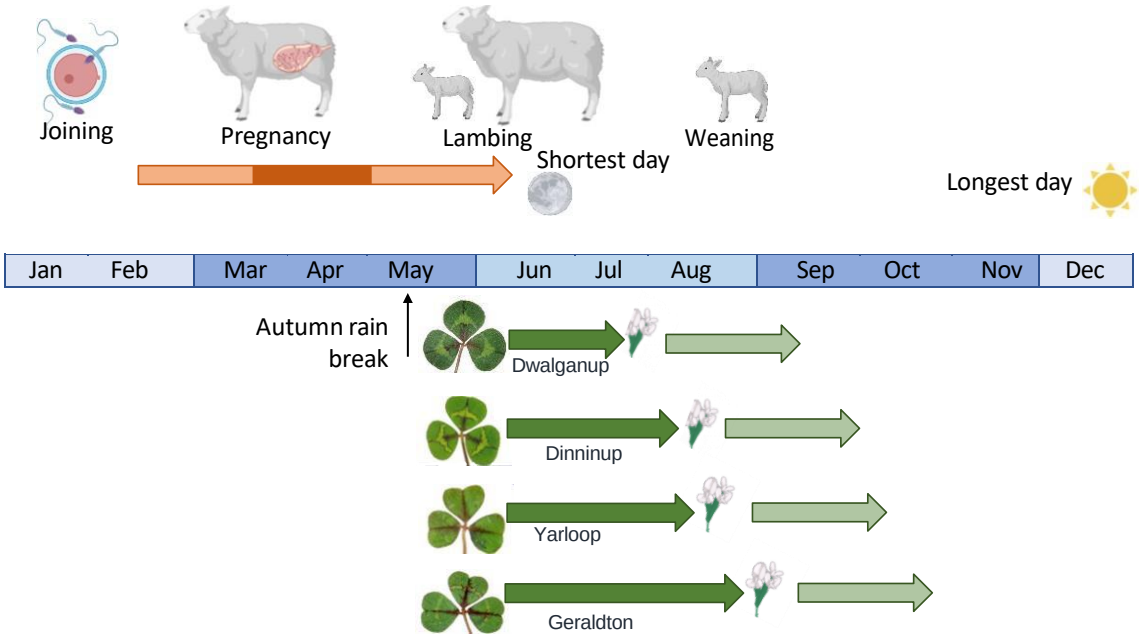


Figure 5. Breeding cycle of the Merino ewe and interaction of pregnancy stage with clover phytoestrogen potency in Western Australia. Ewes begin oestrus cycles as day length begins to shorten, and joining with rams begins early in the year. Ewes are pregnant for ~147 days, with lambing in Western Australia between May and July and weaning ~12 weeks later (Curnow and Conte 2019). Subclover is highly oestrogenic when green and growing (*dark green arrow*), emerging after the autumn rain break. Four cultivars of oestrogenic subclover are present in Western Australia—Dwalgenup, Dinninup, Yarloop and Geraldton (Nichols et al. 2013). Different cultivars will flower at varying intervals in mid–late winter. Isoflavones in subclover diminish rapidly after flowering (*pale green arrow*) and cease detectable levels after 6–8 weeks (Nichols et al. 2013). Farm and flock management, rainfall patterns and other factors can affect the precise dates of these events.

Clover disease and PPI symptoms are general and have multiple potential causative agents

A plethora of additional health issues unrelated to subclover can cause ewe infertility, including failure to ovulate due to poor body condition, undernutrition, infections causing high fever, or other illnesses (Van Lier et al. 2017; Givens and Marley 2008; Silva et al. 2013). Ram health, including infections such as brucellosis, low libido, and lowered sperm count can also affect pregnancy rates (Maquivar et al. 2021). Dystocia often occurs unrelated to phytoestrogen exposure for reasons such as fetal postural abnormalities, incomplete cervical dilation due to congenital malformation, and excessive lamb size for maternal pelvic capacity (Ismail 2017). Neonatal lamb loss occurs in 15–20% of live births within 48 hours due to cold exposure, birthing injuries and predation from wild animals (Flinn et al. 2020). Precise numbers of these incidents are difficult to elucidate, can vary from year to year, and do not always leave identifiable evidence (Hinch and Brien 2013).

Difficulty and variability in quantifying pasture isoflavone levels

Whether subclover cultivars are oestrogenic can be difficult for untrained observers to estimate from visual pasture analysis , especially in early stages of leaf growth (Foster et al. 2022). This is especially confounding when considering preferential grazing of ewes for their favourite pasture species, creating a mismatch between feed on offer and estimated feed consumed (Penning et al. 1997). Formononetin concentrations as low as 0.2% in subclover leaf as dry matter (DM) can affect reproductive function, but intake can be hard to estimate in a mixed pasture (Nichols et al. 2013). As a general estimate, oestrogenic subclovers with more than 1% formononetin DM comprising 20% or more of a pasture—roughly 0.2% formononetin DM—are potentially oestrogenic, but more research is required to estimate safe phytoestrogen doses (Quinlivan et al. 1968).

Estimations of the oestrogenic potential of a pasture are complicated by the changing volumes of subclover available for quantification during the season, and from year to year, depending on rainfall, competition from other pasture fodder species, consumption by livestock, and individual farm management (Marshall 1973). While the genetic potential for formononetin production remains relatively stable over time, the resultant expression of that potential can be influenced from year to year by weather patterns, stress, predation, nutrition and soil health, making it difficult to achieve consensus on the 'safe' amount for grazing sheep (Booth et al. 2006). High rainfall years can significantly increase the proportion of clover in total biomass, resulting in variable pasture oestrogenicity year to year (Piano et al. 1997). Sampling after a false rain break—where a sudden, isolated rainfall event early in autumn is sufficient to initiate seedling germination but insufficient for their continued growth—may result in underestimation of pasture phytoestrogens due to low subclover content in pasture (Dear and Cocks 1997). Additionally, environmental stressors, such as mineral deficiency and waterlogging, can maximise formononetin production in clover, resulting in inaccurate potency estimates if the measurement occurs outside the stressful period (Schoo and Rains 1971). Reports do not often provide the precise concentration of formononetin, with ewes being fed subclover as silage, oestradiol implants in lieu of subclover, or visual confirmation of the oestrogenic cultivars not quantified, leading to a gap in knowledge on the concentration of oestrogenic subclover required for safe consumption, PPI or clover disease (Smith 1971).

Multiple potential mechanisms of endocrine disruption

Phytoestrogens act through multiple methods to exert their effects in different species, and it is reasonable to expect that the same is true for ewes. Phytoestrogens usually act via the classical oestrogen receptors, ERα and ERβ (Hanafy et al. 2005). However, they are also considered selective oestrogen-receptor modulators due to their capacity to antagonise oestradiol in a specific tissue type while concurrently acting as an agonist in another (Oseni et al. 2008). Less commonly, phytoestrogens can act on progesterone and androgen receptors, on tyrosine kinase receptor pathways, and via second

messenger systems, such as dysregulation of intercellular calcium and cAMP channels and g-coupled oestrogen receptors (GPER)—all of which have downstream effects on oestrogen-responsive tissues (Pihlajamaa et al. 2011). The current physiology of the ewe is also impactful, with greater effects in lower weight ewes (Adams 1995). There may be more than one mechanism of phytoestrogen action in ewes.

Phytoestrogen positive effects that may disguise deleterious effects

Phytoestrogens—in appropriate dietary doses—have beneficial effects in several species (Mizutani et al. 2000), including antimicrobial, angiogenic, neuroprotective, anti-inflammatory, cardioprotective and antioxidant properties (Salehi et al. 2018). It is unknown whether these benefits partially or completely inhibit the symptoms of HPG axis dysregulation in exposed ewes. Long-term studies on ewes exposed to low-phytoestrogen subclover (total formononetin concentration < 0.004% of total dry weight, far below the < 0.2% benchmark for safety) did not affect ewe reproduction but had beneficial effects on lamb weight gain and improved carcass and meat characteristics (Pace et al. 2011).

Future research in this space could elucidate the benefits of phytoestrogen ingestion in ewes and whether these benefits are concurrent or independent of endocrine disruption in this species.

Inconsistency applying phytoestrogen research in other species to sheep

While phytoestrogen ingestion has been well-studied in non-ovine mammals, rodents and humans lack ruminal digestion common to cattle and sheep and therefore obtain dietary phytoestrogens via different digestive pathways (Dickinson et al. 1988). Therefore, parenteral injection of phytoestrogens in many rodent studies may not be directly comparable to studies examining dietary consumption in humans or ovine species (Lewis et al. 2003). Cattle share similar ruminal digestion to sheep but do not experience permanent clover disease (Wocławek-Potocka et al. 2013). Humans can produce equol from soy products via intestinal microbia, but significant dietary phytoestrogen exposure also occurs through daidzein and genistein, which sheep cannot metabolise (Setchell and Cole 2006). Humans, like sheep, are also highly variable in their individual ability to metabolise oestrogenic compounds from dietary phytoestrogens, but it is unknown why such variability occurs (Franke et al. 2012).

2.13. Conclusion

Clover disease in Western Australia was historically considered a significant challenge to sheep production systems. Overt physical symptoms—masculinised genitalia, marked reduced fertility, uterine and vaginal prolapse, and increases in ewe deaths during birth—were difficult to ignore and easily correlated with the visual evidence of subclover in pastures. However, prosperity in the decades since, combined with innovation in pasture science, has changed the landscape of Australian farming. Pasture forage systems have moved away from subclover monocultures to include more diversity in rotational crops (Moss et al. 2021). Recommendations were released and adopted on the best

management methods for farms with clover disease issues (Marshall 1973). New cultivars of genetically superior subclover have been released that produce no or low amounts of phytoestrogens (Abdi et al. 2020). Widespread adoption of phytoestrogen-free subclovers correlated with reduced overt physical signs of clover disease in ewes, and the problem was considered eliminated (Adams 1995).

While there has been no research into clover disease in ewes in recent decades, phytoestrogen research on other species has dispelled the notion that a lack of obvious and grandiose symptoms equals a lack of effect (Glover and Assinder 2006; Clotfelter and Rodriguez 2006; Tubbs et al. 2016). Human research has linked maternal phytoestrogen consumption with genital abnormalities in male offspring (North et al. 2000) and consumption of soy-based infant formula with increased rates of adult endometriosis (Upson et al. 2015). With increasing economic uncertainty in farming systems in recent years, due to causes as diverse as the COVID-19 pandemic to increasingly extreme weather from climate change, the importance of reproductive efficiency is gaining research interest (Harle et al. 2007). In addition to the economic stress, ewe infertility is a potential animal welfare concern, so it would be irresponsible to assume that phytoestrogens present in Australian subclover pastures pose zero risk.

Additionally, the mystery of the mechanism behind the immunity of some—but not all—ewes to oestrogenic subclover's effects has not been solved. Even at the peak of clover disease severity, some ewes remained immune to the effects of oestrogenic clover (Marshall 1973). Identifying a subset of immune ewes in an otherwise affected flock could contribute valuable information to the study of endocrine-disrupting agents and provide much-needed clarity on the state of PPI in present-day farming systems. The distinction between clover disease and PPI needs further examination to provide updated advice to researchers, farmers and veterinarians. As the existence of PPI cannot be determined from external signs, histological examination of the reproductive tract of exposed ewes is needed to determine what effect, if any, these pastures have on ewe fertility.

CHAPTER 3. AIMS AND HYPOTHESES

With oestrogenic subclover cultivars still present on southern Australian farms and neonatal lamb losses, infertility and dystocia still presenting ongoing challenges in sheep production systems, it is possible that a critical contributor to reproductive wastage in southern Australia has been overlooked. With significant changes to pasture management contributing to a reduction in subclover phytoestrogens in recent decades, it is unknown if the previously established methodology for diagnosing phytoestrogen exposure (Adams 1986) needs revision. Additionally, there are many unanswered questions from the initial stages of clover disease investigations. The first step in elucidating these mysteries is a detailed analysis of the effects of pasture phytoestrogens, in present-day dietary dosage, on the reproductive tracts of Western Australian ewes. While the lack of reports of visible clover disease indicates it is unlikely to be present on a significant number of southern Australian farms, PPI has yet to be ruled out as a contributor to ewe infertility and perinatal lamb loss.

The experiment reported here is the first histopathology examination of subclover-exposed ewes in recent decades, comparing cervical and uterine pathology of control ewes non-exposed to oestrogenic clover to three treatment groups of phytoestrogen-exposed animals. It was critical to examine ewes *in situ* on working southern Australian farms, consuming as close to a typical diet for the geographical region as possible. The four treatment groups of ewes examined considered the marked differences in the ewe responses to phytoestrogen exposure in previous studies:

- Control (control): ewes unexposed to phytoestrogens
- EX-DD (exposed double-dry ewes): ewes exposed to phytoestrogens that were unable to conceive (‘dry’) with a fertile ram for two subsequent joining seasons
- EX-LL (exposed ewes that lambed but lost lamb): ewes exposed to phytoestrogens that successfully conceived but lost the lamb during the perinatal period
- EX-LW (exposed ewes that lambed and weaned): ewes exposed to phytoestrogens with a history of lambing and raising the lamb to weaning age, indicating no fertility issues.

The design incorporated ewe reproductive tract histopathology, specifically the cervix and uterus, and searched for evidence of differences in exposed ewes compared to non-exposed controls. In addition to previously defined criteria for detecting phytoestrogen exposure in the ewe cervix, I trialled novel methods for potential PPI detection (Adams 1986). Histology staining for tissue type in the uterus and cervix— stains that highlight tissues of different tensile properties, specifically the ratios of ECM to smooth muscle—may shed light on any relationship between phytoestrogen exposure and altered ECM growth in the reproductive tract.

While the precise cause of failure to deliver a live lamb in clover disease ewes was not identified, alterations to tissue types or increased rates of fibrotic structures may prevent the adequate

biomechanical response of the uterus and/or cervix to parturition, which may be a potential mechanism of action for clover disease or PPI stillbirth and neonatal lamb loss. Stereology using grid counting to assess ECM/smooth muscle quantification has been established and used to assess airway ECM/smooth muscle ratios in pulmonary disease (Jones et al. 2014). Additionally, steroid receptors in the uterus have been localised and quantified to determine uterine responsiveness to phytoestrogens. Steroid receptor expression in the ewe uterus has been correlated with tissue-trophic activity and metabolism and is highly responsive to changes in circulating steroid levels (Stone et al. 1978). Increased receptor expression can be a reliable marker of sex steroid activity and has been demonstrated in mammals to also respond similarly to phytosteroid activity, although often at lower intensity (Snochowski and Romanowicz 2003). While absolute quantities of oestrogen receptors have been quantified in ewes already diagnosed with clover disease, it has not been replicated in ewes with suspected PPI, and no studies have examined receptor localisation in either group. This thesis examined oestrogen receptors (ERα and Erβ) as the most likely mechanism of action of phytoestrogens, and the androgen receptor, due to the puzzling and undetermined link between clover disease and masculinisation previously seen in clover-diseased ewes. Hopefully, these markers can provide information on phytoestrogen exposure related to ewe reproductive physiology, with the broader aim of providing producers with specific and relevant information.

Four hypotheses were tested:

1. Ewes exposed to phytoestrogens with aberrant reproductive outcomes display differences in cervix morphology consistent with previous literature, particularly in the uterine region of the cervix, compared to the control.
2. Ewes exposed to phytoestrogens with aberrant reproductive outcomes display significant differences in ratios of tissue types, particularly ECM and smooth muscle, in the uterus and cervix compared to the control.
3. Ewes exposed to phytoestrogens display a more ‘oestrus-like’ expression of sex steroid receptors than the control.
4. Ewes exposed to phytoestrogens with normal fertility outcomes significantly differ from ewes with aberrant reproductive outcomes and more similar to the control.

CHAPTER 4. METHODS AND EXPERIMENTAL DESIGN

4.1. Farms overview and confirmation of subclover phytoestrogens

Control ewes were sourced from Murdoch Veterinary Teaching Farm in Perth, Western Australia (refer to Table 1 for ewe demographic details). Control paddocks contained a mix of predominantly grass, non-oestrogenic white clover (*Trifolium repens*) comprising less than 10% of total biomass, and mixed weeds. No subclovers were present, oestrogenic or otherwise. Pastures were walked on multiple occasions during peak-expected subclover growth (winter–spring 2019) by UWA pasture agronomists for confirmation. It was decided that the oestrogenic subclover content of pastures was negligible to nil.

All three groups of exposed ewes in this study were part of a single commercial flock on a 1450 ha mixed cropping–livestock farm in the mixed farming region of Western Australia. Exposed ewes were sourced from a farm close to the town of Pingelly, 158 km east of Perth at 32.534 °S, 117.086 °E and 297 m above sea level. Average monthly precipitation ranges from 11.6 mm in summer (January) to 81.2 mm in winter (June). Average monthly temperatures range from a winter minimum of 5.5°C to a winter maximum 15.3°C (June) and summer minimum 15.6°C to summer maximum 31.9°C (January) (Bureau of Meteorology 2019) All ewes were rotated among pasture paddocks on the farm. The farm supplying the exposed ewes operates on a self-replacing pure Merino flock stocked at 6189 DSE (dry-sheep equivalent, i.e. carrying capacity for a ~45 kg wether).

Most pasture paddocks contained annual pastures with a legume base of subclover. The ewes had access to a mixed pasture of grass, broadleaf weeds (e.g. capeweed, *Arctotheca calendula*) and subclover from autumn–winter, and supplemental feeding (grain, oats, etc) in summer. Oestrogenic subclover was quantified during autumn–winter 2019 for part of Mia Kontoolas' Honours project titled *Redefining Clover Disease: Effects of oestrogenic clover on the reproductive function in sheep*. These data have been submitted previously; they are included here as an appendix to justify that pasture phytoestrogens were above safe thresholds for the results presented in this thesis (Appendix 1).

Briefly, the three pastures most grazed by ewes on the experimental farm contained subclover as the dominant pasture legume, comprising on average 43% of pasture biomass in the rod-point analysis. Representative samples analysed as dry matter resulted in subclover comprising 12% of the biomass, likely due to the high water content in subclover. The most common cultivars were oestrogenic, with an average formononetin content of 1.09%. The resultant formonetin concentration of total pasture biomass is thus estimated between 0.46% (rod-point analysis) and 0.13% (representative sampling). As these figures are greater than the 0.08% established as an effective dose for symptoms, treatment exposure was deemed sufficient to exert physiological effects.

Ewe selection

This study used 5–6-year-old Merino ewes (*Ovis aries*, n = 47). All exposed ewes were from a single larger flock allocated to one of three experimental groups according to recent historical records of pregnancy and rearing success (Table 1).

Table 1. Ewe demographics for four experimental groups (n = 47)

Group	Age	Weight	Treatment	Birth history	Euthanasia	Location
Control (n = 11)	5–6 years	44.77 ± 6.21 kg	No treatment	No history of birth difficulties	November 2019	Control farm, Perth south
EX-DD (n = 12)	5–6 years	69.8 ± 6.04 kg	Subclover phytoestrogens up to 6 months of year for lifespan	Unable to conceive in 2018 - 2019	October 2019	Experimental farm, Pingelly WA
EX-LL (n = 12)	5–6 years	57.5 ± 8.07 kg	Subclover phytoestrogens up to 6 months of year for lifespan	Conceived in 2019, lost lamb during perinatal period	October 2019	Experimental farm, Pingelly WA
EX-LW (n = 12)	5–6 years	56.54 ± 7.22 kg	Subclover phytoestrogens up to 6 months of year for lifespan	No history of birth difficulties	October 2019	Experimental farm, Pingelly WA

Tissue collected following slaughter

All tissues were collected from two sets of autopsies in October and November 2019, when Merino ewes are least likely to have confounding oestrus cycles. Prior to inclusion, sheep were restrained in a veterinary crush and given a brief health check, weighed and checked for healthy body condition score (Jefferies 1961). Humane euthanasia was performed by a veterinarian following animal welfare requirements and approved by The University of Western Australia Animal Ethics Committee (Approval #RA/100/1657). The reproductive tracts were dissected, with sections excised within 15 minutes of death and 5 mm transverse whole sections immediately preserved from middle and uterine regions of cervix and the right uterine horn immediately adjacent to the uterine body. All samples were immersed in a 4% solution of paraformaldehyde (818715, Merck KGaA, Darmstadt, Germany) in tap water for one week at 4°C.

4.2. Laboratory stains

Haematoxylin and eosin (H&E) staining protocol

Cervix (middle and uterine) sections were processed by first transferring from formaldehyde to 70% ethanol (100983, Merck KGaA, Darmstadt, Germany). Sections were trimmed to fit embedding cassettes (31051105W Hurst Scientific, Forrestdale, Australia), then fixed again in 70% ethanol overnight and embedded in paraffin using a tissue-processing machine (ASP200s, Leica, Wetzlar, Germany). Blocks of tissues were manually sectioned with a microtome (RM2255, Leica, Wetzlar,

Germany) to obtain 8 µm sections. Sections were immersed in a water bath (WB5000D, Ratek Instruments, Boronia, Australia) at 43°C ± 0.5°C and placed on 75 mm × 26 mm microscope slides (WGCF90W, Hurst Scientific, Forrestdale, Australia). Three replicates per animal, a minimum of 200 µm (maximum 400 µm) apart, were taken. Slides were oven-dried at 45°C for 30 mins (Selby Scientific, Melbourne, Victoria, Australia). Slides were dewaxed in toluene (108325, Merck KGaA, Darmstadt, Germany), washed once in 100% ethanol and twice in 70% ethanol in distilled tap water, and then rinsed in water prior to staining.

Staining protocols were established in small test batches and then stained in an automated staining machine (CV5030, Leica, Wetzlar, Germany). Cervix with slides were stained with haematoxylin and eosin according to established protocols (Bancroft and Gamble 2008). Briefly, slides were immersed in haematoxylin solution (75290, ChemSupply, Gillman, Australia) for 90 s for purple colouration of nuclear staining, drained in tap water, and then dipped briefly into acid alcohol (2% hydrochloric acid (HA020-500 ChemSupply, Gillman, Australia) in 98% ethanol) and rinsed in tap water. Slides were briefly dipped 10 times in alkaline solution and removed (25% ammonia (AA005, ChemSupply, Gillman Australia) in 75% ethanol) and then rinsed in tap water. Slides were dehydrated with 70% ethanol and then immersed in eosin (LL007, ChemSupply, Gillman, Australia) for pink colouration of cytoplasmic staining for 45 s.

Slides were then dehydrated in 100% ethanol three times, cleared in toluene, before adhering a 16 mm × 16 mm coverslip (CG11818, Hurst Scientific, Forrestdale, Australia) with dibutyl phthalate polystyrene xylene (DPX) (DPX-SQ, Hurst Scientific, Forrestdale, Australia).

Mason's trichrome staining protocol

Cervix (middle) and uterus (horn) slides were processed, dewaxed and washed as detailed in the H&E protocol above.

Staining was performed following Masson's trichrome protocol (Goldner 1938). Celestine blue dye (195560100, ChemSupply, Gillman, Australia) was applied for 5 mins to stain nuclei dark blue, then washed in water for two mins. Haematoxylin was applied for 5 mins to stain muscle fibres red, and then slides were rinsed in water for 2 mins. Slides were immersed in phosphomolybdic acid hydrate/ dodeca Molybdophosphoric Acid AR (51429, ChemSupply, Gillman, Australia) to clear ECM for 5 mins and then briefly rinsed in water. Slides were finally placed in aniline blue solution (10% aniline blue (142041, ChemSupply, Gillman, Australia) to 90% water) to highlight ECM and rinsed in acetic acid (695092, ChemSupply, Gillman, Australia).

Slides were dewaxed, cleared, mounted and coverslip applied as per the H&E protocol above .

This process was tested manually with smaller batches prior to automation, and then applied to all slides simultaneously in a Leica ASP200s automated staining machine.

Immunohistochemical staining

Twenty-nine animals were selected from the three groups (Control = 9, EX-DD = 10, EX-LW = 10) to balance cost and time efficacy with attaining a representative sample pool. Two replicates ~200 µm apart were included per slide for each animal, yielding 58 samples. All antibodies were optimised manually at test concentrations of 1:100, 1:200 and 1:400 using archival samples for positive controls of AR, ERα and ERβ (rat testis, sheep uterus, and sheep ovary, respectively; Appendix 3). ERα and ERβ final staining used the automated staining protocol below; however, AR was completed using the manual staining protocol due to machine limitations relating to Covid-19 delays on supplies.

Manual staining protocol

Two × 5 µm uterus sections were sliced, 200 µm apart per animal, and placed on a positively charged microscope slide (SCF90W-PC, Hurst Scientific, Forestdale, Australia) before baking for 60 mins at 45°C. Slides were dewaxed and rehydrated with toluene and decreasing ethanol concentrations in tap water.

Sections were manually washed three times with phosphate buffered saline or PBS 0.01 M at pH 7.4 (from powder, P4417, Sigma-Aldrich, St. Louis, Missouri, USA) for 3 mins each. Slides were then incubated in 3% H_2O_2 (HT020, ChemSupply, Gillman, Australia) for 10 mins at room temperature to block endogenous peroxidase. Heat-induced epitope retrieval was performed in sodium citrate buffer pH 6 (PHR1416, Sigma-Aldrich, St. Louis, Missouri, USA) in a pressure cooker (KOS Microwave Histostation, Molecular Machines and Industries, Eching, Germany) at 100°C for 5 mins (Molecular Machines and Industries, Eching, Germany). Samples were blocked for 30 mins with 5% goat serum (G9023 Sigma-Aldrich, St. Louis, USA) in PBS, then incubated overnight at 4°C in a 1:100 solution of rabbit anti-AR antibody (ab3510, ABCAM, Melbourne, Australia) and diluent (S0809, DAKO, Agilent Technologies, Santa Clara, USA). Samples were then washed three times for 3 mins each with PBS before adding HRP-conjugated goat anti-rabbit secondary (ab205718, ABCAM, Melbourne, Australia) 1:500 dilution. Samples were again washed three times for 3 mins each with PBS. Samples were stained with 3,3′-diaminobenzidine (DAB) (5OX DAKO, Agilent Technologies, Santa Clara, USA) followed by 1:10 peroxide substrate buffer (kit 34002, Thermo Fisher Scientific, Waltham, USA). Samples were finally washed three times for 3 mins each with PBS. Slides were then dehydrated in 100% ethanol three times, then dehydrated, cleared, and coverslip applied as per the above protocols.

Automated staining protocol

Samples were sectioned as above, then stained using a Ventana Discovery Ultra SN319363 automatic staining system. Samples were first deparaffinised in EZ prep deparaffin (950102, DAKO, Agilent Technologies, Santa Clara, USA) at 75°C for 8 mins. Cell conditioning using Conditioner #1 (11060, DAKO, Agilent Technologies, Santa Clara, USA) at 95°C was run for 44 mins then rinsed in Reaction

Buffer (200532, DAKO, Agilent Technologies, Santa Clara, USA). Samples were blocked with Inhibitor CM (50561, DAKO, Agilent Technologies, Santa Clara, USA) at 37°C for 4 mins, then rinsed in Reaction Buffer. ABCAM primary antibody (ab75635 anti-ERα 1:100/ ab3576 anti-ERβ 1:200) in diluent was added manually and incubated for 60 mins at 37°C, then washed in Reaction Buffer. Detection (Anti-rabbit HQ 500712, Agilent Technologies, Santa Clara, USA) was applied and incubated for 8 mins. Conjugate 1 (Anti-HQ HRP 39901, Agilent Technologies, Santa Clara, USA) was applied and incubated for 16 mins, then rinsed in Reaction Buffer. Images were incubated in H_2O_2 CM for 4 mins, stained with DAB for 8 mins, and then rinsed with Reaction Buffer. Slides were finally treated with Copper CM (S1967 Agilent Technologies, Santa Clara, USA) for 5 mins before the final rinse.

Slides were removed from the machine and manually dehydrated, cleared, and coverslip applied as per the above protocols.

4.3. Image analysis

Slides were imaged on a Nikon Upright microscope (DSFi2, Nikon Instruments, New York, USA) as indicated below using NiS-Elements BR software (2021 version, Nikon Instruments, New York, USA.) Images were RGB (3 × 8 bit, 1440 × 1024) in Biological Bright Field scene mode with 3 ms exposure. Magnification was set at 10× for morphology and 20× for immunohistochemistry. Analysis was conducted using ImageJ software (version 1.53.k, Madison, USA) after adjusting the scale bar from pixels to µm.

Morphology

The examined markers are detailed below.

Cervix (repeated in uterine and mid cervix):

- Gross anatomical markers of disease, such as multiple cysts, lesions, squamous metaplasia or significant inflammation (Adams 1995)
- Cervical fold architecture: number, area and length of cervical folds (Adams 1986)
- Changes in underlying cervical stroma, measured as the width and area of muscularis (Adams 1986)

Uterus

- Presence of gross anatomical markers of disease, such as cysts, lesions, squamous metaplasia or significant inflammation similar to the cervix examination (Adams 1995)
- Number of uterine glands
- Total size of uterus (area of sample)

The two studies cited as methodology for clover disease diagnosis (Adams 1986; Adams 1995) controlled for differences in ewe size in the selection process, randomly assigned ewes to treatment groups, and then returned to sample several years later. This study differs from the previous two papers in that all measurements were controlled for sample size and, where necessary, ewe weight was specified (Adams 1986; Adams 1995). This is an important consideration, as while it was not possible within the time frame of this thesis to permit 18 months of grazing post-allocation of ewes to groups, this will be similarly true for veterinarians, farmers and researchers who may benefit from future diagnostic recommendations and will require post-hoc diagnostic methods.

Cervical folds were defined as per previous research if they existed between two adequate cervical crypts, defined as "reached more than halfway from the central lumen to the muscle layer, and which also was more than half as large as its nearest neighbour" (Adams 1986). The apex of a fold was the farthest point of a cervical fold away from the base, and the base was the midway point on a parallel line drawn between the terminal crypt on either side of the fold with the freehand sections tool. All demarcation lines were drawn prior to analysis to ensure consistency (Figure 6; Adams 1986). All width measurements were the mean of multiple length measurements at regular intervals along the sample (minimum 10 per image). Individual points for gland counting were quantified using the 'multi-point' tool. A gland was included only if "completely circumscribed with closed borders" (Adams 1986). The irregular smooth muscle layer, longitudinal smooth muscle layer, and transverse smooth muscle layer identified in previous literature (referred to as 'lamina propria') were measured; however, to maintain consistency with previous research, they were collectively referred to as the 'muscularis' layer (More 1984). All results are expressed relative to the total selection surface area.

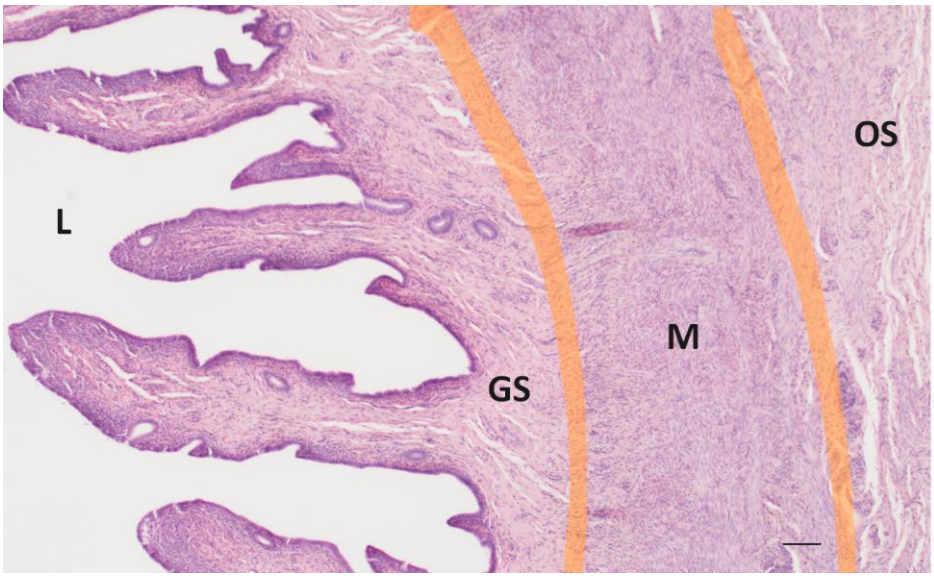


Figure 6. Line showing demarcation of glandular stroma and muscularis layers in ovine uterine, visualised using the hematoxylin and eosin (H&E) protocol. Slides were stained with hematoxylin to highlight cell nuclei (*purple*) and eosin to differentiate extracellular matrix and cytoplasm (*pink*) for differentiating tissue layers. Visible are the lumen (L), glandular serosa (GS), combined muscularis layers (M) and outer serosal membrane (OS), with the demarcation line drawn with the ImageJ freehand selections tool (orange). Images 4x magnification, scale bar = 100 µm

Immunohistochemistry

Uterine epithelia and glands were selected as regions of interest for nuclear steroid receptor intensity. For each section (two per animal), two images of uterine epithelia from opposite sides of the sample were created. For each epithelia image, the entire epithelia was outlined with the freehand sections tool and the coordinates stored in the ROI manager tool prior to image conversion, resulting in four epithelia measurements per animal. This process was repeated for glandular areas, with each section (two per animal) creating two images of uterine epithelia from opposite sides of the sample. The 2–3 clearest representative examples of glands were measured using the same freehand sections and ROI manager tools, resulting in ten glands outlined and selected per animal. Images were then converted in ImageJ to 32-bit greyscale and a threshold preset selected to remove background and leave only clearly stained regions (Figure 7). Images were then converted to 8-bit, and the total pixels contained within each ROI determined. Total pixels were expressed as a fraction of ROI area as arbitrary units (AU). A single mean AU per animal for epithelia and glands was expressed per antibody.

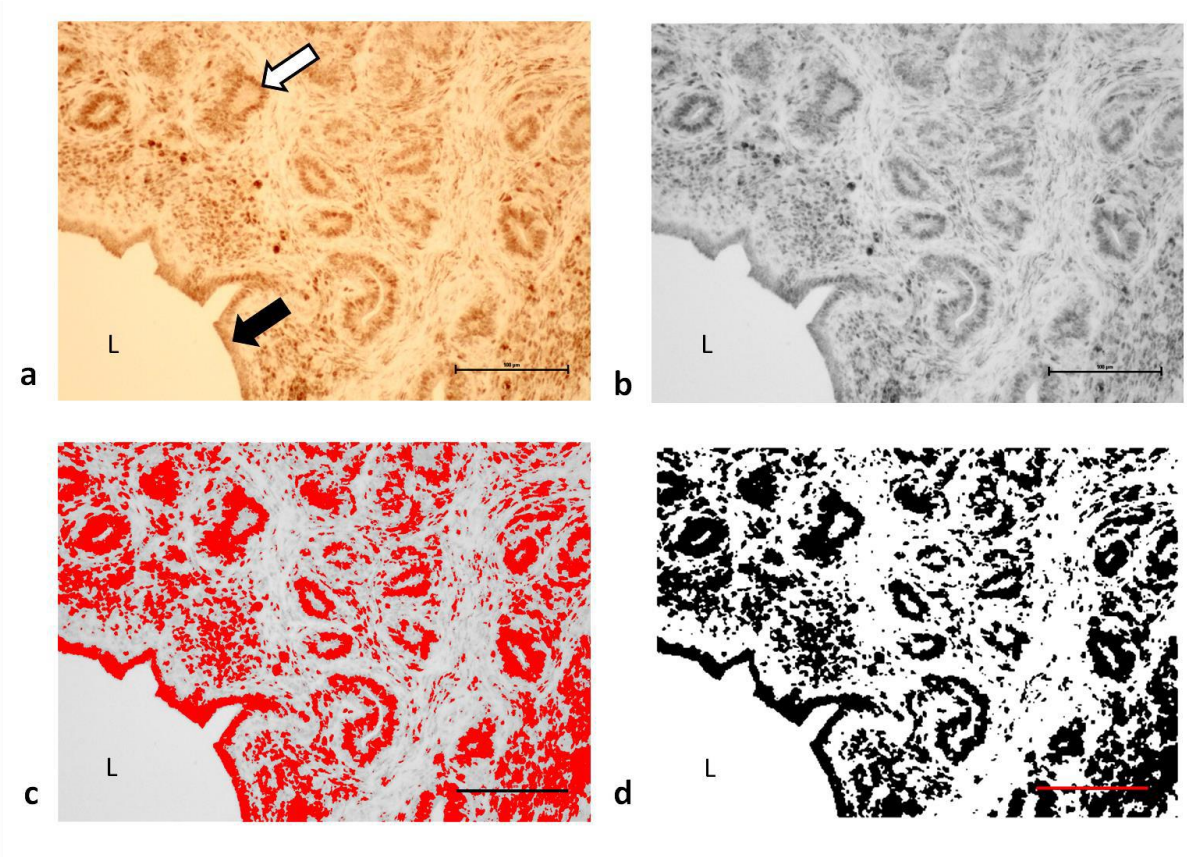


Figure 7. Oestrogen receptor (ERα) expression in ewe uterus (horn), visualised using the immunohistochemistry protocol (a). Slides were stained to differentiate the expression of nuclear receptors for ERα, such as those in the epithelia (*black arrow*) and uterine glands (*white arrow*). Images were then converted in ImageJ to 32-bit greyscale (b) and a threshold preset removed background (c). Final images were then converted to 8-bit (d) to quantify integrated density. Images 20x magnification, all scale bars are 100 µm

Statistical analysis

Statistical tests were performed using XLSTAT (Addinsoft, Paris, France). Outliers were assessed individually and excluded if invalid for reasons unrelated to treatment, such as sample preparation errors. The Shapiro-Wilk test for normality was selected for all experiments based on group sizes.

For all measures on cervix samples, two-way ANOVA examined the effect of phytoestrogen treatment group (control and three exposed groups) and cervix region (mid cervix and uterine cervix) and their interaction. There were ten replicates (sheep) with three replicate measurements from each. All length and width measurements (cervical fold length, cervical fold width, muscularis width) were the mean of a minimum of ten measurements.

For all measurements made on uterus samples, one-way ANOVA examined the effect of phytoestrogen treatment group (control and three exposed groups).

When ANOVA showed a significant effect on phytoestrogen treatment group (significance level $p<0.05$), a Dunnett's post-hoc test was used to compare exposed groups to the control group. For the cervix, this was performed using the means of each treatment group when there was no interaction and separately for each cervix region (uterine cervix and mid cervix) when there was a treatment and region interaction. Differences in animal liveweight were controlled for by expressing results in relation to total sample size (in contrast to previous studies in this area, see appendix 4). Organ sample area is expressed in relation to animal weight as mean of each experimental group, after regression analysis was performed in a previous project examining the same samples (Chazal 2021) determined that animal liveweight was unrelated to organ size ($p = NS$).

CHAPTER 5. RESULTS

5.1. Cervix analysis

Anatomical features

No observations of significant tumours or other lesions in the mid or uterine cervix occurred in any group (mid Figure 8, uterine Figure 9). One to two small cysts per animal presented across a minority of samples in all groups. As they were sufficiently minor in size and number, they were considered histologically normal and unrelated to treatment. Additionally, no obvious stromal or glandular cell proliferation indicative of pathological inflammation was observed in the mid or uterine cervix in any group. As such, no comparisons of the frequency of these markers can be made between groups.

Phytoestrogen exposure does not significantly affect cervical fold number

For cervical fold number, there was a significant effect for cervix region ($p<0.0001$) but no significant effect for treatment or interaction (two-way ANOVA, Table 2, Figure 10a). For cervix region, the uterine cervix had markedly more folds than the mid cervix.

Table 2. Results of two-way ANOVA analysis on the effect of cervix region (mid and uterine) and treatment (unexposed and three exposed treatments) on cervical fold dimensions, including the number of folds, length and area of folds, muscularis area and number of glands in ewe cervix, controlling for sample size. NS = not significant.

		Fold number /sample mm^2	Fold length /sample µm	Fold area /sample mm^2	Muscularis area / sample mm^2	Gland number /sample mm^2	Sample area mm^2 / weight kg
Two-way ANOVA	R^2	0.58	0.21	0.34	0.37	0.22	0.25
	F	35.56	6.52	12.42	14.79	7.32	8.7
	Pr > F	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Cervix region	F	221.67	19.02	63.92	47.53	18.91	28.12
	P value	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Treatment	F	2.59	7.97	4.11	9.32	9.3	7.97
	P value	**0.05**	**<0.0001**	**0.008**	**<0.0001**	**<0.0001**	**<0.0001**
Cervix region x Treatment	F	2.34	0.41	2.03	8.91	0.68	1.61
	P value	NA	NA	NA	**<0.0001**	NA	NA

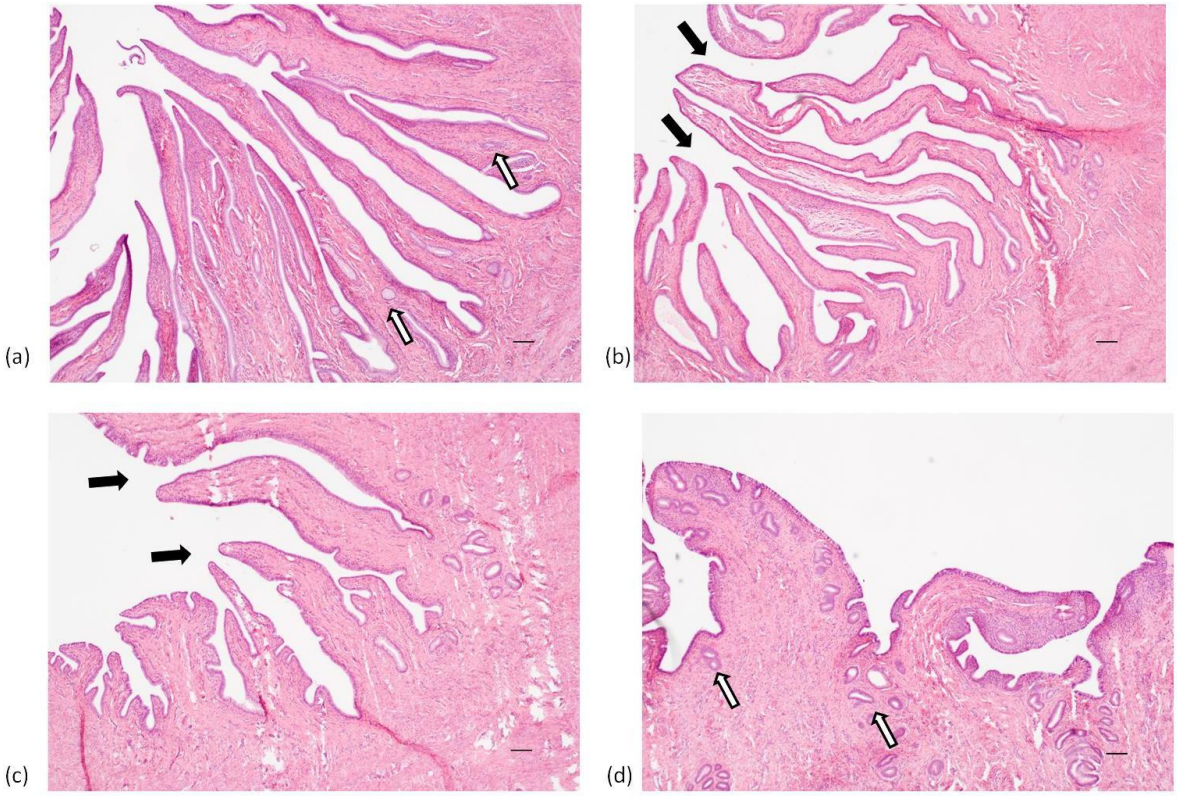


Figure 8. Histological section of ewe mid cervix stained with H&E protocol from four treatment groups: control (a), ewes exposed to subclover phytoestrogens that were double-dry (EX-DD; b), ewes that conceived but lost their lamb at birth (EX-LL; c) and ewes without aberrant reproductive outcomes that conceived, birthed a live lamb and raised the lamb until at least weaning age (EX-LW; d). Cervical folds (*black arrows*) were imaged at 10x magnification to examine histological features, including cervical glands (*white arrows*). All scale bars are 100 µm.

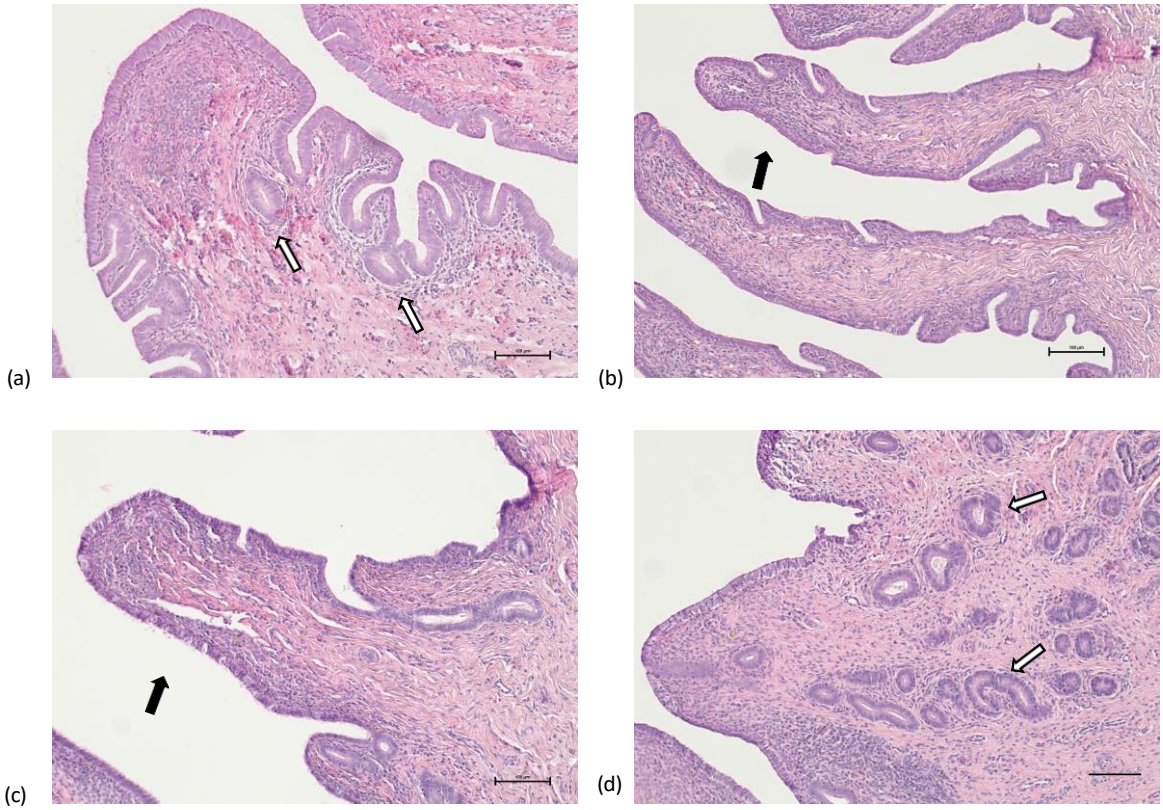


Figure 9. Histological section of ewe uterine cervix stained with H&E protocol from four treatment groups: control (a), ewes exposed to subclover phytoestrogens that were double-dry (EX-DD; b), ewes that conceived but lost their lamb at birth (EX-LL; c) and ewes without aberrant reproductive outcomes that conceived, birthed a live lamb and raised the lamb until at least weaning age (EX-LW; d). Cervical folds (*black arrows*) were imaged at 10x magnification to examine histological features, including cervical glands (*white arrows*). All scale bars are 100 µm.

Phytoestrogen exposure increases the length of folds in mid and uterine cervix in all groups

For cervical fold length, significant effects occurred for cervix region and treatment (both $p < 0.0001$), but not their interaction (two-way ANOVA, Table 2, Figure 10b). For cervix region, the uterine cervix had longer folds than the mid cervix. For treatment, all exposed treatments had significantly longer folds than the control ($p<0.001$). Compared to the control, EX-DD, EX-LL, and EX-LW had 76%, 79%, and 81% greater fold length in the mid cervix and 38%, 32%, and 28% greater fold length in the uterine cervix, respectively.

Phytoestrogen exposure increases cervical fold area in ewes that have lambed but lost lamb only

For cervical fold area, significant effects occurred for cervix region ($p<0.0001$) and treatment ($p=0.008$), but not their interaction (two-way ANOVA, Table 2, Figure 10c). For cervix region, the uterine cervix had greater fold area than the mid cervix. For treatment, exposed treatments had greater fold area than the control; however, this was only statistically significant for EX-LL ($p<0.001$).

Compared to the control, EX-LL had a 50% greater fold area in the mid cervix and a 100% greater fold area in the uterine cervix.

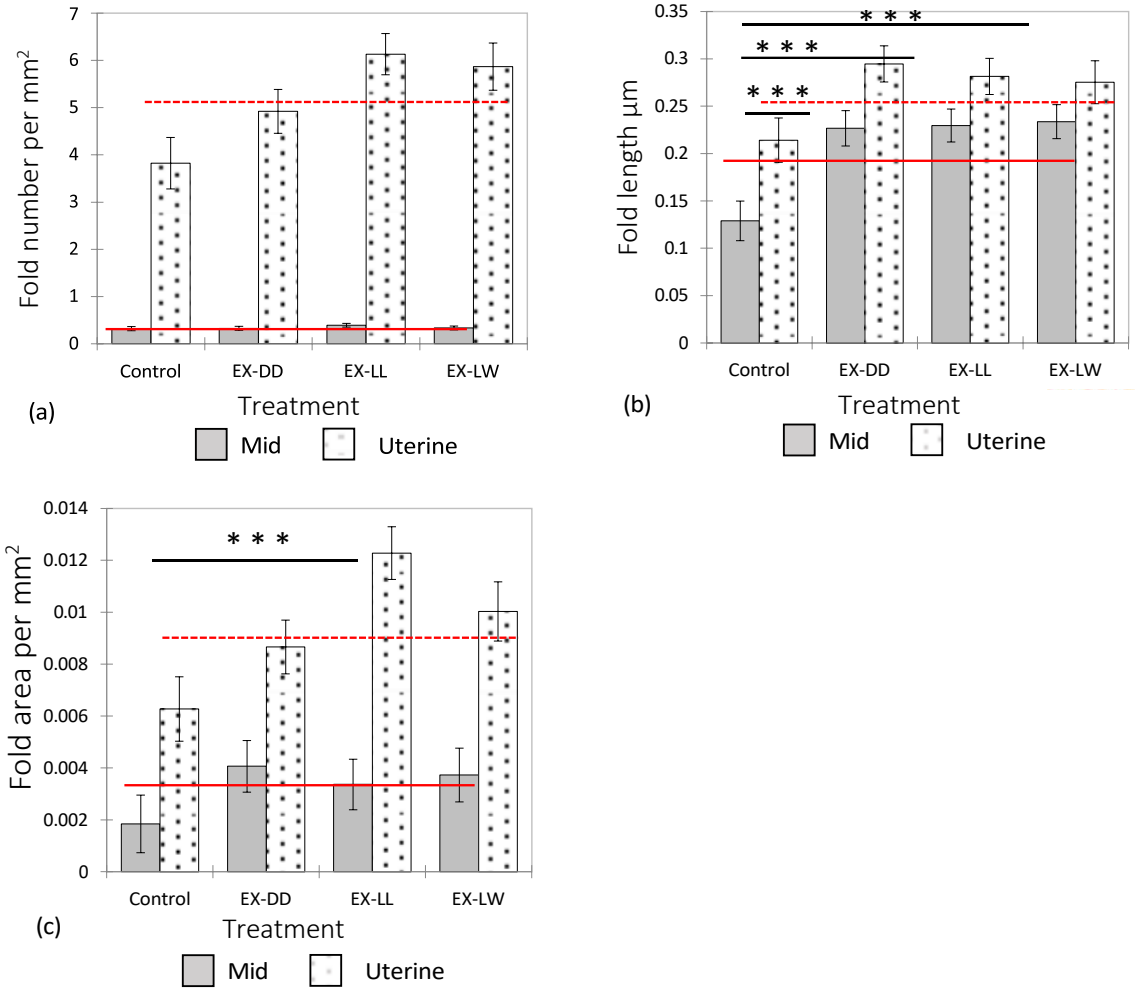


Figure 10. Number of cervical folds (a), fold length per total width (b), and fold area (c) of ewes non-exposed (control, n = 7 in mid cervix, n = 6 in uterine cervix) and exposed to phytoestrogens. Exposed ewes either did not conceive for two consecutive joining seasons (EX-DD, n = 9 in mid cervix, n = 8 in uterine cervix), had conceived but lost their lambs (EX-LL, n = 10 in mid cervix, n = 8 in uterine cervix) or had raised their lambs (EX-LW, n = 9 in mid cervix, n = 8 in uterine cervix). Values are presented as mean ± SEM. The estimated means for cervix region are shown as red lines (mid cervix solid, uterine cervix in dots). Comparisons of treatment means to the control were performed using Dunnett's test at the 0.05 significance level. As no interactions were present, asterisks indicate significant differences between estimated treatment means (i.e. mean of both cervix regions) and the control (* p>0.01, ** p<0.01–0.001, *** p<0.001).

Phytoestrogen exposure increases muscularis area in mid cervix only

For cervical muscularis area, no significant effects occurred for cervix region, treatment, or their interaction (all p<0.0001) (two-way ANOVA, Table 2, Figure 11a). The values for exposed treatments had significantly greater values than control values in the mid cervix, but not the uterine cervix.

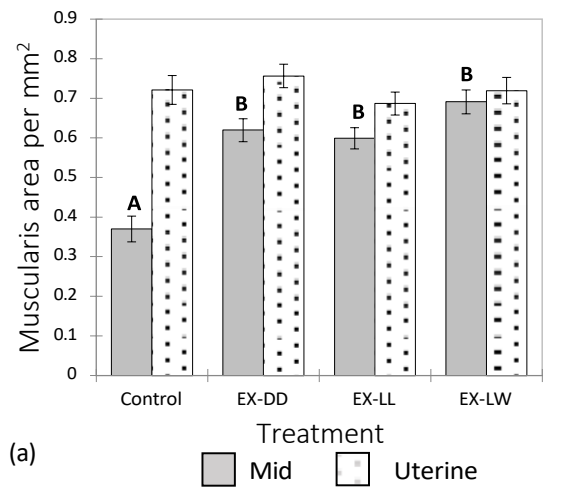
Compared to the control, EX-DD, EX-LL, and EX-LW had 68%, 62% and 87% greater muscularis area in the mid cervix, respectively.

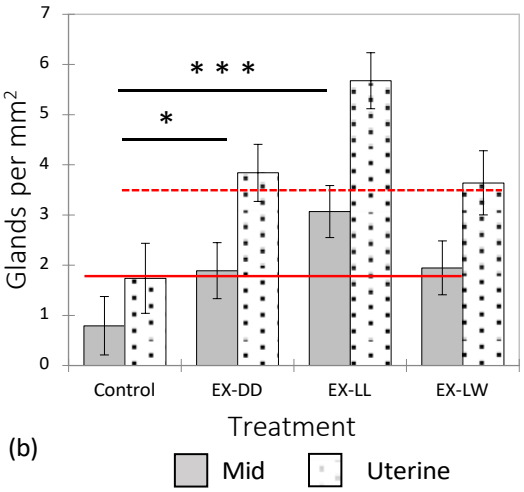
Phytoestrogen exposure increases the number of cervical glands in exposed ewes with aberrant reproductive outcomes

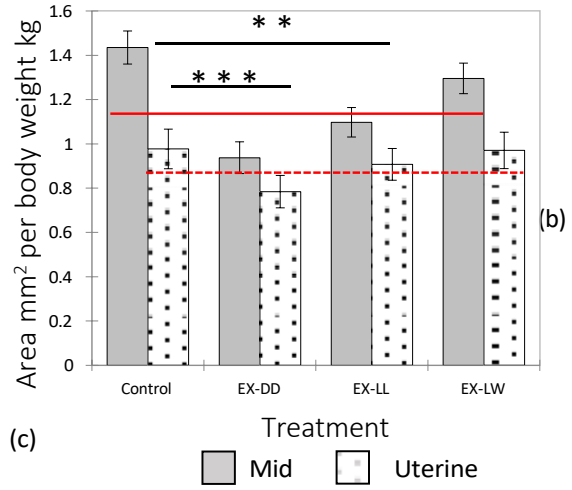
For the total number of cervical glands, significant effects occurred for cervix region and treatment (both $p<0.0001$), but not their interaction (two-way ANOVA, Table 2, Figure 11b). For cervix region, the uterine cervix had more glands than the mid cervix. Exposed groups had more glands than the control. Compared to the control, EX-DD and EX-LL had 139% and 288% greater gland numbers in the mid cervix and 121% and 227% greater gland numbers in the uterine cervix, respectively.

Phytoestrogen exposure decreases cervix area in exposed ewes with aberrant reproductive outcomes

For cervix sample size (area of sample) controlled for body size (weight of ewe), significant effects occurred for cervix region and treatment (both $p <0.0001$), but not their interaction (two-way ANOVA, Table 2, Figure 11c). For cervix region, the uterine cervix was always larger than the mid cervix. For treatment, exposed groups always had a smaller cervix than the control. Compared to the control, EX-DD and EX-LL had 35% and 24% smaller areas in the mid cervix and 20% and 7% smaller areas in the uterine cervix, respectively.







(b)

Figure 11. Cervical architecture measures, including muscularis area (a), gland numbers (b), total size (c) of ewes non-exposed (control, n = 7 in mid cervix, n = 6 in uterine cervix) and exposed to phytoestrogens. Exposed ewes either did not conceive for two consecutive joining seasons (EX-DD, n = 9 in mid cervix, n = 8 in uterine cervix), had conceived but lost their lambs (EX-LL, n = 10 in mid cervix, n = 8 in uterine cervix) or had raised their lambs (EX-LW, n = 9 in mid cervix, n = 8 in uterine cervix). Values are presented as mean ± SEM. The estimated means for cervix region are shown as red lines (mid cervix solid, uterine cervix in dots). Comparisons of treatment means to the control were performed using Dunnett's test at the 0.05 significance level. Asterisks indicate significant differences between treatment means and the control (* p>0.01, ** p<0.01–0.001, *** p<0.001). Due the interaction between cervix region and treatment group for muscularis area, the mid cervix values without common notations (a and b) differ significantly (p<0.0001).

5.2. Uterus analysis

Anatomical features

No observations of polyps, cysts, or other lesions of the uterus were made in any treatment group (Figure 12). Additionally, no apparent hyperplasia or pathological inflammation was observed in endometrial stroma or glands of any group. As such, no comparisons of the frequency of these markers can be made between treatment groups.

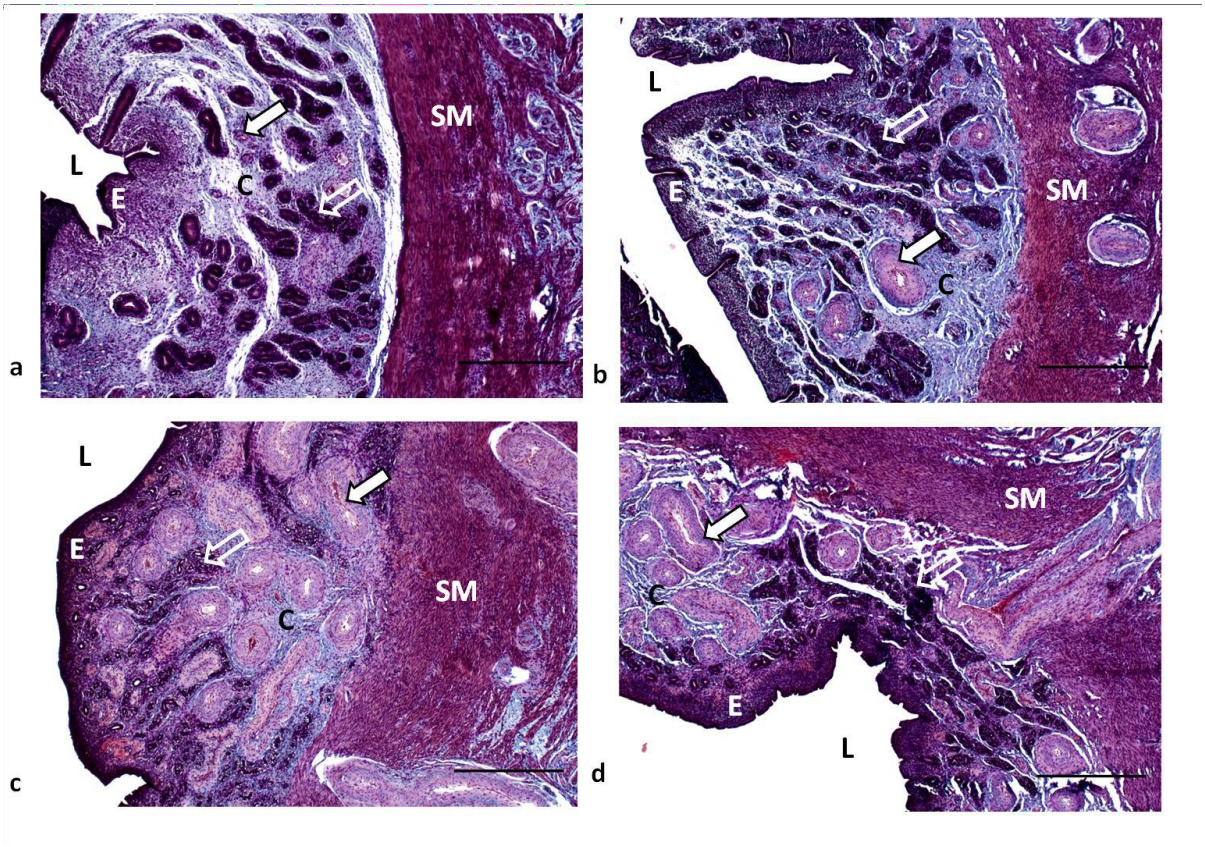


Figure 12. Histological section of ewe uterine horn stained with Masson's trichrome protocol to represent four treatment groups: control (a), ewes exposed to subclover phytoestrogens that were double-dry (EX-DD; b), ewes that conceived but lost their lamb at birth (EX-LL; c), and ewes without aberrant reproductive outcomes that conceived, birthed a live lamb and raised the lamb until at least weaning age (EX-LW; d). Transverse cross-section (4x magnification) highlights contrast between smooth muscle (red-purple, indicated by SM) and extracellular matrix (blue; c). Visible features include luminal space (L), endothelium (E), uterine glands (black arrow) and blood vessels (white arrow). All scale bars are 500 µm.

Myometrial width is not affected by phytoestrogen exposure

Myometrial width per total sample width was not affected by treatment (one-way ANOVA, Figure 13a; Table 3). No significant differences occurred between groups; despite differences in organ size, the ratio of myometrium to individual sample area remained consistent.

Table 3. Results of one-way ANOVA analysis on the effect of treatment (unexposed and three exposed treatments) on uterine myometrial width and gland number, controlling for sample size, uterine size and animal size. NS = not significant.

		Myometrial width / sample mm^2	Gland number / sample mm^2	Sample area mm^2 / weight kg
One-way ANOVA	R^2	0.04	0.25	0.13
	F	1.36	11.97	5.64
	P value	NS	**<0.0001**	**0.001**

Phytoestrogen exposure increases the number of uterine glands in exposed ewes with aberrant reproductive outcomes

The number of uterine glands per sample area was significantly affected by treatment ($p<0.0001$, one-way ANOVA, Table 3, Figure 13b). Compared to the control, EX-DD and EX-LL had 83% and 78% more uterine glands, respectively. EX-LW had 25% more glands than the control, but the difference was not statistically significant.

Phytoestrogen exposure reduces total uterine size in all exposed ewes

The total size of the uterus was significantly affected by treatment when controlled for animal size ($p=0.001$, one-way ANOVA, Table 4, Figure 13c). All exposed groups had significantly larger uterine sizes than the control. Compared to the control, EX-DD, EX-LL and EX-LW had 36%, 24% and 21% smaller uterus size, respectively.

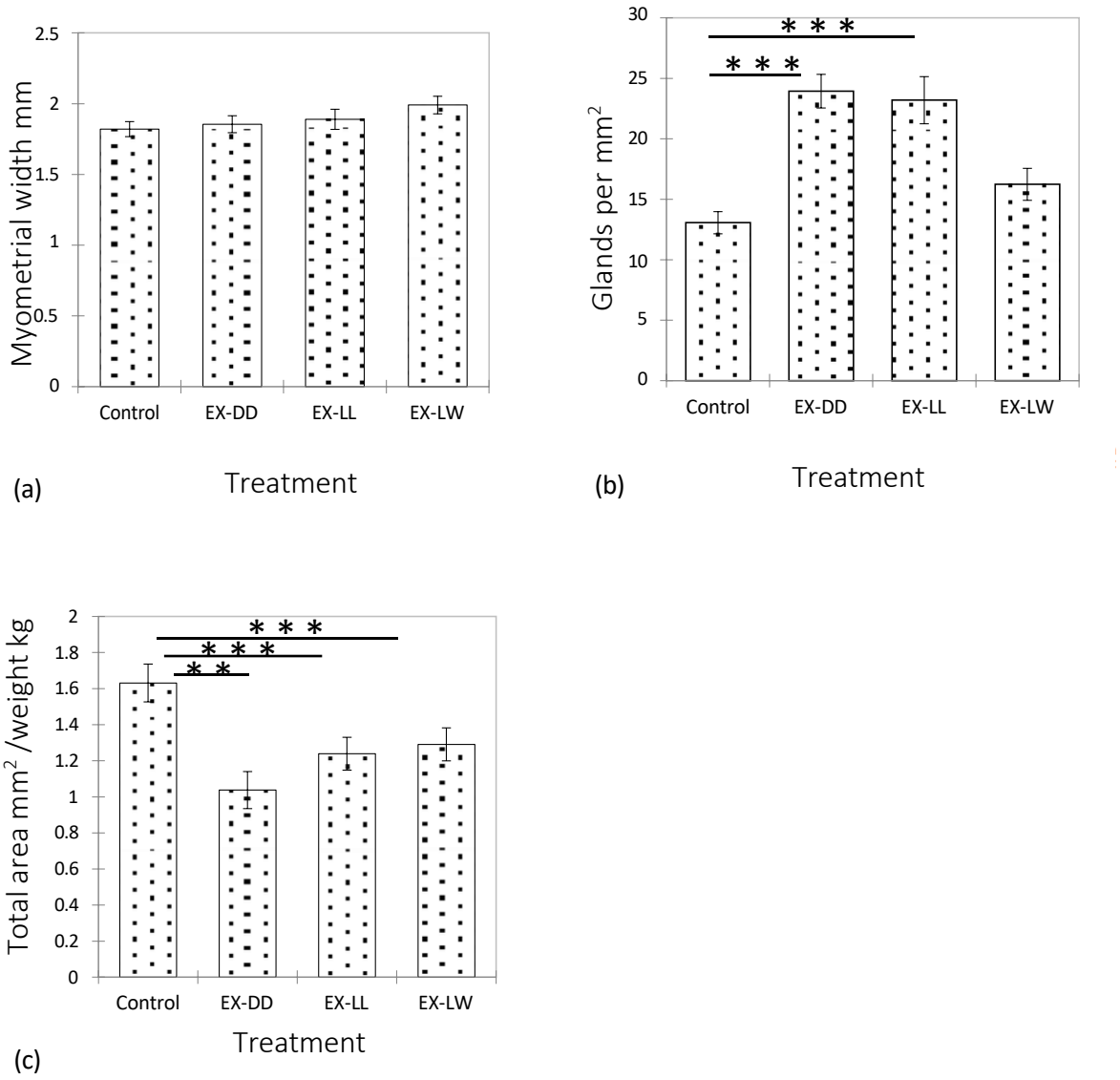


Figure 13. Uterine morphological measures, including myometrial width (a), number of glands (b) and area per kg (c) of the uterus (horn) of ewes non-exposed (control, n = 11) and exposed to phytoestrogens. Exposed ewes either did not conceive for two consecutive joining seasons (EX-DD, n = 9), had conceived but lost their lambs (EX-LL, n = 9) or raised their lambs (EX-LW, n = 11). Values are presented as mean ± SEM. Comparisons of means were performed using Dunnett's test at the 0.05 significance level. Asterisks indicate significant differences between individual exposed treatment groups and the control (* p>0.01, ** p<0.01–0.001, *** p<0.001).

5.3. Uterus quantitative stereology analysis

Phytoestrogen exposure increases smooth muscle volume density in double-dry ewes only

The volume density of smooth muscle in the uterus (horn) was significantly affected by treatment; however, only in double-dry ewes. Compared to the control, EX-DD ewes had 11.5% greater uterine smooth muscle volume density in the uterus (horn) (p=0.005, one-way ANOVA, Table 4, Figure 14a).

Table 4. Results of one-way ANOVA analysis on the effect of treatment (unexposed and three exposed treatments) on the proportion of uterine smooth muscle, ECM, blood vessel, lumen and endothelia as counts per total sample counts. NS = not significant.

		Smooth muscle	ECM	Blood vessel	Lumen	Endothelia
One-way ANOVA	R^2	0.11	0.07	0.25	0.04	0.13
	F	4.53	2.72	11.94	1.52	5.33
	P value	**0.005**	**0.048**	**<0.0001**	0.21	**0.002**

Phytoestrogen exposure increases ECM volume density in exposed, fertile ewes

The ECM volume density of smooth muscle in the uterus (horn) was significantly affected by treatment; however, only in EX-LW ewes. Compared to the control, EX-LW ewes had an 15% greater ECM volume density in the uterus (horn) (p=0.048, one-way ANOVA, Table 4, Figure 14b).

Phytoestrogen exposure increases blood vessel volume density in exposed, fertile ewes

The blood vessel volume density in the uterus (horn) was significantly affected by treatment; however, only in EX-LW ewes. Compared to the control, EX-LW ewes had an 89% greater blood vessel volume density in the uterus (horn) in counts per total sample (p<0.0001, one-way ANOVA, Table 4, Figure 14c).

Phytoestrogen exposure does not affect lumen volume density

The volume density of lumen in the uterus (horn) was not affected by treatment, with no significant effect on counts indicating lumen in the uterus (horn) in EX-DD, EX-LL, or EX-LW compared to the control (one-way ANOVA, Table 4, Figure 14d).

Phytoestrogen exposure decreases endothelial volume density in exposed ewes

The endothelium volume density in the uterus (horn) was significantly affected by treatment in exposed ewes. Compared to the control, EX-DD, EX-LL and EX-LW had 32%, 20% and 31% lower endothelium volume density in the uterus (horn), respectively (p=0.002, one-way ANOVA, Table 4, Figure 14e).

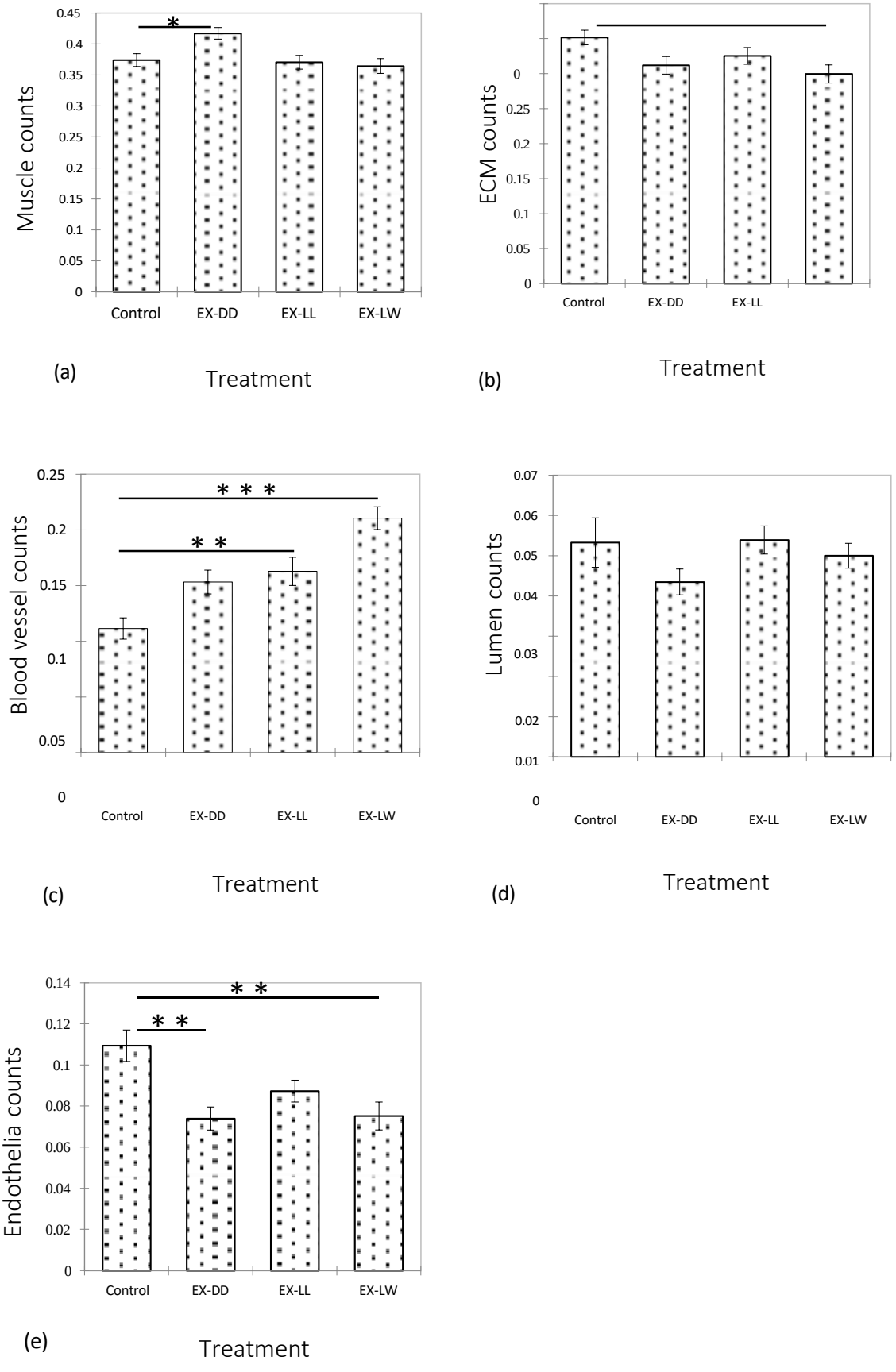


Figure 14. Uterine tissue volume density, including smooth muscle (a), ECM (b), blood vessel (c), lumen

(d) and endothelia (e) of ewes non-exposed (control, n = 11) to phytoestrogens. Counts are presented per total sample. Exposed ewes either did not conceive for two consecutive joining seasons (EX-DD, n = 9), had conceived but lost their lambs (EX-LL, n = 9) or raised their lambs (EX-LW, n = 11). Values are presented as mean ± SEM. Comparisons of means were performed using Dunnett's test at the 0.05 significance level. Asterisks indicate significant differences between individual exposed treatment groups and the control (* p>0.01, ** p<0.01–0.001, *** p<0.001).

5.4. Immunohistochemical analysis of steroid receptors in uterus

Phytoestrogen exposure does not affect ERα expression in ovine uterus

While EX-DD and EX-LL ewes generally had higher ERα expression in the ovine uterus in both epithelia and glands than the other treatments, no significant treatment effects occurred for receptor expression (one-way ANOVA, Table 5, Figures 15a, b, 18a, b). For all groups, ERα was most highly expressed in the endometrial glands, followed by luminal epithelia and the stroma area immediately adjacent to the lumen (Figure 16). Substantial ERα expression also occurred in the nucleus of smooth muscle cells of the myometrium. ERα was the most clearly and intensely expressed of all receptors examined and clearly localised to the nuclei of cells in all treatment groups.

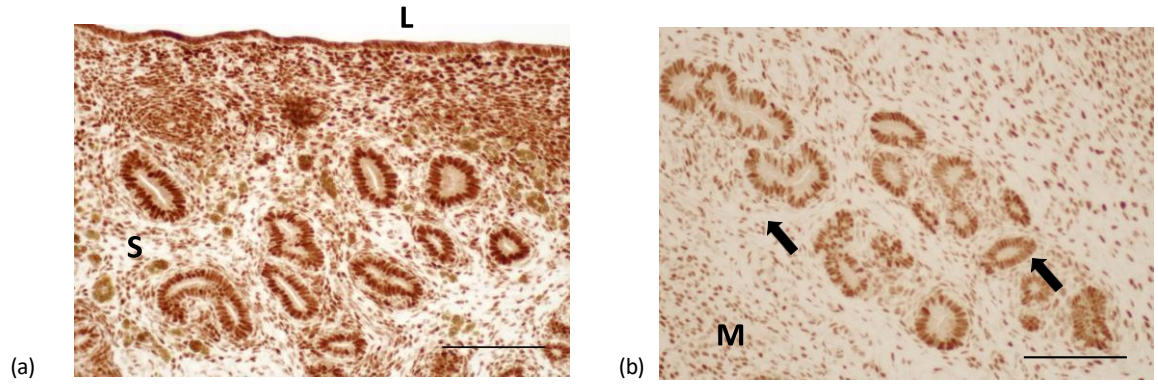


Figure 15. Oestrogen receptor alpha (ERα) expression in ewe uterine horn visualised with immunohistochemical protocol. Transverse cross-section (20x magnification) shows cell nuclei most intensely stained in endothelia (a) and uterine glands (b); however, significant staining also occurred in the uterine stroma (S). Visible feature also shown is lumen (L). Intensity of staining was measured in the endothelia (*white arrow*) and uterine glands (*black arrows*), and mean intensity determined (AU). As no significant differences were observed between exposed groups and the control, the same animal is shown in both images. All scale bars are 100 µm.

Table 5. Results of one-way ANOVA analysis determining the effect of treatment (unexposed and three exposed treatments) on integrated density of ERα, ERβ and AR in epithelia and glands of the uterus, expressed as mean arbitrary units (AU). NS = not significant.

		ERα		ERβ		AR	
		Epithelia	Gland	Epithelia	Gland	Epithelia	Gland
One-way ANOVA	R^2	0.07	0.14	0.1	0.03	0.06	0.07
	F	0.92	2.19	1.39	0.32	0.92	0.92
	P value	NS	NS	NS	NS	NS	NS

Phytoestrogen exposure does not affect ERβ expression in the ovine uterus

Phytoestrogen treatment did not significantly affect ERβ expression in the epithelia or glands of the uterus, similar to ERα, with a consistent expression between groups (one-way ANOVA, Table 5, Figures 15c, d, 16a, b, 18c, d). ERβ followed a similar pattern to ERα, with high expression patterns in endometrial glands, endometrium and luminal epithelia. However, the myometrial ERβ smooth muscle cells had visibly more intense expression than ERβ. The cytoplasm of endothelial cells, on the perimeter of the endometrium, also showed some ERβ expression across all treatment groups.

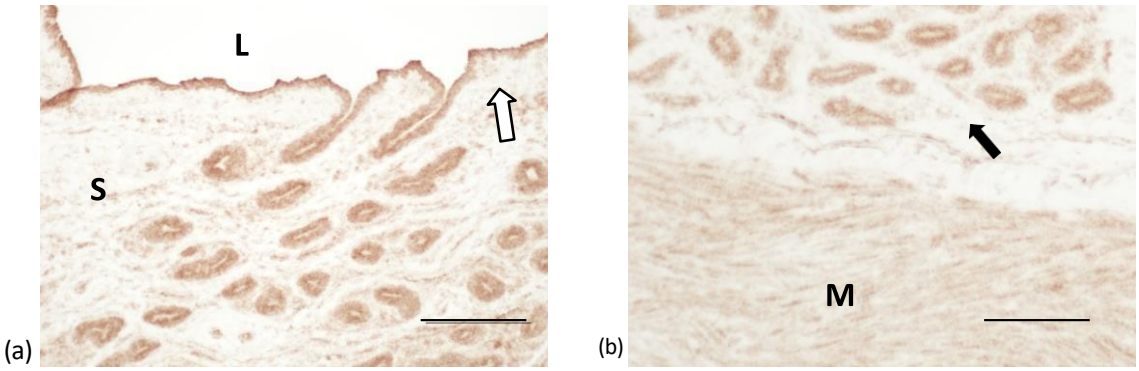
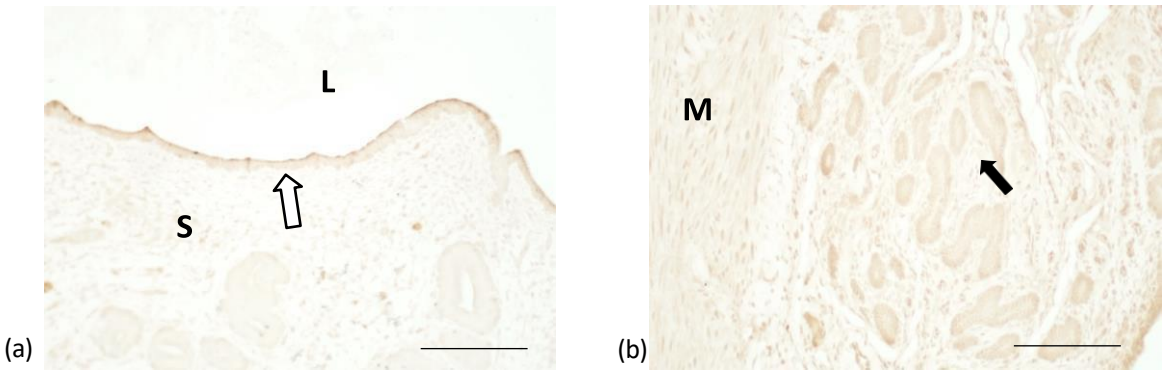


Figure 16. Oestrogen receptor beta (ERβ) expression in ewe uterine horn visualised with the immunohistochemical protocol. Transverse cross-section (20x magnification) shows cell nuclei most intensely stained in endothelia (a) and uterine glands (b). Visible features include luminal space (L), uterine stroma (S) and myometrial smooth muscle (M). Intensity of staining was measured in the endothelia (*white arrow*) and uterine glands (*black arrows*), and mean intensity determined (AU). ERβ staining is more intense in smooth muscle cells (M) than ERα (Figure 16). As no significant differences were observed between exposed groups and the control, the same animal is shown in both images. All scale bars are 100 µm.

Phytoestrogen exposure does not affect AR in the ovine uterus

Phytoestrogen treatment did not significantly affect AR expression in the epithelia or glands of the uterus (one-way ANOVA, Table 5, Figure 17a, b, Figure 18 e, f).

All treatment groups had minimal and faintly expressed AR (Figure 18). Staining of AR appeared most intense in the cell membrane of epithelia rather than nuclei in ewe luminal endometrium; however, AR was also visualised in myometrial smooth muscle cells.



(a) (b)

Figure 17. Androgen receptor (AR) expression in ewe uterine horn visualised with the immunohistochemical protocol. Transverse cross-section (20x magnification) shows cell nuclei most intensely stained in endothelia (a) and uterine glands (b). Visible features include luminal space (L), uterine stroma (S) and myometrial smooth muscle (M). Intensity of staining was measured in the endothelia (*white arrow*) and uterine glands (*black arrows*), and mean intensity determined (AU). Very minimal intensity of AR staining was observed; this appeared most distinctly at the perimeter of endothelial cells (*white arrow*) rather than nuclei as seen in ERα (Figure 16) and ERβ (Figure 17). As no significant differences were observed between exposed groups and the control, the same animal is shown in both images. All scale bars are 100 µm.

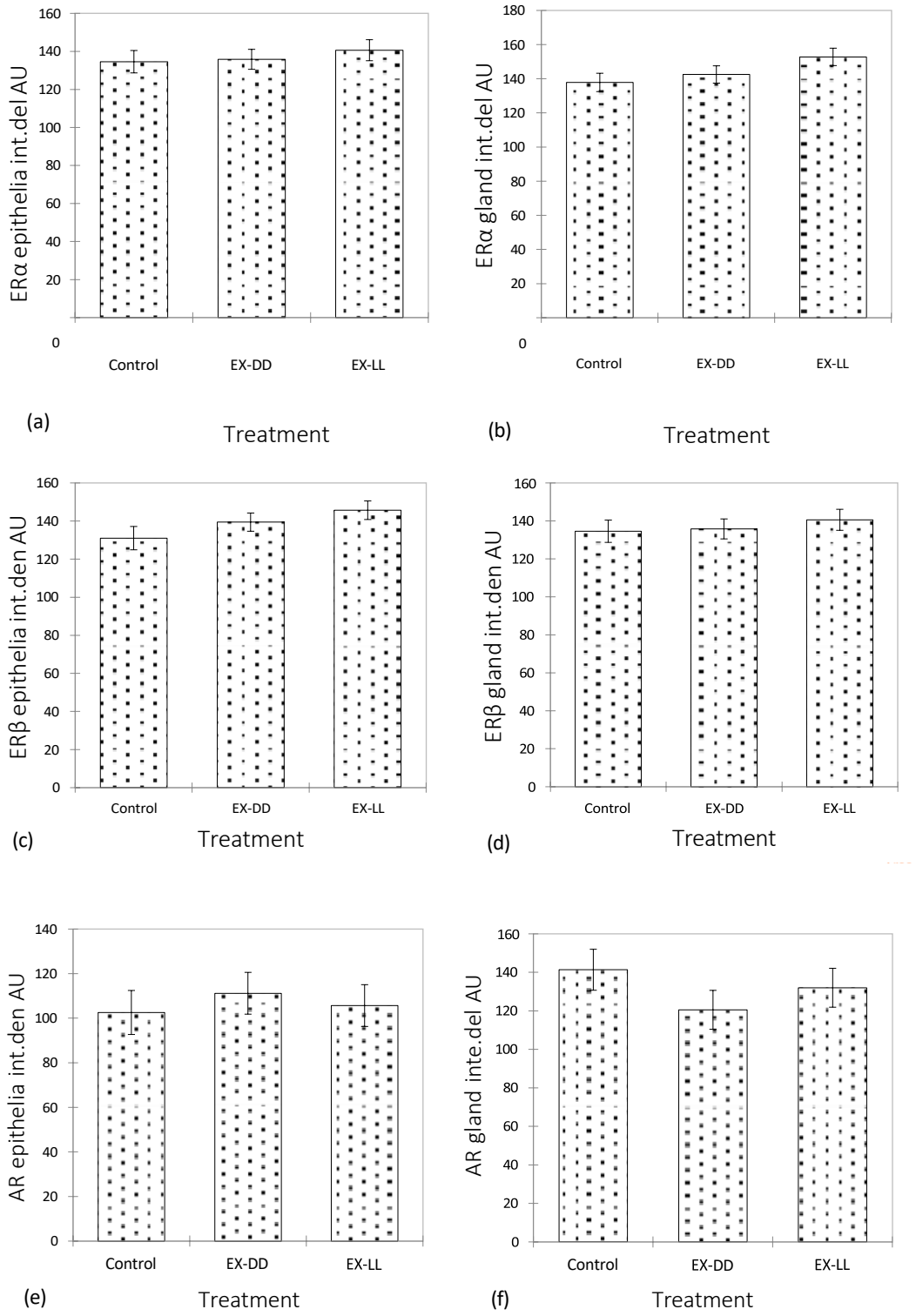


Figure 18. Uterine steroid receptors expression intensity, including ERα (epithelial (a), glandular (b)), ERβ (epithelial (c), glandular (d)) and AR (epithelial (e), glandular (f)) of ewes non-exposed (control, n = 11) exposed to phytoestrogens. Exposed ewes either did not conceive for two consecutive joining seasons (EX-DD, n = 9), had conceived but lost their lambs (EX-LL, n = 9) or raised their lambs (EX-LW, n = 11). Values are presented as mean ± SEM. Comparisons of means were performed using Dunnett's test at the 0.05 significance level. Asterisks indicate significant differences between individual exposed treatment groups and the control (* p>0.01, ** p<0.01–0.001, *** p<0.001).

CHAPTER 6. DISCUSSION

This study is the first in almost 30 years to comprehensively investigate the effect of subclover phytoestrogens on ovine reproductive tract morphology. The study involved a comprehensive histomorphometric analysis of Western Australian ewes, examining cervix and uterus morphology to classify tissue types and sex steroid receptor expression in response to current dietary phytoestrogen exposure due to the presence in mixed pastures comprising outdated subclover cultivars with high phytoestrogen levels. The ultimate goal was to update knowledge of PPI phenotype to reflect changing pasture conditions in the decades since the most recent work, contributing to a broader knowledge base from which relevant recommendations can be delivered to farmers, researchers and veterinarians.

Four hypotheses were tested in this study. Hypothesis 1 stated that ewes exposed to phytoestrogens that had aberrant reproductive outcomes (i.e. ewes that were double-dry (EX-DD) and ewes that had lambed but lost lamb (EX-LL)) differ in cervix morphology compared to the control. Hypothesis 2 stated that ewes with aberrant reproductive outcomes differ in uterine tissue types compared to the control. Hypothesis 3 stated that ewes with aberrant reproductive outcomes have a more 'oestrus-like' (i.e. increased intensity) expression pattern of steroid receptors (ERα, ERβ and AR). Hypothesis 4 stated that exposed but fertile ewes (EX-LW) do not share these changes and is similar to the control. This study saw some morphological parameters present contradictory to those reported in previous works (Adams 1986; Adams 1995). Increases in cervical muscularis were measured in exposed ewes but only significantly differed from the control in the mid cervix, not the uterine cervix as previously demonstrated. These differences were consistent across all exposed ewes rather than isolated to exposed ewes with aberrant reproductive outcomes. Exposed ewes in this study had greater cervical fold length than control ewes, rather than the predicted decrease, and this was consistent across all exposed ewes. However, the most significant finding of this study, consistent with previous clover disease research, was an exponential increase in cervical glands in exposed ewes, in both the mid and uterine cervix, correlated with infertility and perinatal lamb loss.

Cervical gland proliferation is associated with phytoestrogen exposure in ewes with aberrant reproductive outcomes

A marked increase in cervical glands in the cervix of exposed ewes with aberrant reproductive outcomes (EX-LL and EX-DD)—but not in exposed, fertile ewes—compared to non-exposed control ewes is consistent with previous research (Adams 1986). However, glandular proliferation absent any consistent pattern of change in cervical fold morphology is a novel finding. Therefore, Hypotheses 1 and 4 were supported in cervical gland measures. A previous pilot study on different ewes within the current experimental farm drew similar conclusions, with markedly more glands seen in infertile, phytoestrogen-exposed ewes but no change in cervical fold parameters compared to unexposed controls (Kontoolas 2019). The direct association between reproductive outcomes and cervical gland proliferation suggests that abnormal cervical mucus resulting from pathological altered cervical gland

morphology is the main driver of clover disease infertility and/or PPI. In addition, cervical gland morphology may be more sensitive to lower volumes of phytoestrogen exposure than other aspects of cervical architecture (Adams 1977). It is also possible that aberrant cervical mucus production contributes to cervical morphological change in clover disease/PPI ewes through permanently impaired regulation of extracellular matrix remodelling (Kershaw-Young et al. 2009). Cervical glands are highly responsive to permanent morphological and functional change from phytoestrogen exposure, with inhibited functionality persisting even several years after removal from oestrogenic pastures (Adams and Tang 1986). Clover disease ewes in previous literature produced increased volumes of watery, inelastic cervical mucus, which correlated with higher rates of bacterial infection of the cervix, fewer sperm surviving to the fallopian tubes, and ultimately reduced total fertility (Adams and Tang 1986). However, this study is the first to demonstrate such effects in the absence of other morphological changes to cervical tissues. Suppressed cervical gland function from presently available phytoestrogen volumes may be sufficient to cause aberrant reproductive outcomes in a subset of ewes, independent of any changes to cervical folds that may present at more advanced stages of the disease. The contribution of cervical mucus dysfunction to PPI is an area of research recommended for future works.

Cervical fold morphology shows an unclear relationship with phytoestrogen exposure

This study showed no clear pattern of cervical fold response to phytoestrogen exposure connected with reproductive outcomes. Ewes across all exposed groups and cervical regions examined had increased rather than decreased length of cervical folds and no decrease in cervical fold number, with increased fold area only in the EX-LL group. Therefore, Hypothesis 1 was partially supported for cervical fold measures, and Hypothesis 4 was not supported. This contrasts with the paper that identified clear morphological changes in response to phytoestrogen exposure and recommended their method as a diagnostic tool on that basis (Adams 1986). Past literature on clover disease ewes report markedly fewer and shorter cervical folds, with marked muscularis hypertrophy driving increased cervical fold width to span the greater underlying fold area (Adams 1986). The combination of reduced fold length and increased fold width resulted in greater fold area histologically but reduced luminal surface area *in vivo*, which was attributed to ewe infertility (Adams 1986). However, no such pattern exists in the present study, suggesting that cervical muscularis and folds are less sensitive to the effects of lower phytoestrogen doses.

In the present study, all exposed groups increased in muscularis area, with significantly greater values than control ewes for the mid cervix only, so it is unlikely that this contributes to the increased fold area in EX-LL ewes and may be a function of increased fold length. It is unknown what caused the failure to deliver a live lamb in EX-LL ewes who had achieved pregnancy. Future studies should attempt to elucidate cervical contributions to birthing difficulties. While it is unknown whether phytoestrogen exposure contributed to cervical fold length proliferation in these ewes, all exposed groups had longer cervical folds than control ewes on average, and thus did not correlate with aberrant reproductive outcomes.

Adams (1986) recommended counting cervical folds, particularly in the uterine region of the cervix, as the most reliable and easily quantifiable evidence of clover disease; however, the present study did not support this assertion. The cervix region examined interacted with the treatment group for muscularis area only, with muscularis width significantly different from control in the mid cervix only. The mid cervix may therefore be more sensitive to phytoestrogen exposure in current dietary volumes than the uterine cervix; however, this increase is unlikely to be pathological in nature, as it was seen in all exposed groups regardless of the reproductive outcome. Significant differences between exposed and control groups were consistent in each cervix region for all other measures.

Cervical gland counts, rather than fold counts, may more reliably predict PPI

The findings of this study also contradict the assumption of the previous methodology that counting cervical folds is straightforward, with the resulting tally consistent regardless of the level of cervix examined (Adams 1986). This study shows clear variation in the number of cervical folds dependent on the region examined, consistent with more recent work on ovine cervix morphology (Halbert et al. 1990). Cervical folds are not frequently continuous from the uterine to vaginal cervix; rather, folds often overlap and intersect and may begin or cease partway down the cervical tract (Kershaw et al. 2005). This study also found the existing definition of a fold highly interpretative and difficult to apply reliably between samples. The definition of a fold as "at least half as tall as its neighbours" (Adams 1986) is troublingly subjective, especially if the sample was cut on an angle, an error easily committed due to the lack of inherent straight lines in any biological tissue, and exacerbated by preservation. The inclusion or exclusion of a fold for counting also depended on the longest fold in the sample and, therefore, was easily biased as folds varied greatly in size within the same sample. Many same-sized folds could collectively be included in one sample and excluded in the next, depending on the height of their neighbours. This confusion may be due to the high inter-individual variation in cervical morphology among ewes and the lack of apparent differences in exposed ewes compared to the control in previous studies.

Phytoestrogen effects on ovine uterus remain unclear

In the uterus, myometrial hyperplasia was not observed in response to phytoestrogen exposure, as the total myometrial area was consistent across all groups. However, stereology analysis showed smooth muscle volume density increased significantly in EX-DD ewes compared to the control. No differences in ECM volume density per total sample occurred in EX-DD ewes compared to the control ewes, leaving the contribution of phytoestrogen exposure to uterine tissue types inconclusive. Therefore Hypothesis 2 was only partially supported for uterine smooth muscle volume density and not supported for uterine ECM volume density. While oestrogenic stimulation can induce proliferation in uterine smooth muscle via several different mechanisms, including phytoestrogen exposure, the lack of oestrogen receptor expression change in these ewes suggests that further research is needed before correlating uterine smooth muscle increase with the reproductive outcome (Huang et al. 2021). This may be simply due to the examination period being too far removed from the changes that occur during

pregnancy. Ovine uterine involution, the process by which the uterus and surrounding tissues return to a non-gravid state after birth, occurs very rapidly with some markers of pregnancy change undetectable after four weeks or less (Gray et al. 2003). Any changes to tissue types in the uterus and cervix from phytoestrogen exposure that result in altered biomechanical response may only be measurable in the days or weeks following such events. It is advised to examine ewes closer to or during pregnancy and examine changes to the cervix and vagina in addition to the uterus for a more thorough understanding of tissue response to phytoestrogen exposure. The proportion of ECM in the non-pregnant ovine cervix varies throughout the oestrus cycle. However, it correlates with peak oestradiol immediately prior to the LH surge via an oestradiol-oxytocin-mediated pathway that mediates prostaglandin endoperoxide synthase 2 expression and oxytocin production (Kershaw et al. 2007). The impact of phytoestrogen exposure on any of the steps in this pathway is unknown and a potential avenue for future research.

As no significant evidence of prolapse or dystocia was observed in experimental groups, the current phytoestrogen intake may be insufficient to cause ECM aggregation apparent at higher levels of exposure. This is consistent with the assumption that higher rates of dystocia and prolapse, common in clover disease (Beck and Gardiner 1965), are less common as PPI symptoms at lower levels of phytoestrogen exposure (Adams 1990). It is suggested that studies of phytoestrogen exposure during pregnancy, when the maternal endocrine environment is vastly different and additionally mediated by fetal and placental factors, may yield different results to the current study examining ewes in diestrus only (Fowden et al. 2009). The precise ratio of ECM to smooth muscle that any potential deleterious pregnancy outcomes could occur is unknown. However, while the ECM volume per total sample did not significantly differ from the control, the ratio of ECM to smooth muscle still changed in these groups; it is not known if the reduced ECM volume density per total smooth muscle contributed to infertility in phytoestrogen-exposed ewes. Failure to conceive is attributable to endometrial hostility to the trophoblast in up to two-thirds of human miscarriages, with several studies deeming endocrine-disrupting agents responsible (for review, see Pizzorno 2018). While conception failure in clover disease has been linked robustly to impaired sperm transit at the cervix level (Lightfoot et al. 1967), it is not possible from the results of experiments in this thesis to eliminate phytoestrogen influence on endometrial receptivity as a potential pathway of PPI infertility. ECM has important secretory functions in the early stages of implantation and pregnancy, releasing cytokines, proteases, matrix metalloproteinases and other paracrine factors that support blastocyst transit and trophoblast invasion in early pregnancy (Goryszewska-Szczurek et al. 2021). The contribution of subclover phytoestrogens to endometrial hostility is a potential avenue for future research.

An increase in endothelial volume density was unexpected in the exposed populations who had birthed lambs in the past year, partially supporting Hypothesis 2 but not Hypothesis 4 for this measure. Long-term supplementation with dietary phytoestrogens has not been linked demonstrably to endometrial thinning, rather to increased incidence of endometrial hyperplasia in women (Arici and Bukulmez 2004) and prevention of endometrial thinning in patients prescribed anti-oestrogenic medications (Unfer et al.

2004). However, many studies on dietary isoflavone supplementation have been conducted in peri- or post-menopausal women in whom bleeding disorders and endometrial hyperplasia can be more common, so researchers have suggested caution in assuming a causal relationship (Foth and Nawroth 2005). Generally, endogenous oestrogenic exposure unopposed to progesterone increases the mitotic activity of endometrial cells, thus increasing endometrial thickness in a dose-dependent manner (Key and Pike 1988). It is unknown whether the observed phenomenon is fully explained by increased cotyledon regions in the uterine horn from recent placental attachment or a response of these ewes to phytoestrogen exposure; this could be examined more closely in future studies.

Significant differences in blood vessel volume density observed in this study were unexpected, partially supporting Hypothesis 2 but not Hypothesis 4 for this measure. While phytoestrogens are linked to improved vascular function in humans, this is not attributed typically to vascular proliferation. Phytoestrogens exert beneficial change in blood vessel function through vascular relaxation (Nakaya et al. 2007), inhibition of platelet aggregation (Labinskyy et al. 2006), reduction in reactive oxygen species (Mizutani et al. 2000) and suppression of vascular smooth muscle cell proliferation (Dubey et al. 1999). Given the location of the more substantial uterine vessels on the outer perimeter of samples, stark differences in blood vessel volume density may be attributable to sampling error when trimming viscera and not considered for further study. Sampling errors are discussed in a subsequent section on study limitations.

Reproductive organ size reduced in exposed ewes

A surprising finding of this study was the consistently smaller cervix and uterus organ size in exposed ewes than control ewes, in direct opposition to the hypertrophy expected after phytoestrogen exposure (Martin et al. 1973). Hypothesis 1 was supported when examining the results of the cervix and uterus total size measures; however, Hypothesis 4 was not supported as this was also true for EX-LW ewes. A conference paper examining the same experimental ewes reported decreased ovine cervical length in phytoestrogen-exposed infertile ewes relative to control ewes and suggested mechanical stretch from recent pregnancy as a source of difference (Chazal et al. 2021). While cervix length has indeed been shown to increase with parity due to mechanical stretching, the additional and consistent reduction in total organ size across all exposed groups, irrespective of reproductive outcome, suggests a more complex relationship (Kershaw et al. 2005). All ewes in exposed groups had successful pregnancies and raised their lamb to weaning age prior to the experimental recording period, which does not adequately explain the differences in organ size between exposed groups and non-exposed controls, especially in the EX-LW group that successfully birthed and raised a lamb to weaning every year.

Exposure to low, constant volumes of exogenous oestrogenic compounds has been correlated with reduced reproductive organ size in ewes exposed during early fetal life (Hayashi et al. 2004). The effects of disruptive events during fetal or early neonatal life on the development of the ultimate adult phenotype, known as developmental programming, may present a novel avenue for future research on

phytoestrogen-exposed ewes. It is not known how exposure to subclover phytoestrogens during fetal life or the early postnatal period affects adult sheep or the degree to which any genomic or non-genomic effects are inherited. Phytoestrogens can cross the blood–placenta barrier in humans and rodents, and the same may be true for ovine species (Weber et al. 2001). Once sequestered into fetal membranes and amniotic fluid in these species, phytoestrogens may not be excreted effectively due to the immaturity of the fetal kidney, leading to a concentrated dosage effect that is difficult to estimate (Weber et al. 2001).

In addition to genetic trait inheritance, non-genomic heritable changes can occur via epigenetic mechanisms (Sinclair et al. 2016). Epigenetic changes primarily occur via DNA demethylation and chromatin/ histone remodelling, or post-transcriptionally to non-coding RNA that either increases or decreases gene expression (Guo et al. 2019). The epigenetic effects of a hyper-oestrogen environment can alter the expression of DNA that regulates growth and development of reproductive organs and endocrine glands, which can result in reduced fertility, which may be the case for ewes exposed to phytoestrogens (Savabieasfahani et al. 2006). While most previous research on developmental programming effects of oestrogenic compounds indicates increased rather than decreased uterine organ size, previous literature examining lower, continuous doses of oestrogenic compounds in postnatal periods—shows similar results to the present study (Hayashi et al. 2004). Ewe lambs exposed during critical period of uterine development—between postnatal days 14 and 55—saw perturbations in organ maturation and adenogenesis that persisted into adult life (Hayashi et al. 2004). Similarly, ewe lambs exposed to continuous low-dose 17β-oestradiol implants from four weeks of age saw consistently diminished gonadotrophin regulation as adults, and generational inheritance of masculinised play behaviours in second generation offspring (Malcolm et al. 2006). It appears that the concentration, timing and duration of phytoestrogen exposure are critical to adult phenotype (Jefferson et al. 2009). Postnatal days are critical periods for reproductive gland development in ewes, coinciding with the most potent subclover phytoestrogen levels (Nichols et al. 1996). The decreased uterus and cervix size in the present study and unanswered questions around potential developmental programming effects of subclover phytoestrogens is cause for further monitoring and attention in this region. However, in the present study, reduced reproductive organ size was consistent across all exposed groups and not linked with reproductive outcome. Similar results have been reported in rodent studies linking developmental phytoestrogen exposure with reduced reproductive organ size, but no significant loss of fertility or fecundity (Hayashi et al. 2004).

No association demonstrated between ERα, ERβ or AR expression and phytoestrogen exposure

As no significant differences in ERα, ERβ or AR expression were seen in uterine epithelia or glands, no association can be made between steroid receptor expression in the ovine uterus and exposure to phytoestrogens, supporting neither Hypothesis 3 nor 4. However, as our exposed group was sampled in October and phytoestrogen concentration in pastures likely peaked in July/August, it is possible that phytoestrogen levels had sufficiently declined by the time of autopsy, such that any differences in

steroid receptor expression were no longer quantifiable. This suggests that the higher volumes of phytoestrogens ingested earlier in the season do not have long-lasting effects on ERα, ERβ or AR expression in the ovine uterus. It is also reasonable to assume that the lowered phytoestrogen exposure experienced at time of sampling does not affect ERα, ERβ or AR in the uterus. Future work in this area is cautioned as the window for immediate effects of phytoestrogen exposure may be shorter than the current study anticipated.

The clear expression pattern of ERα in the nucleus of glandular cells, luminal endometrium and stromal cells is consistent with previous research examining steroid receptor expression and localisation in healthy ewes without phytoestrogen exposure (Duan et al. 2019). This is similar for the expression of ERβ, which presented comparably to ERα in both studies (Duan et al. 2019). ERα appeared more intensely expressed than ERβ, and localised to the cell nucleus, whereas ERβ was often seen in cytoplasm, especially in the endometrium. This is consistent with another study showing greater expression of ERα in healthy ovine endometrium, especially at lower levels of circulating oestrogen (Yu et al. 2022). Low phytoestrogen levels have a weak positive correlation with oestrogenic activity in human populations with low endogenous oestrogen levels, such as postmenopausal women (Pampaloni et al. 2009). This was seen as a general trend in the observed means; however, no correlation can be drawn as it was not statistically significant. Future research examining phytoestrogen exposure and ovine oestrogen receptor activity should sample ewes earlier in the season, where higher phytoestrogen volumes in pasture may yield significant results. The presence of minimal AR expression localised in the epithelial cell membrane rather than the nuclei indicates minimal AR activation, likely due to low volumes of androgen in circulation, suggesting that current volumes of phytoestrogen exposure do not correlate with increased androgenic expression patterns. The presence of AR expression in uterine smooth muscle cells across all groups supports a role for androgens in smooth muscle cell proliferation during the oestrus cycle, as suggested previously (Duan et al. 2019).

Experimental limitations

The most notable limitation of this study is the sampling of exposed ewes at a single time point in their reproductive cycle on a single farm. Ewes sampled during peak pasture phytoestrogen concentrations may significantly differ in steroid receptor expression patterns, tissue types and other potential markers of temporary phytoestrogen effects that were unchanged in this study. Phytoestrogen exposure during pregnancy or lactation, when maternal circulating steroid concentrations are vastly higher and potentially mediated by fetal and placental factors, may yield different results than the present study that examined ewes towards the end of diestrus (Fowden et al. 2009). Considerable volumes of progesterone, oestrogens, androgens and glucocorticoids, critical to successful implantation and pregnancy, are supplied by the trophoblast, developing embryo, and placenta in varying concentrations through pregnancy and may be inhibited in the presence of increased phytoestrogen (Chatuphonprasert et al, 2018).

It is also unknown whether the level of phytoestrogen exposure observed on the study's experimental farm is typical for the region. Estimates suggest that 20–65% of farms in southern Australia have subclover phytoestrogens contributing 20% or more of biomass; however, there is little recent published data (Foster et al. 2021). In many ways, this is a victory and testament to the hard work and dedication of researchers and farmers in the region to eliminate clover disease (Adams and Croker 1987). Recommendations advising breeding ewes to be removed from grazing clover phytoestrogens, and the continued dilution of oestrogenic subclover pastures with non-oestrogenic fodder, appear to have been highly successful in eliminating serious clover disease (Adams and Croker 1987). However, accurate quantifications of the scope and potency of oestrogenic subclovers across southern Australia are needed for future risk assessments and recommendations of a safe dosage of phytoestrogens and prevention of PPI.

Another limitation of this thesis is sampling error due to the collection of some samples prior to commencing the majority of experimental work when no experimental design was yet determined. While this was necessary for logistical and funding reasons, inconsistencies with sample collection resulted in some samples being removed from the final analysis. This was apparent in the upper cervix H&E histology and disproportionate blood vessel volume density results in uterus stereology. Additionally, some mid cervix samples were also removed due to the cervical os protruding cranially into the mid cervical cavity, effectively doubling the luminal surface area, leading to concerns of sampling bias. As ovine cervical os types highly varied among healthy ewes and afflicted samples across treatment groups, these outliers were considered unrelated to phytoestrogen exposure and excluded (Kershaw et al. 2005). Some protocols were not adequately tested manually prior to large- scale machine staining due to COVID-19 restrictions necessitating sudden periods of university laboratory closure, resulting in darker than expected Masson's trichrome staining in the uterus (see Appendix 4). Ultimately, due to inconsistent staining, Masson's trichrome-stained cervix samples had to be discarded. Larger treatment sizes are suggested for future works due to the relatively small incidence of potential PPI on the studied farm to ensure robust regression analysis.

While all attempts were made to source purebred Merino ewes as experimental animals, it is possible that unknown anatomical differences exist between flocks that were not accounted for in this experiment. While previous studies utilised ewes from the same flock that were randomly assigned treatment groups, this was unfortunately not possible in this experiment due to time and cost constraints. While the possibility of anatomical differences in experimental and control groups unrelated to clover exposure is acknowledged as a limitation of this study, the control group was selected primarily for its documented grazing history. It was considered critical that control ewes had not been exposed to subterranean clover for the duration of their lifespan, which eliminated many commercial ewes in Western Australia whose grazing history was uncertain or unknown.

Avenues for future research

As this study's findings link subclover phytoestrogens and ewe reproductive damage, resulting in reduced fertility outcomes, further research should consider a three-stage approach to treat and prevent PPI. First, further pasture quantification of phytoestrogens across the region is needed, detailing the variability of pasture phytoestrogens across seasons and geographic region, to estimate the extent of phytoestrogen exposure and expected dosages. Second, thorough epidemiological studies are needed to determine PPI histological presentation at current phytoestrogen exposure levels. The timing and duration of exposure could then be linked to subsequent physiological effects at all stages of the ewe reproductive cycle, including pregnancy and critical periods of development when lambs may be more vulnerable to phytoestrogen exposure. Third, after determining the phytoestrogen volumes expected in pastures and expected ewe phenotype in response to observed volumes, updated advice targeting the identification and prevention of PPI can be given to farmers. The ultimate aim of this three-stage process is that PPI prevention and management will reduce ewe and lamb losses and maximise ewe fertility and the number of live lambs born.

Several avenues for future research are suggested from the results of this thesis, including replicating the novel association between pathological cervical gland proliferation and aberrant reproductive outcomes in ewes exposed to phytoestrogens compared to the control to determine the exposure and dosage for glandular proliferation and feasible safe maximum dose of phytoestrogens. Future works in this area should consider how phytoestrogen-mediated cervical mucus becomes hostile to sperm, such as immunological factors, increased anti-sperm antibodies and altered pH, and how this might be treated in breeding flocks (Daunter and Khoo 1984). The relationship between compounds such as glycosaminoglycan secreted in cervical mucus, which may change in clover disease or PPI ewes, and failure to mediate normal cervical remodelling may prove especially interesting (Leethongdee et al. 2007). The prospect that aberrant cervical mucus composition in clover disease ewes mediates cervical remodelling in severe disease manifestations was not demonstrated in this study but presents an exciting opportunity for future research in this area. Strategies for preventing this morphological change, considering its permanent nature as demonstrated in previous works, may be more effective than treating the symptoms once the nature of infertility becomes apparent (Heydon and Adams 1977).

Timing of phytoestrogen exposure is critical for any future research on PPI ewes. Based on the findings of this study, ewes should be sampled closer to peak pasture phytoestrogen concentration than the current study if examining evidence of immediate phytoestrogen ingestion, such as steroid receptor expression. Quantifying oestrogenic subclover volumes would also be useful in linking reproductive tract changes to pasture phytoestrogen levels, as this is expected to vary in the region in response to variable agricultural management and rainfall (Davies et al. 1970). While phytoestrogen concentration in subclover remains relatively stable over time, it can be affected by environmental factors such as insect predation and waterlogging stress, which need to be quantified when assessing the safe phytoestrogen exposure level (Quiroz et al. 2018). The absolute volume of subclover as a percentage of total biomass can also markedly increase in higher rainfall years, resulting in higher total phytoestrogen

exposure (Saba et al. 1974).

Additionally, examinations of uterine ECM as a contributor to birthing difficulties, rather than infertility as mentioned above, would be best examined during or immediately after pregnancy, before tissue volumes have adjusted to pre-pregnancy states, and further examination of the nature of ECM may be required. This study examined ECM total area, assuming that phytoestrogen-mediated proliferation increases ECM volume density by fibrotic masses or general relaxation and disorganisation of collagen bundles comprising a comparatively greater area, as seen from high oestrogen exposure (Aspden 1988). However, a relationship between phytoestrogens and ECM may be more complex when examined histologically. Absolute area of ECM aggregation may fail to account for collagen hydration levels, tensile strength, organisation of collagen bundles, and types of collagen present, particularly the ratios of collagen type III to collagen type I (Moffatt et al. 1993; Iwahashi et al. 2011).

Finally, while experimental pastures in this study were considered highly oestrogenic (estimated 40% oestrogenic cultivars of subclover as percentage of total biomass when sampled in August 2019; Appendix 1), the experimental farm has an average 90% fertility rate, indicating that fewer ewes suffer from PPI than expected from pasture phytoestrogen quantification alone. Pastures may be diluted sufficiently with grasses, weeds and non-oestrogenic subclover to protect the majority of ewes from harm. However, the presence of a small proportion of afflicted ewes within an almost entirely unaffected flock raises an interesting research avenue around the source of resistance of some ewes to subclover phytoestrogens. Previous research demonstrated that ewes exposed to subclover phytoestrogens that remained fertile when bred with unaffected rams grazing the same pastures had a reduced frequency of clover disease in offspring compared to previous generations (Croker et al. 1989). While the source of this resistance was not elucidated, there appeared to be inherited resistance in subsequent generations (Croker et al. 1989). The disappearance of severe clover disease could be partially attributable to genetic selection pressure for increased survival of resistant ewes and removal of susceptible ewes from the gene pool. Future studies could examine the genetic contributions to phytoestrogen resistance, including heritable differences in ruminal microbial populations, which may disproportionately reduce formononetin into insignificant quantities of its oestrogenic metabolite equol (Li et al. 2019).

Conclusion

The general hypothesis of this study that ewes exposed to phytoestrogens with aberrant reproductive outcomes—but not exposed, fertile ewes—show abnormal histopathology compared to unexposed controls was supported. However, significantly altered histopathology was consistent with hypotheses in measures of gland number only, which may be critical for establishing future diagnostic standards for PPI detection. Other measures of cervical and uterine morphology, particularly volume density of ECM in the uterus and cervix, may require further study at different time points in the ewe reproductive cycle. The results of this study emphasise that subclover phytoestrogens, while not the significant threat to ewe life and farm profitability that they once were, are also not harmless to ewe and lamb health.

This highlights a clear need for investigation into PPI phenotype, the best methods for its diagnosis, and its current prevalence across southern Australia as a separate syndrome from clover disease.

While the present research indicates that phytoestrogen exposure as a cause of ewe infertility and neonatal lamb mortality remains a reasonable concern, PPI prevalence is likely very low on the studied experimental farm. However, the experimental farm generally had a very low incidence of infertility and neonatal lamb loss, and further research is encouraged to examine farms containing oestrogenic clover with a greater incidence of reproductive failure. Ewes in this area may have an increased genetic resistance to phytoestrogens, presenting interesting avenues for future research. More detailed quantification of pasture phytoestrogen volumes, including timing and duration of exposure, is necessary. Thorough epidemiological studies are necessary to update the expected PPI phenotype and diagnostic methodology and hopefully provide more detailed and informed recommendations to farmers, veterinarians, and researchers.

CHAPTER 7. REFERENCES

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APPENDIX 1. RESULTS OF PASTURE SURVEY

1 Methods

1.1. Rod-point technique for botanical composition

The three pastures most grazed by ewes in the clover exposure group were sampled using the rod-point technique (Little and Frensham 1993) as it accurately represents pasture components while minimising the time, labour and equipment required (Little and Frensham 1993). Briefly, a 500 mm rod, sharpened to a point at both ends, was placed on the pasture, with the pasture component touching both ends of the rod recorded. The sampling was repeated 100 times at random intervals over the entirety of the pasture and yielded 200 total pasture component recordings per paddock. Weeds and grasses were recorded (Collins et al. 1984). Clovers were identified into cultivars based on leaf shape and individual markings (Nichols et al., 1996; Figure 1).

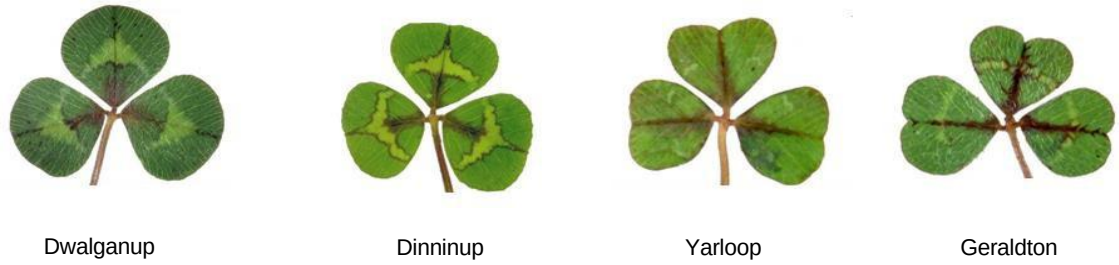

Figure 1. Clover cultivars display distinguishing markings. Clover were identified using these markings combined with leaf size and shape. Expected cultivars included Dwalganup (a), Dinninup (b), Yarloop (c), and Geraldton (d). Images courtesy Dr Kevin Foster UWA and DPIRD (private communications).

1.2. Hand separation of pasture quadrants for biomass proportions

Five representative random samples per pasture measuring 250 mm × 410 mm were collected using Ozito CGH-180K cordless shears (Bangholme, Australia). Each sample was oven-dried at 70°C, and hand separated into individual pasture components to determine relative percentages (Tiwari et al. 1963).

1.3. Thin layer chromatography for formononetin content of green clover leaves

An addition 100 clover samples from each pasture were collected in paper bags for formononetin analysis. Sixty-one samples, a minimum of 20 from each pasture, were later selected for analysis by UWA Pasture Science Laboratories. Quantification and identification of isoflavone components in the leaf blade of the dry matter were assessed using thin layer chromatography (Francis and Millington 1965). Individual clover leaves were extracted with a mortar and pestle and emulsified in 50 mL 100% ethanol, with 0.5 mL of the ethanol extract applied to plates coated in Silicagel 60 F254 (Merck), 15

mm from the edge of the plate on a line drawn by HB pencil. Methanol chloroform (1:9) was used as the mobile phase in the developing chamber (Beck 1964). The clover components were allowed to separate overnight. The plates were dried and observed under a UV lamp at 366 nm, under which formononetin fluoresces green (Camag UV II). Bands representing formononetin components of the clover were measured for length with a ruler against existing standards (Wójciak-Kosior et al. 2014). Formononetin was calculated as a relative percentage of clover composition against total dry weights (Meyers and Meyers 2008). The clover percentage of total food on offer combined with the percentage of formononetin in clover provides a pasture oestrogenicity score of safe (<20%), moderate (20–40%) or potent (>40%) (Quinlivan et al. 1968; Neil et al. 1969).

2 Results

2.1. Quantifying pasture botanical components and clover percentage

All three paddocks contained a mixture of weeds, grass and clover (Table 1). Clover was the dominant botanical component in all pastures (42.8%), followed by weeds and grass (31.2% and 26%, respectively) as determined by rod-point random sampling.

Table 1. Pasture components measured using rod-point random sampling. Values expressed as a percentage of 200 measurements per paddock (mean ± SEM).

	Grass	Weeds	Clover
Paddock 1	32.5 ± 7.9	21.5 ± 7.9	46.4 ± 7.9
Paddock 2	28.2 ± 6.9	24.5 ± 7	47.5 ± 6.9
Paddock 3	33 ± 1.4	32.3 ± 1.4	35.1 ± 1.4

2.1.2 Subclover cultivar identification

Three different clover cultivars, all known oestrogenic cultivars, were identified in all three paddocks (Table 2). Geraldton was the most highly represented cultivar (53.4%), followed by Dinninup and Dwalganup (22.6% and 12%, respectively.) No non-oestrogenic cultivars of clover were located in any of the paddocks.

Table 2. Rod-point clover cultivar as a percentage of total clover per paddock (mean ± SEM).

	Dinninup	Dwalganup	Yarloop	Non oestrogenic
Paddock 1	40.2 ± 3.0	22.1 ± 2.9	38.1 ± 2	0
Paddock 2	16.1 ± 9.2	10.4 ± 9.2	16 ± 8.1	0
Paddock 3	12.3 ± 7.7	3.9 ± 7.7	12.2 ± 7.6	0

2.2. Pasture botanical components measured by representative sample

The hand-dried and counted pasture analysis revealed 11.8% clover, 30.3% weeds and 57.9% grass, as an average across all three paddocks (Table 3).

Table 3. Dry weight of pasture components for three paddocks as kilograms per hectare (mean ± SEM)

	Clover	Grass	Weeds
Paddock 1	62.7 ± 7.9	140.8 ± 2.9	177 ± 2
Paddock 2	69.7 ± 6.9	85.6 ± 9.2	383 ± 8.1
Paddock 3	62.5 1.4	273.5 1.2	393 1.3
Average	65 ± 5.3	166.6 ± 7.7	318 ± 7.6

2.3. Oestrogenic content of pastures measured in formononetin

Formononetin concentration was consistent across all three pastures, with an average of 1.10% of leaf matter averaged for the three paddocks (Table 4).

Table 4. Isoflavone analysis of subclover as a percentage of total leaf sample composition (mean ± SEM)

	Formononetin	Biochanin-A	Genistein
Paddock 1	1.08 ± 0.11	1.10 ± 0.13	1.84 ± 0.16
Paddock 2	1.07 ± 0.12	1.48 ± 0.12	2.00 ± 0.24
Paddock 3	1.15 ± 0.09	1.23 ± 0.15	2.16 ± 0.15
Average	1.10 ± 0.11	1.27 ± 0.13	2.00 ± 0.18

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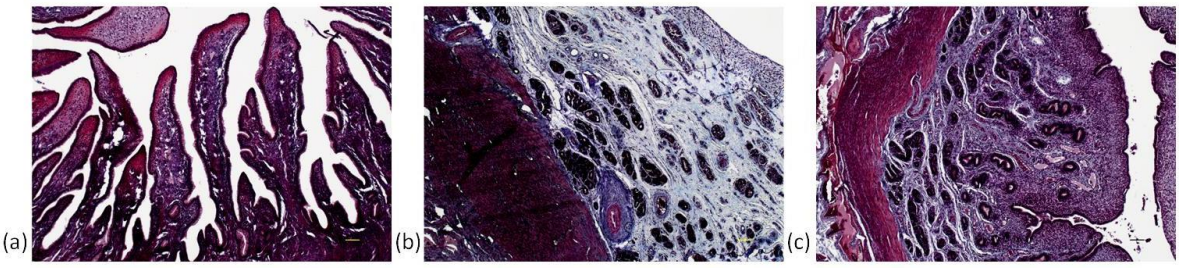
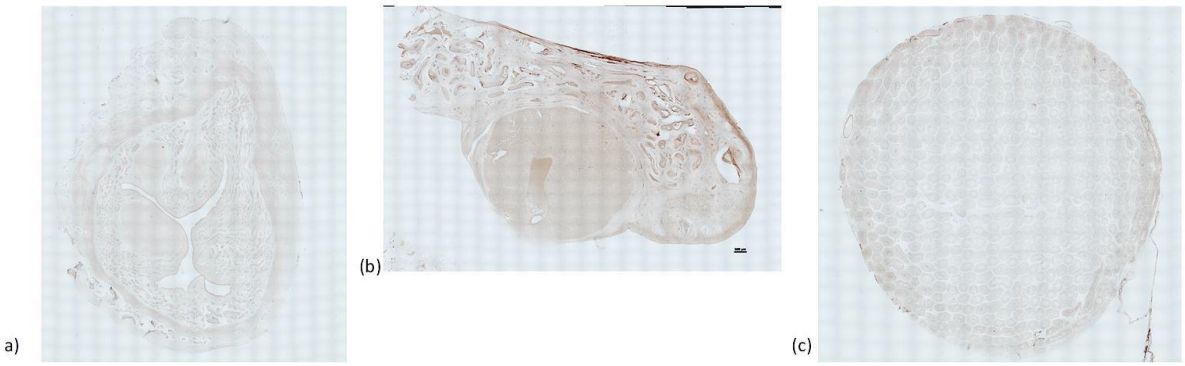
APPENDIX 2. MASSON'S TRICHROME CERVIX



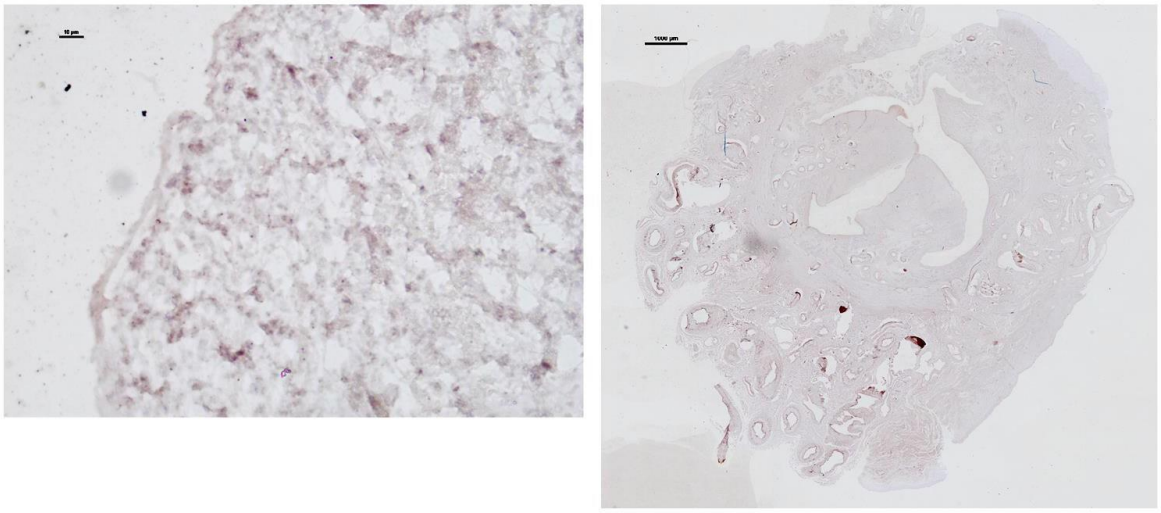
Example of cervix sample stained with Masson's trichrome protocol via automated staining machine that could not be quantified (a), or was much darker than manual testing (b, c). Several automated methods of ECM detection were attempted, including the Colour_Deconvultion2 macro in ImageJ, and Nikkon NiS-Elements Pixel Classifier, which did not prove successful.

For future attempts, assuming more time is available to troubleshoot prior to mass staining, phosphomolybdic could be tested for progressively longer periods to lighten the darker purple sections prior to adding further dyes.

APPENDIX 3. IMMUNOHISTOCHEMISTRY OPTIMISATION



Images showing ERα in ovine uterus (a), ERβ in ovine ovary (b), and AR in rat testis (c).



Hematoxylin counterstain was trialled but ultimately did not improve the final image.

APPENDIX 4. CERVIX DIMENSIONS AND MEASURES WITHOUT CONTROLLING FOR ANIMAL WEIGHT

Previous study (Adams 1986)

	Control			Clover affected		
Area mm²						
muscularis	9.80	±	1.80	36.20	±	6.70
folds	8.90	±	1.90	9.30	±	1.90
Folds mm						
length	0.62	±	0.05	0.39	±	0.04
width	0.35	±	0.03	0.60	±	0.05
number	18.60	±	2.70	3.40	±	0.60
glands	135	±	36	642	±	75

Current study

	Control			EX-DD			EX-LL			EX-LW		
Area mm²												
muscularis	41.44	±	12.67	33.06	±	6.38	33.74	±	10.66	37.57	±	14.08
folds	0.34	±	0.13	0.41	±	0.25	0.44	±	0.23	0.58	±	0.30
Folds mm												
length	0.91	±	0.23	1.02	±	0.21	1.02	±	0.26	1.10	±	0.29
width	0.36	±	0.10	0.33	±	0.11	0.51	±	0.24	0.54	±	0.25
number	16.35	±	5.36	11.05	±	4.72	10.76	±	5.91	11.23	±	6.54
glands	78	±	77	127	±	116	245	±	151	175	±	181

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