AN ABSTRACT OF THE THESIS OF

Clinton Sexson for the degree of Master of Science in Animal Science presented on July 15, 2002.
Title: Effects of Alfalfa on Uterine Growth of Ovariectomized Prepubertal Ewe Lambs.

Abstract Approved: ____________________________________________

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James R. Males

Alfalfa accumulates phytoestrogens and when ingested binds the estrogen receptor and induces morphological changes similar to endogenous estrogens. The objective of this study is to evaluate morphological changes in uteri, vulva, and teats of ovariectomized prepubertal ewe lambs. Eighteen prepubertal ewe lambs were ovariectomized in November 2000 and fed nonestrogenic hay until May 2001. In May, ewes were fed bentgrass straw and cottonseed meal. On day 0 of a 12-day feed trial, ewes were assigned randomly to three treatments (n=6 in each treatment): Estradiol, Control, and Alfalfa. Estradiol treated ewes were fed bentgrass straw and cottonseed meal ad libitum, plus receiving a daily injection of 10 mg estradiol-17β suspended in corn oil. Control ewes were fed bentgrass straw and cottonseed meal ad libitum and received a daily injection of corn oil vehicle. Alfalfa ewes were fed alfalfa ad libitum and received a daily injection of corn oil vehicle. Three blinded observers assigned each ewe a subjective score ranging from 1 (no change) to 4 (significant change) for vulva and teat morphology on
Days 0, 1, 3, 5, 7, 9 and 12. Teat length and circumference were measured on Days 1, 7 and 12. Ewes were slaughtered on Day 13, uteri were weighed, and a cross-section was collected from each uterine horn. Cross-sections were fixed in Lillie’s Neutral Buffered Formalin and embedded in paraffin wax, sectioned at 4-5 μm, and stained with hematoxylin and eosin. An ocular micrometer was used to measure luminal epithelial cell height. Estradiol treated ewes had heavier (p< 0.05) uterine weights and greater (p<0.05) uterine luminal epithelial cell height than that of ewes fed alfalfa or control ewes. Uterine weights and uterine luminal epithelial cell height were greater (p< 0.05) in alfalfa fed ewes than control ewes. Vulva scores for estradiol treated ewes were higher than those of control ewes (p< 0.05). Alfalfa fed ewes had numerically higher vulva scores than control ewes but the difference was not significant statistically (p>0.05). Teat scores or measurements showed no differences (p>0.05) among treatments. Ewes exhibited slight changes in vulva scores due to treatment, but the most noted effects were observed in uterine growth.

This research suggests that uterine weight and uterine luminal epithelial cell height are sensitive to the estrogenic activity of alfalfa and estradiol-17β resulting in morphological changes in estrogen target tissues in the prepubertal ewe lamb.
Effects of Alfalfa on Uterine Growth of Ovariectomized Prepubertal Ewe Lambs.

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The past three years at Oregon State University has been one of the most memorable times in my life. Numerous people have contributed to my success. I want to start by thanking Alix Gitelman, Fred Stormshak, and James Moore for serving as my committee. My major professor, James R. Males, deserved the most credit for providing me an opportunity to research a very practical area of reproduction. His support and mentoring through my graduate experience was second to none. As important, Parviz Kamangar and Oregon Sports Lottery’s financial scholarship support were highly appreciated.

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Introduction

Legume forages have been and will continue to be utilized as an available feedstuff to develop and maintain ruminants. Oregon produces and markets quality legume and legume-mix feeds to producers in the western United States as well as export markets. The feeds can potentially contain phytoestrogens. Phytoestrogens are compounds that have a chemical structure similar to estradiol that allows them to act on estrogen receptors to induce estrus and cause morphological changes in the reproductive tract (Kurzer and Xu, 1997). Phytoestrogens can reduce the reproductive efficiency of domestic ruminants. The reduction in reproductive efficiency may be attributed to reduction of ovulation rates in ewes, cystic ovaries in beef and dairy cattle, and overall reduced ability to conceive. The concern for producers is the potential lower lambing rate in ewes and the irregular estrous cycles that delay conception in beef and dairy cattle. The development of ewe lambs and heifers with alfalfa may be a concern if the phytoestrogen content interferes with the ability of the female to conceive at an early date in the mating season.
Coumestrol along with other phytoestrogens is present in alfalfa and ladino clover (Newsome and Kitts, 1980). Coumestrol has been measured in the plasma of animals consuming these forages (Newsome and Kitts, 1980). Alfalfa (Medicago sativa) generally has a high concentration of coumestans that accumulate in the leaf of the plant. Coumestans are not significantly metabolized in the rumen (Adams, 1995) and coumestrol is the active form that induces the effect on the reproductive tract of the ruminant animal (Saloniemi et al., 1995). Formononetin is considered the most predominant isoflavone influencing reproductive efficiency in domestic ruminants (Adams, 1995; Lundh, 1995; Nwannenna et al., 1995). However, it accumulates to a lesser degree in alfalfa. Formononetin is demethylated and reduced to its metabolite, equol, in the rumen (Nilsson et al. 1967; Livingston, 1978; Adams 1995; Lundh 1995). Equol is the active form of formononetin that evokes morphological changes in the reproductive tract of the ruminant.

Phytoestrogens act much like endogenous estrogens. The chemical structure of phytoestrogens is similar to estradiol. In an active form, its structure allows it to bind with the estrogen receptor. Consequently, the binding causes detectable effects in the reproductive tract of the female. It has been postulated that if alfalfa is fed in high enough concentrations to ewes, then the coumestrol present in plasma could affect uterine events (Newsome and Kitts, 1980).
Phytoestrogens can induce increased vulva and udder development (Oldfield et al., 1966). Research in intact and ovariectomized sheep and rats suggests that uterine weight increase is associated with increasing exposure to an estrogenic diet. The combination of the above mentioned factors have lead some to believe that phytoestrogen can lead to reproductive problems. Problems can be measured by observations of reduced lambing rates and morphological changes in mammary glands and vulva in mature ewes after prolonged exposure to phytoestrogens. Newsome and Kitts (1980) provided one of the few reports on the effects of coumestrol on prepubertal ewes.

Further research is needed to validate the clinical effects phytoestrogens exert on the reproductive efficiency of prepubertal ruminants. Therefore, the objective of this study was to evaluate physiological and morphological changes in ovariectomized prepubertal ewe lambs fed an alfalfa based diet containing known amounts of coumestrol.
Literature Review

Phytoestrogens are compounds that exert estrogenic effects on the central nervous system, induce estrus, and stimulate growth of the female genital tract (Kurzer and Xu, 1997). Over 300 plants are reported to be estrogenic (Kurzer and Xu, 1997). The chemical structure of phytoestrogens is similar to estradiol thus allowing interaction with the estradiol receptor (Adams, 1995a). Phytoestrogens have been most widely studied in laboratory animals, humans, cattle and sheep.

Phytoestrogens can be classified into three groups: coumestans, isoflavones, and lignans. All are diphenolic compounds with structural similarities to natural and synthetic estrogens and antiestrogens (Kurzer and Xu, 1997). Coumestrol is the most common coumestan (Figure 1) and has the greatest activity of the phytoestrogens studied. It has approximately 1/1000 the potency of estradiol-17β (Adams, 1995a). Coumestrol is estimated to be about 1000 times more potent than equol (estrogenic metabolite of formononetin and daidzein) and behaves much like diethylstilbestrol (Kurzer and Xu, 1997). Common examples of isoflavones (Figure 2) include formononetin, biochanin A, and daidzein and genistein. The relative estrogenic activity varies between these isoflavones and the plant species of interest.
Figure 1. Chemical structure of coumestans.

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<th>Coumestans</th>
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<th>R₂</th>
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<tbody>
<tr>
<td>Coumestrol</td>
<td>OH</td>
<td>OH</td>
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<tr>
<td>4-methoxycoumestrol</td>
<td>OH</td>
<td>OCH₃</td>
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From an article by Kurzer and Xu (1997).
Figure 2. Chemical skeleton of isoflavones.

<table>
<thead>
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<td>H</td>
<td>H</td>
<td>OH</td>
<td>OCH₃</td>
<td>OH</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>OCH₃</td>
<td>OH</td>
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<tr>
<td>Daidzein</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Genistein</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
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</tbody>
</table>

From an article by Kurzer and Xu (1997).
Prepubertal ewe lamb

Puberty is defined as the first time an animal can sexually reproduce and is characterized by maturation of genital organs and development of secondary sex characteristics (Knobil and Neill 1994). Several researchers use first estrus as the definition of puberty in sheep (Knobil and Neill 1994). Reproductive organs develop relatively slow and are functionally inactive in early life of the prepubertal ewe lamb (Hafez, 1952). In comparison, rats show slow uterine growth until approximately day 36, then rapid proliferation of the uterus occurs under the increased exposure to estrogen stimulation (Price, 1947). The time prior to and following puberty marks increased growth and activity in reproductive organs and endocrine glands (Hafez, 1952). Genital maturity is important to the progression of puberty (Hafez, 1952). Uterine growth occurs in response to the uterus becoming more sensitive to stimulation by estrogen. Estrogen stimulates increased uterine blood flow, which causes uterine growth, cell proliferation, and hyperemia (Reynolds et al., 1998). Newsome and Kitts (1980) reported that 5-month-old ewe lambs produced mean uterine weights of approximately 0.38 g uterine weight/kg of body weight. This result was significantly lighter than estradiol and coumestrol-treated ewes in the same study (Newsome and Kitts, 1980). This report suggests that the uterus is responsive to estrogen at an early age.
Metabolism of Phytoestrogens

Phytoestrogens accumulate in plants mainly as water-soluble glycosides (Nilsson et al., 1967; Livingston, 1978; Lundh, 1995; Cheeke, 1998). The metabolic fate of the phytoestrogen determines the estrogenic effects of the compound in the ruminant. Ruminants break down the phytoestrogenic compounds differently than monogastrics. Ruminal microorganisms can readily hydrolyze phytoestrogens (Lundh, 1995; Cheeke, 1998).

Coumestrol is not greatly metabolized in the rumen (Adams, 1995a); consequently it is absorbed in an active form and is available to affect the animal (Saloniemi et al., 1995). Coumestrol can be detected in plasma as early as 1 hour after ingestion and this rapid accumulation was thought to be unconjugated coumestrol from the rumen (Kelly and Lindsay, 1978). Free coumestrol accounted for approximately 20 to 40 percent of circulating coumestrol, which was different from 10 percent of unconjugated isoflavones in sheep (Kelly and Lindsay, 1978).

Retention time of feedstuffs is the major reason the bulk of degradation and detoxification of isoflavones (formononetin, daidzein, biochanin A and genistein) takes place in the rumen (Nilsson et al., 1967; Livingston, 1978; Lundh, 1990; Adams, 1995a). As a result, rumen contents tend to be the most estrogenic (Livingston, 1978). The rumen is responsible for the bulk of metabolism, but other organs play a role through the metabolic process. The metabolic pathway of the original phytoestrogen determines the ultimate estrogenic effect of the isoflavones.
In the case of feedstuffs containing the isoflavones, biochanin A and genistein, rumen microorganisms break each down into their nonestrogenic metabolite, or para-ethylphenol (Livingston, 1978; Adams, 1995a; Saloniemi et al., 1995).

Formononetin is the most important isoflavone in ruminants in regards to long-term estrogenic effect (Adams, 1995a; Nwannenna et al., 1995). Formononetin, the 4-methyl ether of daidzein (Kurzer and Xu, 1997), has little to no direct effect on reproduction (Lundh et al., 1990); the estrogenic effect of formononetin is activated through demethylation and reduction (Livingston, 1978). Formononetin is demethylated to daidzein and further metabolized via hydrogenation and ring fusion to equol (Lundh, 1990; Lundh et al., 1990; Saloniemi et al., 1995; Cheeke, 1998). Nilsson et al. (1967) named this metabolic process as the formononetin-daidzein-equol pathway (Figure 3). Equol is more estrogenic than formononetin and can be rapidly absorbed through the ruminal wall (Adams, 1995a). Equol is readily absorbed from the gastrointestinal tract (GIT) and concentrations are shown to increase in plasma after ingestion (Dickinson et al., 1988; Lundh et al., 1990; Wang et al., 1994). Dickinson et al. (1988) has shown that within 6 hours of incubation in bovine rumen fluid, formononetin is almost completely demethylated and the conversion to equol is in progress. Furthermore,
formononetin, daidzein and equol are all available for absorption from the GIT for up to 24 hours after ingestion. Collectively, equol is thought to be the major estrogenic metabolite that initiates estrogenic effects in sheep (Livingston, 1978; Dickinson et al., 1988; Lundh et al., 1990) and, possibly, cattle.
Variation in the sensitivity between ruminant species challenges researchers to analyze how certain species metabolize individual phytoestrogens differently. Early research suggested that the degree of sensitivity in sheep and cattle is due to conjugation ability of the particular species. Conjugation is one of the most important mechanisms in animals to detoxify different ingested foreign substances, including plant estrogens (Lundh et al., 1990). Conjugation activity is found in the epithelial tissues of the rumen, reticulum, omasum and small intestine (Lundh, 1990). Conjugated isoflavones and their metabolite, equol, account for the majority of circulating conjugates while only a few, hydrolyzed, unbound phytoestrogens reach circulation unconjugated. Glucuronic acid is the major detoxification mechanism in conjugation of phytoestrogens. Lundh (1990) postulated that phytoestrogens are conjugated with uridine 5-diphosphoglucuronic acid (UDPGA) in the GIT, which acts as a first line of defense before the substance enters the circulation. The rumen accounts for the major source of conjugation of phytoestrogens. However, unconjugated substances that are absorbed into the circulation are conjugated in the liver. The liver plays a major role in metabolism and detoxification (Lundh et al., 1990; Lundh, 1995), yet only has minor contribution to the total breakdown of phytoestrogens.

Lundh (1995) researched the difference in sensitivity levels of cattle and sheep in response to phytoestrogens. Past research indicated that cattle were affected less by phytoestrogens compared to sheep. Lundh (1995) compared
metabolism between the two species and arrived at the following conclusions. He concluded that conjugation activity takes place in the epithelial tissue of the rumen, reticulum, omasum and small intestine. The rumen and liver were the major organs of metabolism and detoxification. His research indicated that the GIT epithelium should detoxify different substances before reaching the circulation. In comparison to total conjugative activity in the GIT, sheep showed greater activity in the rumen, reticulum, and omasum. However, cattle showed a greater activity in the small intestine. Conjugation of phytoestrogens with glucuronic acid in the GIT accounted for the majority of the degradation.

Hepatic metabolism accounted for a small percentage of phytoestrogen conjugation. The liver conjugates unbound substances that pass into the circulation. Sheep liver can more efficiently conjugate formononetin and daidzein in the presence of UDPGA than cattle (Lundh, 1995). Lundh (1995) concluded that liver microsomes lent no explanation to the species differences.

In vivo metabolism studies by Lundh (1995) suggested that formononetin and daidzein were more rapidly absorbed in cattle than sheep, however sheep conjugated phytoestrogens more efficiently. Also, sheep cleared equol more rapidly than cattle (Lundh, 1995), mainly via urinary excretion (Dickinson et al., 1988). Cheeke (1998) contradicted this statement by stating that absorbed equol was excreted more rapidly in cattle. It was obvious why both Lundh (1995) and Lundh et al. (1990) suggested that, at present, there was no satisfactory metabolic
evidence to explain the species differences between sheep and cattle in their sensitivity to phytoestrogens. At most, conjugation can only be one of many factors needed to explain the differences in phytoestrogen effects between species.

An alternative explanation for differences between species resides in the available number of estrogen receptors (ER) and the ability of the specific phytoestrogen to competitively bind to the ER. The uterine ER concentration is two to four times higher in sheep than cattle (Lundh, 1995).

Phytoestrogens, in a broader sense, refer to chemicals that show effects suggestive of estrogenicity, such as binding to the ER (Kurzer and Xu, 1997). Phytoestrogens must have a chemical structure similar to estradiol in order to bind the ER and cause detectable effects (Adams, 1995a; Cheeke, 1998). Adams (1995a) stressed the importance of demethylization of the phytoestrogen and the position of the two hydroxyl groups to allow binding to the ER. More specifically, the binding characteristics of the uterine ER allow coumestrol and genistein to bind the ER and act as weak estrogens in a way similar to dimethylstilbestrol and estriol (Newsome and Kitts, 1980). Coumestrol, daidzein, genistein, equol and O-desmethylangolensin (O-DMA) have been reported to bind the ER in cytosol preparations of sheep uterus with relative binding affinity of 5, 0.1, 0.9, 0.4, and 0.05% of estradiol, respectively (Kurzer and Xu, 1997). This suggested that phytoestrogens might be able to exert weakly estrogenic effects even in the absence of endogenous estrogens (Kurzer and Xu, 1997). Also, Cheeke (1998) pointed out
that when phytoestrogens act in conjunction with endogenous estrogens, significant biological effects could result. Newsome and Kitts (1980) stated doses greater than biological needs may cause tissues to become refractory to estrogen stimulation.

This research has been represented in the post-pubertal adult as well as the prepubertal female. Prepubertal ewe lambs have been reported to have sensitive and functional uterine ER even before cyclic ovarian activity (Garofalo and Tasende, 1996). This would suggest that the ovariectomized prepubertal ewe would have the available ER to respond to stimulation by exogenous estrogens.

Effects of Phytoestrogens in Ruminants

Sheep:

Field studies in Australia and New Zealand correlated increased phytoestrogen content in legumes to decreased fertility in ewes grazing these legumes (Livingston, 1978; Adams, 1995a). It has been shown that both coumestans and isoflavones can play a role in the estrogenic effects of feedstuffs. Both coumestans and isoflavones are estrogenic in sheep (Adams, 1995b). Research on the infertility associated with “clover disease” in mature ewes dates back to the 1970’s (Adams, 1977, 1995b; Adams et al., 1989). More recently temporary infertility has been reported to occur in prepubertal rats and ewe lambs (Newsome and Kitts, 1980; Nwannanna et al., 1995). “Clover disease” is the most
noted cause of infertility in ewes after long term exposure to phytoestrogens; also reported levels of coumestrol from 20 to 50 ppm (Newsome and Kitts, 1980) may reduce the reproductive efficiency of sheep. Livingston (1978) reported that coumestans have a greater capacity to inhibit estrus in ewes than isoflavones. Cheeke (1998) suggested that temporary infertility is especially pertinent to coumestans.

Infertility attributed to phytoestrogens can be classified into two forms: 1) temporary and 2) permanent.

Temporary:

Although phytoestrogens have relatively weak activity when compared with endogenous estrogens, their high plasma concentrations accounts for their significant estrogenic effects in animals (Livingston, 1978; Lundh et al., 1990). Producers found the most obvious effects to be reduced lambing rates and multiple births, decreased first service conception, irregular estrus, and reduced conception rates (Newsome and Kitts, 1980; Valderrabona et al., 1988; Adams, 1995b). Also, producers reported that their livestock experienced abortions, neonatal mortality, dystocia, and uterine prolapse in their flocks (Vetter, 1995). Classically, "clover disease" has been associated with the observed signs of infertility, however, alfalfa and other legumes may induce similar effects. Several clinical observations are used to identify phytoestrogens in feedstuffs including increased teat size in
wethers and ewes, milk secretion in wethers and nonlactating ewes, and swelling and reddening of the genitalia associated with estrus (Valderrabona et al., 1988; Adams, 1995a; Nwannenna et al., 1995). Adams (1995b) observed mammary gland enlargement and milk or yellow fluid discharge from the teat. Mammary gland enlargement was associated with significant increases in mammary gland weight (Valderrabona et al., 1988). Valderrabona et al. (1988) also commented that mammary tissue appeared more glandular and had little connective and adipose tissue. Adams (1977) reported that a normal alveolar pattern was not established and secretions were apparently derived from ducts and alveolar buds. With the exception of Merino ewes, ewes exhibit vaginal reddening, vulva swelling and increased thickness and keratinization of the vaginal epithelium (Adams, 1977, 1995b). Adam’s (1977) study demonstrated that the basal layer of the stratum germinativum in the vagina became hyperplastic and irregular. Mild inflammations were also widely spread throughout the vagina (Valderrabona et al., 1988). Nwannenna et al. (1995) suggested that reddening of the vulva and other changes were associated with the hyperemia that accompanies hyperplasia and hypertrophy of the reproductive organs under estrogen influence.

Other morphological changes occur in the reproductive tract when sheep consume phytoestrogens. Adams (1995b) observed cervical enlargement and mucous cell accumulation in the luminal epithelium. Adams (1990) suggested that
there was an altered responsiveness to estrogen stimulation resulting in abnormal function of the cervix and vagina. He further commented that estrogen exposure after puberty led to the cervix becoming shorter and broader.

Adams (1977) conducted two short-term studies. In experiment 1, the reproductive tracts were recovered from ewes that had grazed estrogenic pasture for 60 days. Adams (1977) observed that the epithelium of the caudal cervix developed stratified squamous metaplasia, and mucous and goblet cells were not abundant. In experiment 2, reproductive tracts were recovered from ewes grazing estrogenic pasture for 0, 3, 6, 10, 20, and 40 days. From day 6 to 20, large amounts of mucous had accumulated in the cervix and anterior vagina and were accompanied by an increased number of active goblet cells. However, by day 40, results were similar to experiment 1, where fewer goblet cells were observed and mucous decreased. Valderrabona et al. (1988) observed hyperplasia of cervical secretory cells and changes in the chemical composition of cervical secretions, namely the presence of neutral mucopolysaccharides. Valderrabona et al. (1988) suggested that neutral mucopolysaccharides decreased the amount of acidic radicals, thereby decreasing the viscosity of the cervical mucous and lowering sperm migration efficiency in the cervix. A principal function of the cervix is to serve as a sperm reservoir and mucous, with the appropriate viscoelasticity, allows sperm to penetrate and migrate through the cervix (Adams, 1995b). This process is hindered when the cervix is under the influence of phytoestrogens.
Uterine weight increases in a linear fashion with increasing doses of estrogen (Adams, 1977, 1995b; Newsome and Kitts, 1980). Newsome and Kitts (1980) concluded that this was real growth and not just the result of edema. Johnson et al. (1997) suggested that the influence of ovarian steroids on uterine growth in ewes appears to result from effects on cellular hypertrophy rather than cellular hyperplasia. Murray (1992) showed that under estrogen treatment uterine luminal and glandular epithelium become more columnar in shape verses the cuboidal shape of the control. Estradiol-17β administration to long-term ovariectomized ewes induced hypertrophy of the uterine epithelium (Murray, 1992). One may assume this response would be similar between endogenous and exogenous estrogens. Edema can develop in the uterine submucosa and myometrium and cases of subacute endometritis can appear when ewes graze phytoestrogenic pasture (Adams, 1977; Valderrabona et al., 1988). The oviductal ampulla also experiences an increase in epithelial cell height (Adams, 1977).

In addition to uterine histological changes, there may be altered estrogen responsive changes that are essential for proper embryo implantation. Leukemia inhibitory factors (LIF) are essential for embryo implantation and are synthesized in the oviduct (Reinhart et al., 1999). LIF synthesis is reported to be estrogen
regulated in the oviduct and compounds with structural similarities, such as phytoestrogens, may act as endocrine disrupters resulting in possible reproductive failure (Reinhart et al., 1999). Reinhart et al. (1999) reported that LIF is involved in implantation in sheep, cattle, and mice.

Ovaries are accordingly affected by the phytoestrogen content of the plant. Newsome and Kitts (1980) concluded that ovarian weights were smaller in estrogen treated ewes versus control ewes. Adams (1977) observed that ewes grazing estrogenic pasture developed excessive numbers of small and medium sized ovarian follicles, many of which were deficient in antrum formation, and this abnormality was accompanied by early atresia. Cells of the rete ovarii appeared larger and more were sloughed into the lumen.

Ovulation rates for ewes under the influence of phytoestrogens also appear to be reduced. Smith et al. (1979) observed a reduction in the percentage of ewes having multiple births which was caused primarily by a decreased ovulation rate. There appears to be a close correlation between coumestan levels and ovulation rate (Smith et al., 1979). Pasture containing high coumestrol content (1000 ppm) inhibited estrus and ovulation, although moderate coumestrol content (200-400 ppm) only depressed ovulation (Kelly and Lindsay, 1978). Cheeke (1998) stated that inhibition of ovulation might be due to lowered ovarian estrogen secretion.
Newsome and Kitts (1980) stated sheep infertility might be caused by depressed neuroendocrine centers of the brain that control the reproductive cycle. After short-term exposure to phytoestrogens, Adams (1977) reported groups of small, shrunken, hyperchromatic neurons in the dorsal part of the hypothalamus. These neuronal changes may be associated with impaired hypothalamic function and the development of permanent infertility. The degranulation of pituitary δ basophils observed suggested that gonadotropin metabolism was affected in ewes on estrogenic pasture causing an alteration in ovarian/pituitary function (Adams, 1977). In agreement, Smith et al. (1979) suggests that reduced ovulation could be due to a reduction in the supply of gonadotropin, most likely follicle stimulating hormone (FSH), available in the later period of estrus. This impaired function may account for the changes in the ovary and the reduction in ovulation rates and estrus.

*Permanent:*

Prolonged exposure to feeds containing phytoestrogens can cause permanent infertility in the ewe. Producers have noted suppressed flock fertility for repeated years after ewes have grazed estrogenic pastures (Cheeke, 1998).

Signs associated with temporary infertility persist and further morphological changes occur. The vulva undergoes masculinization accompanied by fusion of the ventral labia and fewer layers of stratified squamous epithelium are found in the vaginal mucosa (Adams, 1995b).
In ewes grazing estrogenic pastures for several years, infertility is characterized by cystic glandular hyperplasia of the cervix and uterus (Dickinson et al., 1988). The cervix is altered the most when subjected to long periods of phytoestrogen exposure and irreversible tissue differentiation occurs. The cervical folds become blunter and fuse together leaving behind coiled tubular glands that tend to become cystic (Adams, 1995b). The area of the lamina propria in the endocervix increases and stromal cells accumulate under the epithelium (Adams, 1995b). The luminal epithelium becomes predominately plain columnar cells with fewer recognizable mucous cells, ciliated cells, or stratified squamous cells (Adams, 1995b). The resulting cellular transformation of the cervix leaves the organ resembling the uterus (Adams and Sanders, 1988; Adams, 1995b). These conditions persist and are accompanied by neutral mucopolysaccharides in the cervical secretions. Neutral mucopolysaccharides are associated with reduced viscoelasticity of cervical mucus and reduced sperm transport. Long term exposure to phytoestrogen may stimulate reorganization and redifferentiation of cervical tissue causing the changes in the neutral mucopolysaccharides (Adams and Tang, 1986). Finally, the cervix no longer responds to estrogen stimulation (Newsome and Kitts, 1980; Adams, 1995b).

Uterine histology remains relatively similar to the normal ewe. Uterine horns may coil and become sharply stretched (Adams, 1995b) and because histological changes of the cervix no longer protect the integrity of the uterus, it is
exposed to the hostile environment of the vagina. As a result, mild endometritis is common and the uterine glands may become cystic thereby hindering sperm transport and contributing to the major cause of infertility.

These morphological changes occur as part of a general sexual transdifferentiation in affected ewes, and are accompanied by slight defeminization and masculinization of sexual behavior and an impairment of the sex-dependent mechanism controlling the preovulatory surge of LH (Adams and Sanders, 1988). In most mammalian species, the genes for estrogen-directed morphogenesis are switched off after the completion of organogenesis (Adams and Sanders, 1988). However, in the ewe they may be reactivated to a slight extent during adult life by plant estrogens, thereby producing permanent infertility (Adams and Sanders, 1988).

**Cattle:**

Red clover and alfalfa both have been reported to cause infertility in cattle. Livingston (1978) stated that as early as the 1960’s hyperestrogenic syndrome was reported in dairy cattle herds in Israel as a result of an intake of phytoestrogens. The typical signs of estrogenism included mammary gland development, swollen vulvas, discharge of cervical mucus, and uterine growth (Adams, 1995a; Shore et al., 1998). Decreased infertility may be a result of cystic ovaries, persistent
nymphomania, irregular estrus, and even anestrus (Adams, 1995a; Shore et al., 1998). Shore et al. (1998) reported that high estrogen concentrations on the day of insemination are associated with increased early embryonic loss in cattle. Collectively, reproductive efficiency from the standpoint of conception is reduced. It appears that ovarian function returns to normal several weeks after removal from estrogenic feeds (Adams, 1995a) and no permanent infertility has been reported in cattle (Lundh, 1995).

Livingston (1978) reviewed the research of Adler and Trainin which suggested that hyperestrogenic syndrome in dairy cattle was caused by consumption of alfalfa. Their research suggested checking for cystic ovaries, irregular estrous cycles, and noting any precocious mammary and genital development in heifers.

Rats and Mice

Coumestrol induced morphological changes in neonatally treated mice including persistent vaginal cornification, hemorrhagic ovarian follicles, and premature vaginal openings (Burroughs, 1995; Medlock et al., 1995). Sustained exposure to phytoestrogens during the neonatal period caused persistent vaginal cornification, cervico-vaginal pegs and downgrowths, and uterine squamous metaplasia in rats (Markaverich et al., 1995). In immature, ovariectomized rats, coumestrol stimulated cellular hypertrophy and perhaps protein synthesis.
Coumestrol induced increased uterine wet and dry weight in ovariectomized rats reflecting increased water and protein content (Markaverich et al., 1995). Coumestrol administered on postnatal day 1-5 caused increased uterine weight, increased uterine luminal epithelial cell height, and caused premature uterine gland development in intact rats (Medlock et al., 1995; Kurzer and Xu, 1997). Estradiol caused sustained uterine wet weight beyond 24 hours after injection and was associated with the stimulation of cellular DNA synthesis and true uterine growth (Markaverich et al., 1995). Estrogenic response and uterine growth induced by coumestrol or equol may involve binding to the ER, increasing the ER binding capacity of the uterus by causing ER activation or phosphorylation or by enhancing ovarian release of estrogen (Markaverich et al., 1995).

**Measurements of Estrogenic feeds:**

Obviously, phytoestrogens interfere with normal reproduction in ruminants (Wang et al., 1994). Number of services per conception may be a method to track reproductive efficiency. Still, this reproductive failure cannot be pin-pointed to delayed conception because many factors affect the reproductive efficiency of sheep and cattle. However, researchers have suggested that the estrogenic content of a pasture can be assessed by increased teat length and secretion of fluid in wethers (Oldfield et al., 1966; Livingston, 1978; Nwannenna et al., 1995). In the
case of subterranean clover, teat length of wethers is directly correlated to the concentration of formononetin in the pasture (Cheeke, 1998). The increase in length of teats on wether lambs can serve as a measure of plant estrogen potency (Oldfield et al., 1966). This test is most accurate on low estrogenic pastures. Teat length returned to normal approximately 25 days after removal from the pasture (Nwannenna et al., 1995). Adams (1977) stated that vaginal cornification in ewes may accompany these outward signs.

An increase in uterine weight is a predictor of estrogenic activity (Cheeke, 1998). The relationship between uterine weight responses and temporary infertility appears to be slightly better than that between teat length and infertility (Nwannenna et al., 1995). Initial analyses of forage plant estrogens were by means of the mouse uterine weight bioassay (Livingston, 1978). After further research, sheep were considered to be more sensitive as animals for plant estrogen bioassays than laboratory animals (Livingston, 1978). Bioassays used for phytoestrogens are summarized in table 1 (Adams, 1995a).

Nwannenna et al. (1995) demonstrated that differences in uterine diameters were distinguishable in a noninvasive manner with ultrasonography. The method showed no significant difference in preliminary studies, but with further utilization ultrasonography might prove to be a valuable source for identifying uterine growth. Livingston (1978) reported that nucleic acid content in uterine tissue was a more sensitive measurement than uterine weight. Histological examination of affected
ewes proved to show more tubular glands and increased epithelial cell height in uterine glands and lumen. The infertility syndrome in ewes grazing estrogenic pastures for several years is characterized by cystic glandular hyperplasia in the cervix and uterus (Dickinson et al., 1988). Measurement of the uterus-like histological changes in the cervix are the most specific diagnostic test for sheep (Adams, 1995a). The simplest criterion for examining ewes is the reduction in the number of cervical crypts reflecting a reduced number of cervical folds and an increase in lamina propria (Adams, 1977). A test important for measuring

Table 1. Comparison of bioassays for phytoestrogens

<table>
<thead>
<tr>
<th>Bioassay</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teat length of wethers</td>
<td>Sensitive, simple</td>
<td>Imprecise, accurate only for lowly estrogenic pasture</td>
</tr>
<tr>
<td>Uterine weight of ovex. ewes</td>
<td>Accurate, well validated</td>
<td>Expensive; RNA/DNA ratio in uterine biopsy may reduce cost</td>
</tr>
<tr>
<td>Weight of cervical mucus</td>
<td>Rapid</td>
<td>Inaccurate after 2 days of exposure, due to cervical refractoriness</td>
</tr>
<tr>
<td>Uterine weight of lab animals</td>
<td>Accessible</td>
<td>Inaccurate guide for ruminants, due to differences in metabolism</td>
</tr>
</tbody>
</table>

From Adams (1995a)
permanent infertility is the spinnbarkeit test. The spinnbarkeit, which is the length to which a strand of mucus can be drawn out before it breaks, is a measure of the viscoelasticity of the cervical mucus (Adams, 1995b). Alterations in cervical mucus impede sperm transport through the cervix (Livingston, 1978) and ultimately hinder the arrival of sperm to the site of fertilization. A reduced spinnbarkeit is associated with a cervical malfunction and is common in permanent infertility. As stated above, changes that accompany the reduced spinnbarkeit are changes in cervical histology that resembles uterine morphology (Adams and Sanders, 1988). Table 2 outlines Adams (1995a) suggested diagnostic tests for permanent infertility.

Table 2 - Comparison of diagnostic tests for subclinical permanent infertility in ewes.

<table>
<thead>
<tr>
<th>Test</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical histology</td>
<td>Accurate, specific</td>
<td>Labor-intensive</td>
</tr>
<tr>
<td>Macroscopic cysts in endometrium</td>
<td>Simple</td>
<td>Imprecise, not specific, need abattoir material</td>
</tr>
<tr>
<td>Cervical mucus spinnbarkeit</td>
<td>May test living animals</td>
<td>Varies with season, need Accurate detection of estrus</td>
</tr>
<tr>
<td>Masculinization of vulva</td>
<td>Cheap and rapid</td>
<td>Not specific or quantitative</td>
</tr>
</tbody>
</table>

From Adams (1995a)
Chemical Analysis

Chemical analysis of feeds for estrogenic content has become popular, especially to prevent harmful reproductive failure. Thin-layer chromatography, gas chromatography-mass spectrometry, and high-pressure liquid chromatography (HPLC) have all been used to measure phytoestrogen content (Wang et al., 1994). HPLC is commonly used to measure concentrations of coumestans and isoflavones in forage, as well as, animal tissue, and both animal and human urine (Adams, 1995a). Phytoestrogen content of rumen fluid, urine and plasma can be determined by radioimmunoassay (Wang et al., 1994).

Variation in phytoestrogen content of plant:

Accumulation of phytoestrogens in legumes varies depending on season, environmental factors, amount and variety of fertilizer, and stresses involved with growth. Oldfield et al. (1966) stated that the potency of these "plant estrogens" has been shown to vary with species of plant, time of cutting, location, fungal infestation and other factors. Table 3 outlines many of the common estrogenic feeds studied in livestock research.
Table 3. Estrogenic Legumes and their associated Phytoestrogen.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Scientific name</th>
<th>Major Phytoestrogen</th>
<th>Reason for accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa</td>
<td><em>Medicago sativa</em></td>
<td>Coumestrol, some formononetin</td>
<td>Foliar disease</td>
</tr>
<tr>
<td>Annual Medic</td>
<td><em>Medicago spp.</em></td>
<td>Coumestrol</td>
<td>Fungal disease</td>
</tr>
<tr>
<td>White Clover</td>
<td><em>Trifolium repens</em></td>
<td>Coumestrol</td>
<td>Foliar disease</td>
</tr>
<tr>
<td>Subterranean Clover</td>
<td><em>Trifolium subterraneum</em></td>
<td>Genistein, formononetin, and biochanin A</td>
<td>Depends on genotype</td>
</tr>
<tr>
<td>Red Clover</td>
<td><em>Trifolium pratense</em></td>
<td>Mostly formononetin</td>
<td>Depends on genotype</td>
</tr>
<tr>
<td>Soybean</td>
<td><em>Glycine sp.</em></td>
<td>Genistein, daidzein, coumestrol</td>
<td></td>
</tr>
</tbody>
</table>

From Adams (1995a)

*Medicago sativa*

Alfalfa's resistance to plant pathogens is affected by factors including variety, fungal infection, season (time of harvest), irrigation and age of plant (Smith et al., 1979; Adams, 1995a; Shore et al., 1995). In alfalfa, spontaneous growth is normally richer in coumestrol than cultivated ones, and perennial species
accumulate more coumestrol than annual ones (Galvano et al., 1993). Irrigation with sewage water containing steroid estrogens, primarily estrone, caused increased coumestrol content in legumes (Shore et al., 1995).

Medicago sativa: Disease

Hawk et al. (1967) suggested that certain foliar diseases of Medicago sativa (alfalfa) may be responsible for the estrogenic effect of alfalfa on the reproductive tract of ewes. Spring black stem induced by Phoma medicaginis is prevalent in alfalfa and is characterized by foliar and stem lesions (Rhodes and Myers, 1986). It has been shown that coumestrol accumulates to significant levels in alfalfa infected with foliar diseases, and that its concentration is directly correlated with the degree of infection (Smith et al., 1979; Adams, 1995a; Saloniemi et al., 1995). Although, this was a common thought among most researchers in this field, Saloniemi et al. (1995) stated there was no indication of disease in their study. Livingston (1978) commented that the degree of infection is higher in older, established plants than new cultivated ones.

Stress associated with attack by aphids or fungal pathogens increases coumestrol concentration (Adams, 1995a). The injured or stressed plant may develop lesions caused by common leafspot organisms (Bickoff et al., 1967). The increase of coumestans have been shown to be correlated with injured and or diseased plants and many fungal, bacteria, and viral diseases cause the formation of
aromatic compounds, such as coumestan derivatives, in plant tissue (Bickoff et al., 1967). Bickoff et al. (1967) stated that coumestrol accumulates in the leaf around the immediate area of the lesion and concentration is highest in the leaves of the plant compared to the stalk (Smith et al. 1979; Galvano et al., 1993). Livingston (1978) commented that the incidence of foliar disease increased in older plants.

*Medicago sativa: Season (Time of harvest)*

Livingston (1978) reported that estrogenic activity in the spring coincides with high concentrations in forage legumes. Estrogenic activity of alfalfa was low during early spring (vegetative stage) and increased to maximum levels at full bloom or seed head stage (Livingston, 1978). In spring-autumn legume species (e.g.: alfalfa), the final cutting tends to become higher in coumestrol than that from initial cuttings (Livingston, 1978; Galvano et al., 1993). Winter growth prior to blossom also accumulated high estrogens (Livingston, 1978). Saloniemi et al. (1995) reported that the coumestrol concentration was remarkably higher in the spring (early blossom) and fall (second aftermath). Kitts et al. (1959) suggested, in the case of alfalfa, the time at which first cutting was made appeared to influence the estrogenic activity of subsequent cuttings. For example, when the first cutting was made in May and was followed by cuttings in June, July, and August, the activity tended to closely follow that of plants allowed growth without
interference (Kitts et al., 1959). On the other hand, when the first cutting is made in June, July, and August subsequent cuttings possess little or no activity (Kitts et al., 1959). Alfalfa harvested as high moisture silage seems to be more estrogenic than fresh cut or low moisture silage (Livingston, 1978).

*Trifolium*

*Trifolium repens* (white clover) is most commonly infected with coumestrol and the concentration increases in close relationship to foliar disease (Adams, 1995a). Formononetin may also be present in the plant, but accumulation of coumestrol is the highest. Cold autumn weather and associated night frosts may increase coumestrol concentrations in white clover (Saloniemi et al., 1995).

*Trifolium subterraneum* (subterranean clover) can accumulate up to 5% dry weight of isoflavones, such as formononetin, genistein, and biochanin A (Adams, 1995a). Formononetin causes the greatest impact in ruminants. Deficiency in phosphates, sulphate or nitrogen fertilizer can result in less accumulation of formononetin (Adams, 1995a). Livingston (1978) reported that sulfur deficiency increased concentrations of estrogenic isoflavones in subterranean clover and severe sulfur deficiency resulted in nearly two times the concentration of total isoflavones. There appears to be an exchange between deficiencies of phosphate,
sulfate or nitrogen fertilizers (which decrease isoflavone concentration) and plant yields (Adams, 1995a). Adams (1995a) observed that isoflavones were present only in green clover and rapidly dried hay retained isoflavone concentrations.

*Trifolium pratense* (Red clover) is affected by environmental factors causing it to be more estrogenic in the spring and less estrogenic after flowering (Adams, 1995a). Saloniemi et al. (1995), in a Finnish study, reported that isoflavones accumulate during rapid spring growth and during fall aftermath. Cool weather increases the amount of phytoestrogens (Saloniemi et al., 1995) and fertilizer deficiency causes an increase in formonononetin and decreased plant yield (Adams, 1995a). In comparative studies, red clover cultivated in phosphorus poor soils tended to be higher in formonononetin concentration than soils fertilized with phosphorus and increased nitrogen fertilizer decreased phytoestrogen content (Saloniemi et al., 1995).

Glycine species, mainly soybeans, have shown estrogenic effects in swine, lab animals, and humans (Adams, 1995a). Although monogastrics are affected, ruminants show no symptoms (Adams, 1995a). Soybeans accumulate genistein, daidzein, glycetin and coumestrol. Soybeans have been studied extensively for their potential benefits in hormonal therapy for postmenopausal women, cancer treatment, reducing the risk of heart disease and alleviating osteoporosis (Kurzer and Xu, 1997; Cheeke, 1998).
Materials and Methods

Plant phytoestrogenic content

Samples of alfalfa, orchardgrass, and bentgrass were collected from across Oregon and tested for phytoestrogen content with HPLC. Alfalfa proved to be the plant source chosen as the phytoestrogen treatment and bentgrass straw was the only feed that tested nonestrogenic. Cottonseed meal, a nonestrogenic feed source was used to supplement the protein quality of the bentgrass straw. Feeds were chopped and random batch samples were extracted from each feed. HPLC was utilized to determine the phytoestrogen content of each feed. Feed samples were analyzed by Murphy, Iowa State University, using HPLC (Murphy et al., 1997, 1999).

Experimental Animals: Pretrial Period

Twenty-four crossbred prepubertal ewe lambs were selected from the spring lamb crop. Ewes were weighed and grouped according to weight and age. Lambs for the study were born in late March and weighed approximately 41 kg. Twins were removed from the group. Ewe lambs were grazed on pasture until November; thereafter they were confined to a dry lot and fed native grass hay until late November.
During the last week of November, eighteen ewe lambs were ovariectomized through a mid-ventral laparotomy under general anesthesia. Ewe lambs were fasted 30 hours prior to the ovariectomy procedure to ensure the efficiency and ease of the surgery because of the diminished volume of the viscera. Anesthesia was induced with a 15 ml injection of five-percent sodium pentothal (Abbott Laboratories, North Chicago, IL) into the jugular vein. During the surgery, the lambs were subjected to a halothane (Halocarbon Laboratories, River Edge, NJ) -oxygen gas mixture through inhalation to maintain anesthesia. The lambs were laid dorsal side down on an elevated surgery table, which allowed the viscera to fall forward. After the operating area was clipped and disinfected, a three-inch incision was made midventral and anterior to the mammary glands. The incision passed through the fascia and linea alba, peritoneum was penetrated with blunt dissection, and the peritoneum was widened to allow the reproductive organs to be exteriorized. Ovaries were exteriorized, hemostats were used to clamp off the ovarian arteries where they enter the hilus and the ovaries were removed. Ovaries were examined for presence of corpora lutea and follicles. Uterine body and horns were returned through the peritoneum, the peritoneum was closed with number two catgut, and the connective tissue was sutured with number two–O catgut. Finally, the exterior skin layer was closed with vetafil.
Post surgery, ewe lambs were administered 10 ml intramuscular injection of both penicillin and banamine to combat infection and pain, respectively. They were placed in a recovery area and monitored every 4 hours for approximately 24 hours. Ewe lambs displaying edema and seepage received a second 10 ml intramuscular injection of penicillin.

Following surgery, lambs were allowed ad libitum access to native grass hay until late April. In March, jugular vein samples from each ovariectomized ewe were collected into 10-ml heparinized vacutainer vials. Plasma was separated from each sample and placed into a separate vial. Samples were sent to Oregon Health Sciences University for radioimmunoassay (RIA) of progesterone and estrogen concentrations (Hess et al., 1981). Analysis was used to ensure the surgeries had successfully removed all ovarian tissue. In May, lambs were fed nonestrogenic bentgrass straw and cottonseed meal for 25 days. On day 26, lambs were weighed and assigned randomly to one of three treatment groups. Lambs were placed in individual feeding crates and allowed ad libitum access to bentgrass straw and cottonseed meal for a 4-day acclimation period. Prior to feed trial diets were balanced isonitrogenously using the protein content of alfalfa as the base for calculation.
Experimental Animals: Feed Trial Period

On Day 0 of a 12-day feed trial, ewes were assigned randomly to one of three treatment groups (n=6): Estradiol, Control, and Alfalfa. Estradiol treated ewes received ad libitum access to bentgrass straw and cottonseed meal and a daily injection of 10 mg of estradiol-17β (Estradiol-17β; Sioux Biochemical, Inc., Sioux Center, IA) suspended in corn oil. Control ewes received ad libitum access to bentgrass straw and cottonseed meal and received a daily injection of corn oil vehicle. Alfalfa treated ewes were fed first cutting alfalfa ad libitum and received a daily injection of corn oil vehicle. Diets were designed to supply similar levels of protein and energy. Lambs were fed at 0700 and a second feeding at 1700. Orts were collected at the morning feeding and fresh diet was fed including any increases for ewe fed below ad libitum in previous feedings. Lambs were allowed free access to salt and water, and were weighed on Days 0, 8, and 13.

Clinical examination:

Clinical examinations were carried out throughout the experimental feeding period to monitor changes in external genitalia and mammary gland development. Three unbiased and independent examiners scored vulva and teat morphology on Days 0, 1, 3, 5, 7, 9, and 12 for each ewe. Ewes were presented in a newly randomized order for each day of observations. Arbitrary scores were assigned to changes in the external genitalia (Appendix 1) and mammary glands (Appendix 2).
Vulva scores ranged from 1 (no change in size or color) to 4 (swollen and bright red). Teat scores were designed for both teat length and circumference; hence, the range of scores was between 1 (no change) to 4 (significant increase in both). Examiners noted any secretions from the vulva and/or teat and any unusual behavior.

Teat length and circumference was measured on Days 1, 7, and 12. Fabric tape calibrated in mm was used to measure length and circumference of both the left and right teat. Length was measured from the base to apical tip of the teat. Circumference was measured by circling the tape around the base of the teat, and the circumference was recorded. There was no space between the teat and the tape as well as no undue pressure on the teat.

All animal procedures were approved by Oregon State University Animal Care and Use Committee.

Tissue Fixation and Preparation:

On Day 13 of the experimental feeding period, ewes were humanely slaughtered and the uteri were removed and weighed. A 25 mm cross-section was collected from the curvature of each uterine horn for histological preparation. All samples were rinsed with physiological saline and fixed in Lillie’s Neutral Buffered
Formalin. Each cross-section was fixed, processed and embedded in paraffin wax, sectioned at 4-5 μm, and stained with hematoxylin and eosin by the Veterinary Diagnostic Laboratory (Oregon State University, College of Veterinary Medicine, Appendix 3).

Slides for each uterine horn were prepared; each consisting of three independent sections of tissue per slide. Cell heights of the uterine luminal epithelial were examined under light microscopy and measured with an ocular micrometer as described by Murray (1992). Luminal cell height was measured in a block of 10 cells at three random coordinates in the plane of a cross-section so that 30 cells were collectively measured in each cross-section. Three cross-sections were examined per uterine horn resulting in a total of 90 individual measurement per horn and 180 per ewe.

Statistical analysis:
The statistical software program NCSS 2000 (Number Crunch Statistical System; J. L. Hintze; Kaysville, UT) was used to analyze all data. MANOVA was used to test for overall significance for both clinical scores and measurements and uterine morphology. After a treatment was significant with MANOVA, individual explanatory variables were analyzed with ANOVA. Clinical scores and measurements were analyzed with ANOVA for repeated measures. Duncan’s Multiple-Comparison Test was used to analyze differences between means if the
probability level for the calculated F-ratio was \((p < 0.05)\). The full model was utilized in all repeated measures analysis. Uterine weight was analyzed with one way ANOVA to test for significance of treatment. Uterine weights were calculated as a percentage of live body weight at the time of slaughter. Uterine luminal epithelial cell heights were analyzed with ANOVA using Duncan’s Multiple-Comparison Test to determine significant differences among means. A backward elimination technique was used to reduce the full model. When an explanatory variable was found not significant, the data were pooled and reanalyzed until the model only contained the most significant information.
Results

It has been previously reported that legume species, in particular alfalfa, may accumulate phytoestrogens in association with fungal disease (Bickoff et al., 1967; Smith et al., 1979; Adams, 1995a; Saloniemi et al., 1995). Grass species, such as timothy (Lundh et al., 1990; Nwannenna et al., 1995), orchardgrass (Kitts et al., 1959), and ryegrass (Braden et al., 1971; Valderrabona et al., 1988), were reported to be nonestrogenic. In search of feed for this feed trial, alfalfa with phytoestrogen content was the easiest to locate. Alfalfa, which had suffered stress of freezing and was infected with spring black stem, was purchased from a producer in Oregon. Rhodes and Myer (1986) reported that spring black stem was accompanied by foliar and stem lesions, which may allow accumulation of phytoestrogens. Alfalfa tested with approximately 138 ppm coumestrol and traces of formononetin.

Nonestrogenic feed for the control and estradiol treatments was the most challenging to locate. Orchardgrass was the initial feed selected as the control diet. After testing 4 samples that contained from 39 to 156 ppm coumestrol (Table 4), it was decided to select a nonestrogenic bentgrass straw and cottonseed meal as a
Table 4. Phytoestrogen content of feeds sampled for feed trial.

<table>
<thead>
<tr>
<th>Feed Sample</th>
<th>Coumestrol content (ppm)</th>
<th>Formononetin content (ppm)</th>
<th>Biochanin A content (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Orchardgrass 2</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Orchardgrass 3</td>
<td>38.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Orchardgrass 4</td>
<td>156</td>
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<td>0</td>
</tr>
<tr>
<td>Bentgrass straw 1</td>
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<td>0</td>
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<tr>
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<td>0</td>
</tr>
<tr>
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<td>0</td>
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<tr>
<td>Alfalfa 2</td>
<td>112</td>
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<td>0</td>
</tr>
<tr>
<td>Alfalfa 3</td>
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</tr>
<tr>
<td>Alfalfa 4</td>
<td>62</td>
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<td>0</td>
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<tr>
<td>Alfalfa 5</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Alfalfa 6</td>
<td>66.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Alfalfa 7</td>
<td>124</td>
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<td>0</td>
</tr>
<tr>
<td>Alfalfa 8</td>
<td>62.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Alfalfa 9</td>
<td>137.5</td>
<td>72</td>
<td>0</td>
</tr>
<tr>
<td>Cottonseed meal</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

control diet. These findings for orchardgrass contradict previous research (Kitts et al., 1959). The difference may be caused by undetected amounts of alfalfa or clover in our sample, regardless; the assumption that these grasses are nonestrogenic needs further investigation.
Mean vulva score was different (p<0.05) among treatments and over time. The mean vulva score of 1.61 for estradiol treated ewes was greater (p<0.05) than mean vulva scores of 1.16 for control ewes. Alfalfa fed ewes (Figure 5) exhibited a mean vulva score of 1.45, which trended to be greater than that of control ewes, but scores were not different (p>0.05).

A significant (p<0.005) day effect indicates that mean vulva scores increased over time with day 12 scores greater (p<0.05) than initial day (Figure 4). There was a significant interaction (p<0.005) between treatment and day of observation (Figure 5). Estradiol treated ewes showed an increase (p<0.05) in mean vulva score by day 1. The increase remained different (p<0.05) from initial scores with the exception of day 5. Mean vulva scores reached a maximum (p<0.05) score on day 3 (Figure 6), but the decrease (p<0.05) from day 3 to day 5 (Figure 6) was difficult to explain. The mean vulva scores tended to be higher after day 5 and were different (p<0.05) than initial scores by day 12. Alfalfa fed ewes showed an increase in mean vulva score by day 3 (Figure 7), remained higher than initial day, and attained a maximum score by day 9. Mean vulva scores for control ewes trended to be lower over time, but not different (p>0.05) from initial scores (Figure 8).
Figure 4. Mean vulva score of all treated ewes on Days 0-12.
   a, b Days bearing different letters are different (p<0.05) from each other.

Figure 5. Mean vulva score of control and treated ewes over days of observation.
   a, b Mean vulva score bearing different letters are different (p<0.05) from each other on Day 3 and 12.
Figure 6. Mean vulva scores of estradiol treated ewes. Day 3 score of 1.83 in the photo above and a Day 5 score of 1.33 in the photo to the right.
Figure 7. Mean vulva scores of alfalfa fed ewes. Day 3 score of 1.33 in the photo above and a Day 5 score of 1.5 in the photo to the right.
Figure 8. Mean vulva scores of control ewes.
Day 3 score of 1.0 in the photo above and a Day 5 score of 1.0 in the photo to the right.
There was an observer effect shown (p<0.05) for mean vulva scores. Independent observer 1 (IO1) recorded lower (p<0.05) mean vulva scores than either independent observer 2 (IO2) or independent observer 3 (IO3). A significant day by observer interaction (p<0.05) was shown (Figure 9). IO1 consistently scored lower than IO2 and IO3. IO1 apparently did not observe any major changes from the initial day when compared to day 12. Mean vulva scores for IO2 and IO3 show a trend to increase over time indicating they may have been more sensitive to the slightest changes in vulva color or size. Many factors could explain this interaction. Observers may have had some preconceived idea that scores needed to increase over time, or they may have had previous knowledge of the predicted outcome that could have prejudiced their scores.

Mean teat scores were not different (p>0.05) among treatments. There was a significant (p<0.005) day effect (Figure 10). By day 7 the overall mean teat score had peaked and was higher (p<0.05) than previous days. The overall mean teat score decreased the two remaining days but remained relatively high (Figure 10).

A significant (p<0.05) observer effect (Figure 11) and a day by observer interaction (p<0.05) were noted. IO3 recorded a higher (p<0.05) overall mean teat score of 1.10 than the mean teat score of 1.01 and 1 for IO2 and IO1, respectively (Figure 11). This effect was noted in the analysis as significant, but the practical significance of nine hundredth of a score may not be convincing. It was interesting to note that IO1 scored consistently as indicated by the absence of standard error.
Figure 9. Mean vulva scores of control and treated ewes scored over days by three independent blinded observers. a,b Observer bearing different letters are different (p<0.05) from each other.

Figure 10. Mean teat score of all treated ewes on Days 0-12. a,b Days bearing letters are different (p<0.05) from each other.
Figure 11. Observer effect on mean teat score for all treated ewes on all days of observation.

Observer bearing different letters are different (p<0.05) from each other.

Figure 12. Mean teat scores of control and treated ewes scored over days by three independent blinded observers.

Observer bearing different letters are different (p<0.05) from each other on Days 7, 9, and 12.
bars. The resulting interaction (Figure 12) was hypothetically a product of an observer seeing an increase due to differences in daylight, observer mood, or other factors distracting the observer during the evaluation. The overall increase was probably an artifact and was independent of treatment.

Mean teat circumference (measured in cm) was not different (p>0.05) for treatments. Yet, there was a trend for mean teat circumference to increase (p<0.05) from day 1 to day 7, and again increased (p<0.05) to a high at day 12 (Figure 13). Mean teat circumference tended to be higher but not different (>0.05) for both estradiol treated and alfalfa fed ewes in comparison to control ewes.

There was a significant (p<0.05) day by treatment interaction for mean teat circumference (Figure 14). Estradiol treated ewes increased (p<0.05) in mean teat circumference by day 7 with no further increase (p>0.05). Alfalfa fed ewes increased (p<0.05) in teat circumference by day 7 and had an additional increase (p<0.05) by day 12. Control ewe's mean teat circumference decreased (p<0.05) by day 7 and continued to measure lower than day 1 upon final measurement. This decrease may be mostly due to measurement error.

There was no difference (p>0.05) in mean teat length (measured in cm) due to treatment. There was a clear day effect (p<0.05); (Figure 15) and an interaction (p<0.05) between day and treatment (Figure 16). There was a trend for overall mean teat length to increase (p<0.05) from day 1 to day 7 and again increased (p<0.05) by day 12 (Figure 15). Estradiol treated ewes and alfalfa fed ewes both
Figure 13. Mean teat circumference (cm) of all treated ewes on Days 1, 7, and 12. 

a,b,c Days bearing different letters are different (p<0.05) from each other.

Figure 14. Mean teat circumference (cm) of control and treated ewes scored over days by three independent blinded observers.
Figure 15. Mean teat length (cm) of all treated ewes on Days 1, 7, and 12. Days bearing different letters are different (p<0.05) from each other.

Figure 16. Mean teat length (cm) of control and treated ewes scored over days by three independent blinded observers.
followed a steady increase in mean teat length. Both treatment groups showed longer (p<0.05) mean teat length on day 7 and 12 than day 1. Furthermore, mean teat length significantly increased (p<0.05) from day 7 to day 12 in both groups. On day 12, control ewes measured a significant increase (p<0.05) in comparison to day 1. There was a strong correlation between teat length and teat circumference (r = 0.71). As a result there was an identical increasing trend observed between circumference and length of teat.

Two ewes were removed from further analysis at this point. An estradiol treated ewe showed a hydrosalphinx at the time of slaughter, which increased her uterine weight substantially and under light microscopy showed a larger than average luminal epithelial cell height for one uterine horn. A control ewe was removed because approximately one-third of her uterus could not be recovered and only the uterine body could be fixed for microscopic analysis.

A one-way ANOVA test showed that treatment (Figure 17) had a significant effect (p<0.0005) on mean uterine weights (expressed in g uterus/kg body weight). Estradiol treated ewes recorded an average mean uterine weight of 0.67 g/kg, alfalfa fed ewes mean uterine weigh was 0.35 g/kg, and control ewes had a mean uterine weight of 0.19 g/kg. Estradiol treated ewes achieved heavier (p<0.05) mean uterine weights than alfalfa fed ewes and also were heavier (p<0.05) than control ewes. As well, alfalfa fed ewes mean uterine weights were heavier (p<0.05) than control ewe mean uterine weights.
Figure 17. Mean uterine weight expressed as a percentage of live body weight of ovariectomized prepubertal ewe lambs.

a,b,c Means bearing different letters are different (p<0.05) from each other.

ANOVA was applied to analyze the effect treatment had on uterine horn luminal epithelial cell height. Using backward elimination, individual cell heights within a field of measurement were found not different (p>0.05) so the mean cell height was calculated for the field. A second ANOVA revealed that the individual
field of measurement was not different (p>0.05), so mean cell height for each
cross-section was calculated. The last ANOVA found no difference (p>0.05)
between uterine horns, so the final model was reduced to include treatment as the
main effect and overall mean uterine horn epithelial cell height as the response.

The reduced model showed differences (p<0.005) among treatments for
mean uterine horn luminal epithelial cell heights. After exposure to alfalfa and
estradiol treatments, mean uterine horn luminal epithelial cell height increased
(p<0.05) and measured approximately 13.67 \( \mu \text{m} \) and 23.18 \( \mu \text{m} \), respectively
(Figure 18). Estradiol treated ewes exhibited taller (p<0.05) epithelial cell heights
than alfalfa fed ewes or control ewes. Alfalfa fed ewes were different (p<0.05)
from estradiol treated ewes and control ewes. Uterine horn luminal epithelial cell
height was highly correlated to mean uterine weights (r = .88) resulting in very
similar trends in the results.

Anatomically, the treatment caused differences among control ewes and
estradiol treated and alfalfa fed ewes. Luminal epithelial cells appeared to be more
columnar in estradiol treated and alfalfa fed ewes versus the cuboidal shape of
ovariectomized controls (Figure 19). Uterine glands appeared to increase in
number and in diameter.
Figure 18. Mean uterine horn lumen epithelial cell height of ovariectomized prepubertal ewe lambs.

Treatments bearing different letters are different (p<0.05) from each other.
Figure 19. Uterine horn luminal epithelial cell height. H & E X 1000. ----------- equals approximately 40 μm.
Discussion

Daily injection of estradiol-17β and daily ingestion of alfalfa containing approximately 138 ppm coumestrol and 72 ppm formononetin stimulated morphological changes associated with increased mean vulva scores. Estradiol treated ewes had an average score of 1.60 and were higher (p<0.05) than mean vulva score for control ewes with an average score of 1.16. Mean vulva score for alfalfa fed ewes was 1.45, which was higher than that of control ewes, but not significantly so. These trends for treated ewes to be higher than control ewes should be in conjunction with a score that described slight changes in the size and the color change from pale to reddish or pink in the scoring system. Oldfield et al. (1966) reported similar results with their arbitrary scoring system. They observed a score of 2.04 for high estrogen treatment (99 ppm coumestrol) versus a score of 1.07 for low estrogen treatment (35 ppm coumestrol) for a 10 week treatment; they noticed differences as early as 3 weeks in the trial (Oldfield et al., 1966). Although our scores did not coincide, the difference may be due to a cumulative effect of coumestrol in the extended time period in the study conducted by Oldfield et al. (1966).
It has been previously reported that coumestans and isoflavones may induce morphological changes similar to endogenous estrogen, such as estradiol-17β, which causes swelling and reddening of the vulva (Adams, 1995b). After treatment with estradiol-17β or alfalfa containing coumestrol, the associated morphological change of the vulva to change color and swell may be induced by the increased blood flow that accompanies hyperplastic and hypertrophic enlargement of the reproductive organs (Nwannenna et al., 1995).

In comment to the 72 ppm formononetin in the alfalfa treatment, Nwannenna et al., (1995) indicated that ovariectomized ewes exposed to red clover containing formononetin showed differences in vulva scores by day 4 when compared to initial scores. In the same study, estradiol-17β implants caused increased vulva scores by day 1 of treatment. Our research and previous studies only validate that an arbitrary scoring system that rates swelling of and the associated color changes of the vulva induced by estrogenic feed or estradiol-17β in ovariectomized ewes may be used to detect phytoestrogenic activity of feeds.

Previous research shows that increased teat length in nonpregnant ewes and wethers is a sensitive bioassay of phytoestrogen activity in legume feeds (Oldfield et al., 1966; Livingston, 1978; Adams, 1995a). Teat length of wethers is directly correlated to the concentration of formononetin in the pasture (Cheeke, 1998). The study is in disagreement with previous research. All parameters measuring morphological teat changes were not affected by treatment. Although, figures
4,7,8, 9, and 10 show that scores and measurements do show interaction over time. Treatment over time caused estradiol treated ewes and alfalfafed ewes to increase (p<0.05) in all parameters from the initial day to day 12. The discrepancy between results of this study and previous studies may be due to the sensitivity of observer to detect noticeable effects or measurements of morphological teat change may not have been precise enough to detect a difference in such a small sample size.

Estradiol and alfalfa treatment stimulated uterine growth when compared to a control diet. Previous research reported that the additional increases in uterine weights are due to real growth, not just accumulation of water (Newsome and Kitts, 1980; Reynolds et al., 1998). Mean uterine weight of 0.67 g/kg of body weight for estradiol treated ewes in response to a daily injection of 10 mg estradiol-17β is similar to the weight of approximately 0.6 g/kg of body weight reported by Newsome and Kitts (1980) to be a maximum response. Alfalfa fed ewes mean uterine weight and control ewes mean uterine weight are lower than reported by Newsome and Kitts (1980). It is reported in the same study that 2.5 mg per day is close to physiological dose and that coumestrol levels of 132 ppm induces a similar response in intact ewes (Newsome and Kitts, 1980). Coumestrol level of 138 ppm in this study stimulated a mean uterine weight of 0.35 g/kg of body weight. One may postulate that this level of coumestrol content may be enough to stimulate uterine response in ovariectomized prepubertal ewes similar to intact prepubertal ewes. There is a need for comparison of uterine growth in ovariectomized ewes
induced by coumestrol content in alfalfa and physiological doses of progesterone versus intact prepubertal ewe lamb uterine growth. The jury is still out in determining if coumestrol is affecting uterine growth in a positive manner or is an antagonist that may delay puberty. It has been suggested that coumestrol may have the ability to delay puberty at this level (Newsome and Kitts, 1980). More information is necessary to determine the true biological effect of phytoestrogens on puberty.

Uterine horn lumen epithelial cell height increased after a 12 day treatment with estradiol-17β and alfalfa. Murray (1992) reported a uterine gland epithelial cell height of 23 μm after only 2 days of serum levels of 5-10 pg/ml of estradiol-17β. Estradiol treated ewes had a mean uterine horn lumen epithelial cell height of 23.2 μm. Alfalfa fed ewes’ uterine lumen epithelial cell height was 13.7 μm, which is intermediary when compared with estradiol treated ewes and control ewes, which had the shortest cell height of 10.3 μm. This result might suggest that coumestrol or formononetin may not bind the available estrogen receptor with the same affinity as estradiol-17β to induce similar cell growth.

Orchardgrass has served as a nonestrogenic control in studies examining phytoestrogen content of legume feeds (Kitts et al., 1959). Oregon agriculture produces high quality orchardgrass that one might assume would serve as a perfect control diet for the present study, but after testing several samples with HPLC and having the results return positive for phytoestrogen content (Table 4) it was decided
to utilize bentgrass straw for our study. Difficulty arises in explaining why this study is the first to report coumestrol content in grass species. Field records show that each stand of orchardgrass had been sprayed to eliminate broad leaf plants such as clovers or alfalfa, which would increase the estrogenic content of the grass. It may be possible that certain stresses due to environment, fertilizer, or timing of harvest may have stimulated accumulation of phytoestrogens. Further testing of fields in Oregon and across the country is needed to validate the finding of the study.

A select few articles have described the effects of phytoestrogens on prepubertal ewe lamb. Newsome and Kitts (1980) showed that coumestrol in conjunction with endogenous estrogen may delay puberty. Nwannenna et al. (1995) presented research results that confirm that morphological changes occur in the vulva in relation to estrus and that teat length increased after treating prepubertal ewe lambs with formononetin or estradiol. The present study illustrates that vulva scores increase, uterine wet weights increase, and uterine horn lumen epithelial cell heights increase after treatment with coumestrol and estradiol. Data in this study coincides with Nwannenna et al. (1995) in stating that uterine responses may be a more sensitive measure to detect differences due to exogenous estrogens than clinical measures.
Many questions remain to be answered. Do phytoestrogens inhibit or delay puberty and does this effect persist to damage the life long fertility of the ewe lamb? Further research is needed to more closely examine the effects of phytoestrogens on puberty in the ewe lamb and answer these among other possible questions.
References


Appendix 1. Subjective scoring system to monitor morphological changes in the vulva of an ovariectomized prepubertal ewe lamb.
All ewes will undergo clinical examination to monitor changes in external genitalia. Three independent examiners that are blinded towards the results of the data will score the ewes. The primary investigator will be responsible for teaching the examiners the scoring system prior to the beginning of the experiment. In an effort to eliminate any serial correlation between subsequent observations for the same ewe, the primary investigator will present the ewes in a random order for each scoring session. The vulva is expected to increase in size and change color with increasing exposure to phytoestrogens. The subjective scoring system is shown in the table below.

<table>
<thead>
<tr>
<th>Score</th>
<th>Expected changes associated with score.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>appears normal and has no change in color or size</td>
</tr>
<tr>
<td>2</td>
<td>slight change: increased size and slight change from pale toward reddish or pink color</td>
</tr>
<tr>
<td>3</td>
<td>definite increase in size and has swollen appearance; red in color</td>
</tr>
<tr>
<td>4</td>
<td>very swollen and extremely inflamed with bright red appearance (may have discharge from the vulva)</td>
</tr>
</tbody>
</table>

* If one of the parameters shows no change, then the evaluator shall use a half point score. (Example: no change in size, but change from pale to red would receive a score of 1.5)

* It will be important for the examiner to note any unusual changes that are not included in the scoring system.
Appendix 2. Subjective scoring system to monitor morphological changes in teat length and circumference of an ovariectomized prepubertal ewe lamb.
All ewes will undergo clinical examination to monitor changes in teat length and circumference. Three independent examiners that are blinded towards the results of the data will score the ewes. The primary investigator will be responsible for teaching the examiners the scoring system prior to the beginning of the experiment. In an effort to eliminate any serial correlation between subsequent observations for the same ewe, the primary investigator will present the ewes in a random order for each scoring session. The diet of estrogenic feed is expected to cause increased teat length and increased teat circumference. The subjective scoring system is outlined in the following table:

<table>
<thead>
<tr>
<th>Score</th>
<th>Expected changes associated with score.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>no change</td>
</tr>
<tr>
<td>2</td>
<td>slight increase in both length and circumference</td>
</tr>
<tr>
<td>3</td>
<td>moderate increase in length and an appearance of fullness or filling of the teat</td>
</tr>
<tr>
<td>4</td>
<td>significant increase in both length and circumference; definite full and swollen appearance (may be able to secrete milk)</td>
</tr>
</tbody>
</table>

* If one of the parameters shows no change, then the evaluator shall use a half point score. (Example: no change in circumference, but change in length would receive a score of 1.5)
Appendix 3. Routine haematoxylin and eosin stained slides submitted by Oregon State University, Veterinary Diagnostic Laboratory, College of Veterinary Medicine
Routine H & E Stained Slides:

Tissues were fixed in 10% neutral buffered formalin.

If the tissues had to be decalcified, Cal-Ex II (Fisher Scientific - Houston, TX) would have been used.

Processed on LX 300 Tissue Processor (Fisher Scientific - Houston, TX).

Embedded in Paraffin Type 9 (Richard-Allen Scientific - Kalamazoo, MI).

Sections cut at 4-5 microns on microtome.

Stained on the S/P Automatic Slide Stainer GLX set up for H & E.

Gill-3 Haematoxylin (Shandon, Inc. - Pittsburgh, PA).

Eosin Y Alcoholic (Shandon, Inc. - Pittsburgh, PA).

Coverslip with Shur/Mount Mounting medium (Triangle Biomedical Sciences - Durham, NC).