EFFECTS OF LINSEED MEAL ON GROWTH AND REPRODUCTIVE PERFORMANCE IN RUMINANTS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

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ABSTRACT

Ilse, Breanne Rose, M.S., Department of Animal Sciences, College of Agriculture, Food Systems, and Natural Resources, North Dakota State University, June 2011. Effect of Linseed Meal on Growth and Reproductive Performance in Ruminants. Major Professor: Dr. Kimberly Vonnahme.

Linseed meal (LSM) was fed to ovariectomized ewes exposed to estradiol 17-β (E2) implants over time, and LSM was supplemented to beef cows during late gestation and early lactation to evaluate the estrogenic potential of the phytoestrogen secoisolariciresinol diglycoside (SDG) found in LSM. Forty-eight ovariectomized ewes were fed a diet containing 12.5% LSM for 0, 1, 7, or 14 d and implanted with estradiol-17β (E2) for 0, 6, or 24 h before tissue collection. Uterine cellular proliferation, vascularity, and the expression of the angiogenic factors and their receptors were recorded. There was an interaction of LSM and E2 on uterine mass ($P = 0.05$). At 24 h of E2 exposure, proliferation was reduced ($P < 0.001$) when ewes were fed for 14 d compared to being fed 0 or 1 days. There was a LSM x E2 interaction ($P \leq 0.03$) on *VEGF receptor-2 (KDR)* and *basic fibroblast growth factor receptor (FGFR2)* decreasing by 24 h E2 exposure. Exposure of LSM and E2 may impact the estrogenic response of sensitive tissue.

Two studies were conducted to examine the effects of supplementing beef cows with LSM during late gestation or early lactation on calf growth and development. In Experiment 1, multiparous cows received either LSM or a control (CON) supplement (approximately 10% diet dry matter) for the last 60 d of gestation. Offspring weights, ADG, heifer age at puberty, conception percentage and steer carcass characteristics were recorded. In Experiment 2, multiparous cows received LSM or CON supplements (approximately 10% diet dry matter) during the first 60 d of lactation, and only heifer calf

development performance was observed and recorded. Heifer calf weight, average daily gain, and attainment of puberty were assessed. For both experiments, birth weight, weaning weight and ADG were not affected ($P > 0.31$) by LSM supplementation. While final body weights were heavier ($P = 0.04$) for steer calves in Experiment 1, there was no effect ($P = 0.09$) of supplementation on carcass characteristics. In Experiment 1 and 2, attainment of puberty in heifer calves was not influenced ($P > 0.58$) by supplement type. Linseed meal supplementation during late gestation or early lactation does not appear to have a negative impact on calf growth, onset of puberty in heifer calves, or steer carcass quality, implicating that it may make a good supplement choice in cattle.

Keywords: beef cattle, sheep, growth, angiogenesis, cellular proliferation, phytoestrogen, puberty, linseed meal

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LIST OF ACRONYMS AND ABBREVIATIONS

α	Alpha
ADG	Average Daily Gain
ADF	Acid Detergent Fiber
ANGPT1	Angiopoietin-1
ANGPT2	Angiopoietin-2
AVG	Average
β	Beta
BCS	Body Condition Score
BrdU	Bromodeoxyuridine
BW	Body Weight
Ca	Calcium
cm	Centimeter
CON	Control Treatment
CP	Crude Protein
CREC	Carrington Research Extension Center
°C	Degrees Celsius
d	Day
DM	Dry Matter
ED	Enterodiol
EL	Enterolactone
NOS3	Endothelial Nitric Oxide Synthase

ERα	Estrogen Receptor alpha
ERβ	Estrogen Receptor beta
et al	And others
E2	Estradiol-17β
FGF	Fibroblast Growth Factor
FGFR2	Fibroblast Growth Factor Receptor 2
FLT1	VEGF Receptor-1
FSH	Follicle Stimulating Hormone
g	gram
GLM	General Linear Model
h	Hour
hd	Head or Number of Animals
HCW	Hot Carcass Weight
i.e.	That is
i.d.	Inter diameter
o.d.	Outer diameter
IFG-I	Insulin-Like Growth Factor-1
IVDMD	In Vitro Dry Matter Disappearance
IVOMD	In Vitro Organic Matter Disappearance
kcal	Kilocalorie
KDR	VEFG Receptor-2
kg	Kilogram
KPH	Kidney Pelvic Heart

LH	Luteinizing Hormone
LSM	Linseed Meal
m	Meter
Mcal	Megacalorie
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mo	Month
mRNA	Messenger Ribonucleic Acid
µm	Micrometer
n	Number
NASS	National Agricultural Statistics Service
NDF	Neutral Detergent Fiber
NDSU	North Dakota State University
NE	Net Energy
NFE	Nitrogen Free Extract
ng	Nanogram
NRC	National Research Council
OM	Organic Matter
P	Phosphorus
P	P value Related to F test
PROC MIXED	Mixed Procedure of SAS

P_4	Progesterone
REA	Ribeye Area
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
®	Registered Trade Mark
SAS	Statistical Analysis System
SDG	Secoisolariciresinol diglycoside
SEM	Standard Error Mean
SFM	Sunflower Meal
GUCY1BC	Soluble Guanylate Cyclase
TBA	Trenbolone acetate
TDN	Total Digestible Nutrients
Tie-2	ANG receptor
TMR	Totally Mixed Ration
USDA	United States Department of Agriculture
VEGF	Vascular Endothelial Growth Factor
vs	Verses

CHAPTER 1. GENERAL INTRODUCTION

Flax (*Linum usitatissimum*) has been cultivated since early civilization for its use in oil and fiber production and dietary health benefits. The northern plains of the United States are the primary region of flax production with North Dakota ranking number one in flax production in the United States (NASS, 2010). Flaxseed in the United States is predominantly grown to produce linseed oil for industrial products such as paint, flooring, and lubricants. However, interest in consumption of flaxseed for human health has increased in recent years, with emphasis on the health benefits of omega-3 fatty acids, lignans, and antioxidative properties of flax as a dietary supplement for prevention of cancer, heart disease, and diabetes.

Industrial processing of the flaxseed for linseed oil results in the remaining co-product known as linseed meal (LSM) which is a valuable livestock feed. Morrison (1946) described LSM as a high quality protein supplement for several classes of livestock, including ruminants (Bethke et al., 1928; Weber, 1934), and LSM continues to be used in modern livestock production.

The flaxseed hull contains the plant lignan; secoisolariciresinol diglycoside (SDG), therefore LSM in which the hull remains after the removal of the oil is a rich source of SDG. Secoisolariciresinol diglycoside is converted into the mammalian lignans, enterodiol and enterolactone, by the colon bacteria in monogastrics (Thompson and Ward, 2006; Clavel et al., 2006) and by microorganisms in the digestive tract including ruminal microorganisms (Gagnon et al., 2009; Zhou et al., 2009). Enterodiol and enterolactone are classified as mammalian lignans which have the ability to mimic the steroid hormone estrogen and bind to estrogen receptors in mammals (Gagnon et al., 2099; Ward et al.,

2001). When estradiol-17β (E2), a steroid hormone, binds to its receptors in the uterus, resulted in increased uterine weight (Ford et al., 1975), however uterine size is not increased in weight alone, but also by increasing cellular proliferation and angiogenesis in the ovariectomized ewe (Johnson et al., 1997; Johnson et al., 2006; Reynolds et al., 1998a; Reynolds et al., 1998b). O'Neil et al. (2008) reported an increase in cellular proliferation jejunum in ovariectomized ewes exposed to E2 implant. However, when LSM is fed to ewes, the response to E2 in the jejunum is negated (O'Neil et al., 2008). The estrogenic impact of LSM on the uterus in the presence or absence of E2 is currently unknown.

While the impacts of LSM, or lignan components of LSM, have not been studied exclusively in the uterus, others have demonstrated that lignans can impact the developing offspring in rats (Tou et al., 1998; Chen et al., 2003). The altered development of the offspring born from rats fed LSM is of interest since LSM is a readily available feed for livestock.

The objective of this thesis is to determine the effect of supplemented LSM in ruminant diets during critical times of development on uterine physiology and offspring growth and reproductive performance. This thesis includes a literature review, a chapter investigating the impacts of LSM and E2 on uterine cellular proliferation and angiogenesis in ewes, and a chapter investigating the impacts of supplementing beef cows LSM during late gestation or early lactation on development of their calves, as followed by a general discussion.

Review of the Literature

Flaxseed and linseed meal

Flax (*Linum usitatissimun*) has been cultivated since the birth of agriculture originating in the Fertile Crescent dating back 7,000 years (Zohary and Hopf, 2000; Allaby et al., 2005). Cultivation of flax continued throughout history for fiber use in textiles, paper, oil, and for its nutritional properties and medicinal therapy (reviewed in Martin et al., 1976). According to the USDA, National Agriculture Statistics Service (USDA-NASS), flax, an annual oilseed, has been produced in North Dakota since 1889. Since this early establishment of flax, North Dakota ranks number one in the nation for flax production. Current production of flaxseed is primarily for the industrial use of linseed oil for paint bases and floor coverings, soap and lubricants (Wiesenborn et al., 2005).

Flaxseed contains between 32 and 44 percent oil on a DM basis (Martin et al., 1976). Linseed oil is extruded through mechanical (crush) or chemical (solvent) extraction. The remaining fraction after extraction of the linseed oil is referred to as linseed meal (LSM). Since the 1920 and 1930s, LSM has been included in rations fed to ruminants (Bethke et al., 1928; Weber, 1934). Moreover, the popularity of LSM as a high quality protein supplement in several classes of livestock was described in (Morrison, 1946). Nutrient value of solvent extracted LSM is 90% DM; 78% TDN; 38.3% CP; 1.5% Ether Extract, dry matter basis (NRC, 1984). The excellent nutrient profile of LSM allows for application in domestic cattle production.

Phytoestrogens and lignans

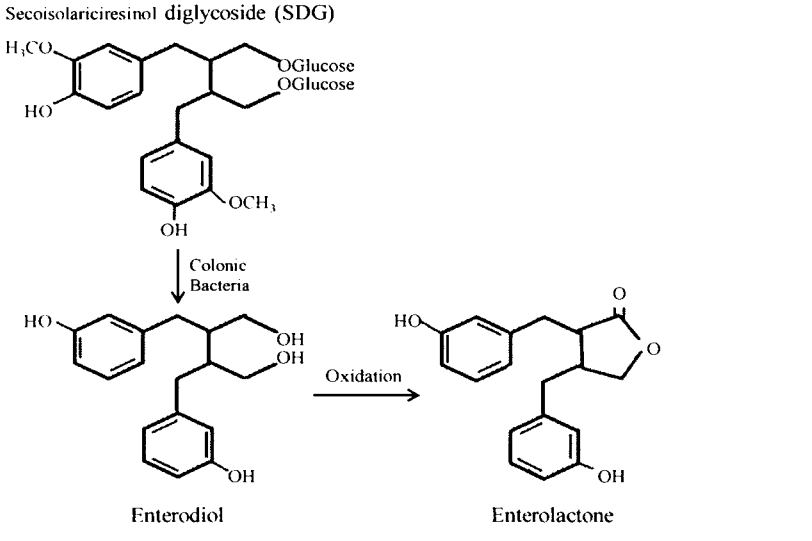
Phytoestrogens are polyphenolic compounds with chemical structure similar to steroidal sex hormones (Sprando et al., 2000; Gagnon et al., 2009). Phytoestrogens have the ability to mimic endogenous estrogens when converted to mammalian lignans (Gagnon et al., 2009; Ward et al., 2001; Thompson et al., 1991). The competitive binding of lignans mimicking estradiol 17-β may result in estrogenic or antiestrogenic activity.

The primary lignan of flaxseed is secoisolariciresinol diglycoside (SDG). Flaxseed contains 976 g/kg SDG (Gagnon et al., 2009; Liu et al., 2006), which is located in the hull of the flaxseed. The SDG located in the hull is 46 times greater than SDG in the cotyledons of flaxseed (Wiesenborn et al., 2005). Secoisolariciresinol diglycoside is converted into the mammalian lignans, enterodiol and enterolactone, by the bacteria in the colon of monogastric mammals (Thompson and Ward, 2006; Clavel et al., 2006) and the ruminal microorganisms in ruminants (Figure 1.1) (Gagnon et al., 2009; Zhou et al., 2009). Gagnon et al. (2009) reported in a ruminal metabolism trial with dairy cows that the metabolism of lignans occurs in the rumen rather than abomasum or the intestinal tract. Enterodiol and enterolactone are detectable in urine, plasma and milk of ruminants, but enterolactone is the primary mammalian lignan converted from SDG in the rumen (Petit and Gagnon, 2009 and Zhou et al., 2009).

Further, estrogen receptor α and β have a different C-terminal lignan-binding and N-terminal transactivation (Kuiper et al., 1998; Rosselli et al., 2000) and lignans may have different affinity due to structure of the receptor site of specific tissues. Therefore mammalian lignans may have the potential to have estrogenic or antiestrogenic activity by

competitively binding to estrogen receptor sites in a tissue specific manner (Kuiper et al., 1998: Rosselli et al., 2000). However the effects of enterodiol and enterolactone may also be due to the ratio of enterodiol and enterolactone circulating the blood serum in relation to endogenous estrogens also circulating in serum (Adams. 1995).

Figure 1.1. Structure of secoisolariciresinol diglycoside and conversion to enterodiol and enterolactone.



Adapted from Thompson and Ward 2006. Food- Drug Synergy and Safety

Interestingly, mammals with low levels of circulating endogenous estradiol- 17β such as luteal phase of the estrous cycle, the influence of phytoestrogens on reproductive physiology would most likely be estrogenic rather than antiestrogenic.

Developmental programming

Developmental programming can be defined as when a stimulus or insult occurs at a critical point in development that impacts the health, growth and performance of the

offspring throughout its lifetime (Barker and Osmond, 1986). The relevance for producers is the lifetime productivity of livestock directly affects profitability.

Recent research by Stalker et al. (2006; 2007) reported that protein supplementation in the during late gestation had a long term effect on calf growth with significant increase in calf weaning weight and live calves at weaning (Stalker et al., 2006). Further Larson et al. (2009) reported that maternal diet protein supplementation during late gestation increased calf birth weight, calf weaning weight and steer carcass traits, therefore increasing the overall value of the steer carcass at harvest.

Maternal diet also affected reproductive parameters in heifer offspring. Protein supplementation (42% CP) of beef cows during late gestation resulted in heifers exhibiting increased pregnancy rates due (Martin et al., 2007). This may not be solely attributed to late gestation alone as Gardner et al. (2008) reported that the lifetime reproductive performance is not directly affected by maternal nutrition during pregnancy but rather by early postnatal growth during lactation.

Heifers with increased growth rates or greater weaning weight reach earlier puberty due to increased nutrition, and therefore are able to reach maturity and conceive earlier (Martin et al., 1992). Alternatively Wiley et al. (1991) reported prepartum nutrition levels of first-calf beef heifers did not affect calf performance through breeding. Wright et al. (2002) reported exposure of estrogen mimicking compounds during gestation or lactation resulted in advanced onset of puberty and first progesterone (P_4) rise and follicle stimulating hormone preovulatory surge was elevated for longer than those born to dams whom were not exposed. Age at puberty is an effective indicator of lifetime performance of beef females because it is an independent measurement of fertility before conception and

lactation (Martin et al., 1992). Maternal nutrition during gestation does affect tissue and organ development in offspring in utero, therefore long term postnatal efficiency could affect the offspring lifetime performance (Long et al., 2009). Heifers that reach puberty at an early age are more productive over their lifetime in a cow herd (Lesmeister et al., 1973; Martin et al., 1992; Ciccioli et al., 2005).

The influence of estradiol on angiogenesis and proliferation

Growth and development of tissue requires extensive blood supply thus, formation of blood vessels to propagate tissue growth is necessary. Angiogenesis is the development of new blood vessels to support the growth of new tissues (i.e. embryonic development, tumors) or tissue maintenance and repair (Mosby's Medical Dictionary, 8th ed. 2009). In mature female animals normal angiogenesis occurs extensively during reproductive cycles and pregnancy. Estradiol 17-β stimulates blood flow (Kuiper et al., 1998; Magness et al., 2005; Rosselli et al., 2000) and cellular proliferation in the uterus (Johnson et al., 1997; Reynolds et al., 1998a; Magness et al., 2005) for preparation for pregnancy and to nourish a developing fetus. Vasodilation is stimulated by the binding of estradiol 17-β to both subtypes of estrogen receptors ERα and ERβ with great affinity to reproductive tissues via nuclear binding or membrane fractions of uterine cells (Kuiper et al., 1998; Rosselli et al., 2000; Magness et al., 2005). Binding of estrogen to ERα or ERβ in both cattle and sheep increased the expression of several angiogenic factors (Johnson et al., 1997; Reynolds et al., 1998a; Magness et al., 2005), including: vascular endothelial growth factor, vascular endothelial growth factor receptor-1 (FLT1) and receptor-2 (KDR), soluble guanylate

cyclase (a nitric oxide receptor), basic fibroblast growth factor, endothelial NO synthase, angiopoietin1, angiopoietin 2, and angiopoietin receptor 2 (Tie-2).

Reynolds et al. (1998b) reported that in the endometrium of ovariectomized ewes exposed to exogenous estradiol 17-β resulted in an increase in heparin-binding angiogenic factors, such as vascular endothelial growth factor as well as basic fibroblast growth factor and their respective receptors. Estradiol resulted in uterine tissue proliferation and vascularization (Redmer and Reynolds, 1996; Reynolds et al., 1998a; and Johnson et al., 2006). Reynolds et al. (1998a) hypothesized that the increase in cell size in addition to cell number was a direct result of the angiogenic factors forcing both increased vascular development and blood flow.

Linseed meal and SDG supplementation: influence on growth and reproduction of offspring

Exposure to estrogen mimicking compounds such as the SDG lignan found in flaxseed, and LSM has been reported to be beneficial to reproductive performance of offspring born from, or suckling, supplemented dams due to the conversion of SDG into the mammalian lignans enterodiol and enterolactone. Further, females born from rat dams supplemented with 10 percent flaxseed during gestation and/or lactation were younger and lighter at puberty and displayed lengthened estrous cycles (Tou et al., 1998; Whitten et al., 1995).

In contrast, Collins et al. (2003) reported flaxseed or linseed meal fed at high percentage (20%- 40%) of the diet during gestation did not affect fetal growth parameters but did affect lifetime reproductive indices of both offspring sexes in rats. Furthermore,

Ward et al. (2001) found no effects in rats on reproductive or growth parameters of offspring when 10% flaxseed or SDG was fed to dams during lactation.

Offspring could be exposed to the lignan effects during the postnatal period. Tou et al. (1998) confirmed that mammalian lignans were detectable, by radioactively tagging of lignan diet in supplemented rat dams diet during lactation exposing the offspring to synthetic estrogen levels during the suckling period. Female offspring suckling lignan supplemented dams had reduced age and lighter body weights at reproductive maturity and greater length of estrus and male offspring had greater prostate and sex gland weights (Tou et al. 1998).

Petit et al. (2009) reported that in ruminants, only enterolactone was present in the milk of cows supplemented linseed meal during lactation, which increased linearly with level, while enterodiol was not detected in significant quantities. Further Petit et al. (2006) reported beneficial effects of supplementing phytoestrogens in ruminant diets.

Statement of the Problem

Maternal supplementation of flaxseed, LSM, or SDG has resulted in changes in the physiological or reproductive characteristics of offspring. Tou et al. (1998) reported female rats born from dams supplemented with 10 percent flaxseed during gestation and/ or lactation resulted in earlier age and lighter body weight at puberty and displayed lengthened estrous cycles. Furthermore, Collins et al. (2003) reported when flaxseed or linseed meal was fed to rats at 20 percent to 40 percent of the diet during gestation supplementation affected the lifetime reproductive indices of both male and female offspring.

In ruminants, Petit and Palin (2006) reported dairy cows fed flaxseed from calving to day 50 of gestation reduced embryo mortality and cows had larger corpora lutea compared to the unsupplemented controls. The lignan SDG which is found in flaxseed and LSM, which is converted to mammalian lignans has the potential to mimic E2. Reynolds et al. (1998b) reported in the endometrium of ovariectomized ewes, exposed to exogenous E2 resulted in increase of heparin-binding angiogenic factors. Heparin-binding angiogenic factors such as vascular endothelial growth factor and basic fibroblast growth factor and their respective receptors resulted in uterine tissue proliferation and vascularization (Redmer and Reynolds, 1996; Reynolds et al., 1998a; and Johnson et al., 2006). However O'Neil et al. (2008) reported an interaction between LSM feeding and E2 implants in ovariectomized ewes which resulted in decreased jejunal mass and cellular proliferation.

Therefore, this thesis investigated how feeding LSM in the presence or absence of E2 would impact uterine cellularity and angiogenic factor expression. It was hypothesized that if E2 in the presence of LSM would negate the impacts of E2, then feeding LSM during a time when E2 is physiologically high such as late pregnancy or low during early lactation may have detrimental impacts on offspring growth and reproductive performance.

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CHAPTER 2. IMPACTS OF LINSEED MEAL AND ESTRADIOL-17β ON CELLULARITY, ANGIOGENIC FACTORS mRNA EXPRESSION, AND VASCULARITY OF THE UTERUS IN OVARIECTOMIZED EWES

B. R. Ilse, M. R. O'Neil, G. P. Lardy, L. P. Reynolds, and K. A. Vonnahme

Abstract

To determine the estrogenic potential of the phytoestrogen secoisolariciresinol diglycoside (**SDG**) found in linseed meal (**LSM**) on uterine cellular proliferation, vascularity, and angiogenic factor mRNA expression, 48 ovariectomized ewes were fed a diet containing 12.5% LSM (14.53 mg of SDG/g of feed on DM basis) for 0, 1, 7, or 14 d and implanted with estradiol-17β (**E2**) for 0, 6, or 24 h before tissue collection. There was an interaction of LSM and E2 on uterine mass (grams; $P = 0.05$; percent change; $P < 0.003$). Exposure of E2 for 24 h increased uterine mass ($P \leq 0.02$) on d 1, 7 and 14 of LSM feeding, with greatest mass occurring in ewes exposed to E2 for 24 h and 1 d LSM feeding. Regardless of days fed LSM, after 24 h of E2 exposure, uterine mass was greatest. Cellular proliferation within the uterine luminal epithelium was greatest ($P < 0.01$) at 24 h E2 exposure compared to 0 h and 6 h. When expressed as the percentage change in uterine cellular proliferation, feeding LSM for 14 d negated these effects. Only length of E2 exposure impacted vascularity with capillary number density ($P = 0.02$) with 6 h of E2 exposure being greater than 24 h. While several the expression of mRNA angiogenic factors were influenced by E2, there was only a LSM x E2 interaction ($P \leq 0.03$) on *vascular endothelial growth factor receptor 2* and *fibroblast growth factor receptor 2C*

decreasing expression by 24 h. It appears that E2 sensitive tissues may be influenced by the duration of LSM feeding.

Key words: angiogenesis, cellular proliferation, estrogen, linseed meal, phytoestrogen

The material in this chapter was co-authored by B. R. Ilse and M. R. O'Neil, G. P. Lardy, L. P. Reynolds, and K. A. Vonnahme. B. R. Ilse prepared and analyzed the samples for laboratory analysis and conducted the statistical analysis. B. R. Ilse developed this manuscript and summarized results as the primary author. G. P. Lardy, L. P. Reynolds, and K. A. Vonnahme assisted in manuscript reviews and statistical interpretation of data.

Introduction

Estradiol 17-β (E2) is a known modulator of many reproductive parameters, including increased endometrial angiogenesis and angiogenic factor expression (Johnson et al., 1997a; Johnson et al., 2006), uterine blood flow (Kuiper et al., 1998; Rosselli et al., 2000; Magness et al., 2005), and uterine growth (Reynolds et al., 1998a). Specifically, Johnson et al. (1997a; 2006) demonstrated that E2 increases the expression of several angiogenic factors that were hypothesized to orchestrate the alterations in uterine vascularization (Reynolds et al., 1998b). The role that E2 elicits for proper cyclicity and to promote pregnancy has been long recognized and continues to be studied.

Linseed meal (LSM) is a byproduct of flax where the oil has been removed for industrial purposes. The protein rich LSM has been utilized in livestock diets. The flax hull, and thus LSM, contains the plant lignan, secoisolariciresinol diglycoside (SDG). Secoisolariciresinol diglycoside is converted into the mammalian lignans, enterodiol and enterolactone, by the colon bacteria in monogastric (Kuiper et al., 1998; Thompson et al.,

1991; Chen et al., 2003) and by microorganisms of the rumen in ruminants (Gagnon et al., 2009; Petit et al., 2009; Zhou et al., 2009). Enterodiol and enterolactone mimic E2 and can bind to estrogen receptor (ERα and ERβ) (Kuiper et al., 1998) and therefore potentially act in an estrogenic or antiestrogenic manner depending upon the duration of exposure or the tissue of interest (Zhou et al., 2009; Thompson and Ward, 2006; Clavel et al., 2006).

Recently, our laboratory demonstrated that when E2 is administered to ovariectomized ewes fed LSM, LSM negates the E2-induced increase in jejunal cellular proliferation and weight of the gastrointestinal tract and liver (O'Neil et al., 2008).

We hypothesized that LSM would negate the positive influence of E2 on the reproductive tract in the ewe. Our specific objectives were to determine the length of LSM feeding and E2 exposure would affect uterine luminal epithelial cellular proliferation and caruncular vascularity as well as the mRNA expression of angiogenic factors: vascular endothelial growth factor (*VEGF*), VEGF receptor-1 (*FLT1*), VEGF receptor-2 (*KDR*), basic fibroblast growth factor (*FGF2*) and receptor (*FGFR2*), angiopoietin 1 (*ANGPT1*), angiopoietin 2 (*ANGPT2*), ANG receptor (*Tie-2*), endothelial nitric oxide synthase (*NOS3*), and soluble guanylate cyclase (*GUCY1B3*) in caruncles from ovariectomized ewes.

Materials and Methods

Animals and treatments

Animal procedures were approved by the North Dakota State University Animal Care and Use Committee. Ewes were housed indoors at the North Dakota State University Animal Nutrition and Physiology Center for the duration of the experiment. Forty-eight multiparous ewes (54.6 ± 1.1 kg initial BW) of mixed breeding were fed in groups of 9 to

11 in pens measuring 3.04 × 3.73 m, ovariectomized via midventral laparotomy (Reynolds et al., 1998a), and allowed to recover for at least 28 days before treatments were initiated. After ovariectomy, ewes were fed an SDG-free diet (Control diet; Table 2.1) until initiation of treatment. The feeding of a SDG-free diet following ovariectomy was carried out to ensure that any circulating endogenous estrogens as well as any dietary SDG was cleared from the body before treatments were initiated. Ewes were fed to meet their requirement for maintenance for each pen based on their metabolic body weight (NRC, 1985). Ewes were weighed every two weeks, had *ad libitum* access to water, and were fed once daily at 0800 throughout the experiment.

Immediately before initiation of treatment, ewes were moved to individual pens measuring 1.52 × 1.87 m. Ewes were weighed and were assigned randomly to each treatment group. Treatments were arranged as a 3 × 4 factorial. Main effects consisted of length of exposure to E2 via subcutaneous implant (0, 6, or 24 h) and length of feeding a diet containing 12.5% LSM (0, 1, 7, or 14 d; as-fed basis; Table 2.1). The LSM diet was designed to be SDG free, with the exception of the inclusion of 12.5% LSM. Laboratory analysis of the LSM used to formulate the LSM diet, indicated the SDG content of the LSM diet to be 14.53 mg SDG per g of feed on a DM basis (O' Neil et al., 2008). Control and LSM diets were formulated to be similar in CP (13.7 and 13.6% CP, respectively) and NE (1.80 and 1.79 Mcal/kg of diet, respectively). Both diets were pelleted and ewes were limit fed to meet the net energy requirements for maintenance based on ewe metabolic body weight (56 kcal/$kg^{0.75}$; NRC, 1985). Ewes were either fed the Control diet throughout the experiment (i.e. 0 days LSM feeding) or were fed the LSM diet for either 1, 7, or 14 days before tissue collection. Ewes within each treatment of LSM feeding were implanted

with E2 for 0, 6, or 24 hours before tissue collection. Thus, each treatment group consisted of 4 ewes.

Ewes receiving E2 implants were locally anesthetized and implanted subcutaneously in the axillary region as validated by Johnson et al. (1997a). Implanted ewes each received two Silastic implants (3.35 mm i.d. × 4.65 mm o.d. × 15 mm length; Dow Corning, Midland, MI) each containing 50 mg of E2 (Sigma-Aldrich, St. Louis, MO). After implantation, the incision was closed with surgical staples and a topical antibiotic was applied to the incision site. The implants remained until tissue collection, at which time their presence was confirmed.

Tissue collection

Exactly 1 h before tissue collection, ewes were injected via jugular venipuncture with bromodeoxyuridine (BrdU; Aldrich, Milwaukee, WI; 5 mg/kg BW; Zheng et al., 1996; Johnson et al., 1997a, b). At time of tissue collection, ewes were stunned via captive bolt and exsanguinated. The reproductive tract was removed. The broad ligament, cervix, and oviducts were removed and the uterus was weighed. Next a portion (~ 1 cm wide) of the uterus just above the uterine body was immersed and fixed in Carnoy's fixative.

Following fixation, the tissues were dehydrated with increasing concentrations of ethanol, xylene, and then embedded in paraffin. Moreover, from the remaining fresh tissue, caruncles were dissected from the uterus and snap-frozen in liquid N_2 and stored at -70°C for later mRNA quantification.

Diet and tissue analyses

Diet analyses have been previously published (O'Neil et al., 2008). Briefly, diet samples were analyzed for DM, ash, N, Ca, P (Methods 930.15, 942.05, 990.02, 968.08, and 965.17, respectively; AOAC, 1990), ADF, and NDF (Ankom, Fairport, NY).

Table 2.1. Diet composition and analyzed dietary composition of ewes fed a control diet or linseed meal diet for 0, 1, 7, or 14 days (O'Neil et al., 2008).

Item	Control Diet	LSM Diet
Ingredient[1]	-	-
Beet pulp, %	81.25	87.5
Dried Distiller's grain, %	17	-
Sunflower meal, %	1.75	-
Linseed meal, %	-	12.5
Analyzed dietary nutrient content[2] 100%, DM basis		
DM, %	93.65	93.24
OM, %	11.77	12.31
CP, %	13.70	13.60
NDF, %	43.18	41.34
ADF, %	24.13	24.78
Fat, %	2.27	1.22
IVDMD, %	85.86	86.91
IVOMD, %	84.76	86.11
Ca, %	0.64	0.92
P, %	0.23	0.24

[1]Formulated using as fed values.
[2]All values are based on percentage of DM.
[3]In vitro dry matter disappearance.
[4]In vitro organic matter disappearance

In vitro OM digestibility was determined on diet samples by a modified procedure of Tilley and Terry (1963), in which samples were centrifuged and the supernatant fluid was discarded before the addition of pepsin.

Caruncular mRNA expression of *VEGF, FLT1, KDR, NOS3, GUCY1B3, FGF2, FGFR2, ANGPT1, ANGP 2*, and *Tie-2* was determined using quantitative RT-PCR as adapted from Redmer et al. (2005) and Vonnahme et al. (2006) following capillary electrophoresis of total cellular RNA using an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE) to determine quantity and quality of extracted total cellular RNA. Fixed, embedded uterine tissues were sectioned to 4 μm and affixed to glass slides. Staining protocols to determine uterine vascularity and cellular proliferation were performed as previously described (O'Neil et al., 2008). Briefly, slides evaluated for cellular proliferation were deparaffinized prior to immunohistochemistry. Slides were incubated with primary antibody (Envision mouse anti-BrdU diluted 1:150 with antibody diluent; Dako), and Vector SG substrate (Vector Labs, Burlingame, CA).

Nuclear fast red was used for counter-staining. Digital images of tissues were collected for both vascularity and proliferation data using a Nikon DXM 1200 digital camera (Fryer, Chicago, IL) and an image analysis software package (Image-Pro Plus, version 5.0; MediaCybernetics, Inc., Silver Spring, MD). Proliferation was determined by image analysis of BrdU stained cells in the uterine lumen (luminal) region with 6 fields for each ewe. The percentage of BrdU stained nuclei of all nuclei present in the luminal region was determined to obtain a proliferation index. Caruncular areas within the endometrial tissue were used to determine measurements of uterine vascularity.

Measures of vascular area density, number density, surface density, and area per capillary were obtained from the caruncular areas of the endometrial lumen 10 fields per ewe and determined via image analysis.

Statistical analysis

Data for ewe uterine cellular proliferation, vascularity, and angiogenic factor expression were analyzed as a 3 × 4 factorial in a randomized complete block design using the PROC GLM procedures of SAS (SAS Inst. Inc., Cary, NC). Model statement included effects of block (based on initial weight), length of LSM feeding, length of E2 exposure, and the interaction of LSM feeding and E2 exposure.

Data are presented as least squares means ± SEM. Mean separations were performed via LSD which were protected by an overall treatment *F*-test at $P = 0.05$. Differences were considered significant if $P \leq 0.05$ unless otherwise stated.

Results

Ewe weights were previously published (O'Neil et al., 2008) and were not different (52.1 ± 0.99 and 51.5 ± 0.97 kg, respectively $P > 0.37$. There was an interaction of LSM and E2 on uterine weight ($P = 0.03$; Figure 2.1A) and percentage change of uterine mass ($P < 0.003$; Figure 2.1B). For uterine mass, when ewes were not exposed to E2, there was a tendency ($P = 0.07$) for uterine mass to be lower in 1 d LSM fed ewes compared to ewes not fed LSM with ewes fed LSM for 7 and 14 d being intermediate. Estradiol-17β exposure for 24 h tended ($P = 0.09$) to increase uterine mass compared to 0 or 6 h on d 0, and did increase uterine mass ($P \leq 0.02$) after 1, 7 and 14 d of LSM feeding. In ewes fed LSM for 1 day, E2 exposure for 6 h increased ($P < 0.0001$) uterine mass compared to 0 h of E2 exposure.

There was no difference ($P > 0.63$) in uterine mass in ewes exposed to E2 for 0 or 6 h in ewes fed LSM for 0, 7, or 14 d. Moreover, uterine mass was greater ($P < 0.01$) in ewes exposed to E2 for 24 h and 1 d LSM feeding compared to all other ewes. Exposure to E2 for 24 h did not differ ($P > 0.83$) in uterine mass between ewes fed LSM for 0, 7 or 14 d.

When expressed as percentage mass change from the 0 h E2 exposure, within a day, uterine mass at 6 h of E2 exposure was similar ($P > 0.16$) to 0 h, except in the ewes fed LSM 1 d. In ewes fed LSM for 1 d, percent change in uterine mass increased ($P = 0.01$) with increasing E2 exposure. In all ewes, regardless of day fed LSM, 24 h E2 exposure increased ($P < 0.001$) uterine mass compared to 6 h exposure. Moreover, ewes fed LSM for 1 d had increased ($P < 0.001$) percentage mass change with 24 h E2 exposure compared to ewes on d 0, 7, and 14.

There was no interaction between LSM and E2 ($P = 0.43$) or main effect of LSM supplementation ($P = 0.81$) on uterine dry weight, but there was an E2 effect ($P < 0.001$). With increasing length of E2 exposure, there was a decrease in percentage dry weight (19.45 vs. 18.64 vs. 15.59 ± 0.20 % for 0, 6, and 24 h, respectively). While there was no LSM x E2 interaction ($P = 0.61$) or main effect of LSM ($P = 0.15$) on uterine cellular proliferation there was a main effect on E2 with uterine luminal epithelium having an increased ($P < 0.01$) proliferation at 24 h (17.84 ± 2.55%) compared to 0 h (6.66 ± 2.65%) and 6 h (8.47 ± 2.55%) estrogen exposure, which did not differ ($P = 0.63$; Figure 2.1A).

There was, however, tendency ($P = 0.07$) toward an interaction between LSM and E2 on the percentage change in cellular proliferation. When LSM was fed for 0 or 1 d, the percentage change in uterine luminal proliferation was similar ($P > 0.60$) between 0 and 6 h

E2 exposure, but increased ($P < 0.01$) by 24 h (Figure 2.2B). However, by d 7 of LSM feeding, uterine luminal proliferation was not influenced ($P > 0.16$) by E2 exposure.

Moreover, at 14 d of LSM, there was no effect ($P > 0.88$) of E2 on uterine luminal cellular proliferation, regardless of any duration in E2 exposure. At 6 h of E2 exposure, proliferation tended to be greater ($P = 0.09$) when LSM was fed for 7 d compared to 1 d with d 0 and 14 d being intermediate. At 24 h of E2 exposure, proliferation within the uterine lumen was reduced ($P < 0.001$) when ewes were fed 14 d of LSM compared to days 0 and 1, with d 7 being intermediate ($P = 0.14$).

Linseed meal days fed and hours of E2 exposure did not affect ($P > 0.23$) capillary surface area, or area per capillary (Table 2.2). There was a tendency ($P = 0.06$) for length of E2 exposure to affect capillary surface density where 0 h was similar ($P = 0.12$) to 6 h and 24 h, however 6 h of E2 exposure was greater ($P = 0.02$) than 24 h (40.79, 43.23, and 35.42 ± 2.35, for 0, 6, and 24 h, respectively). There was an effect of length of E2 exposure ($P = 0.02$) on capillary number density where 0 h was similar ($P > 0.13$) to 6 h and 24 h, however 6 h of E2 exposure was greater ($P < 0.01$) than 24 h (1083.0, 1278.9, and 859.72 ± 100.4, for 0, 6, and 24 h, respectively. There was no effect ($P \geq 0.07$) of LSM, E2, or their interaction on mRNA expression of *FGF2, Tie2,* and *ANGPT1* (Table 2.3).

There was a LSM x E2 interaction ($P \leq 0.03$) for *KDR* and *FGFR2*. On d 0, 1 and 7, when ewe were exposed to E2 for 24 h, *KDR* mRNA expression decreased ($P < 0.01$) compared to 0 and 6 h which did not differ. Ewes fed LSM for 0 d and exposed to E2 for 24 h had increased ($P \leq 0.06$) *KDR* mRNA expression compared to all other days fed LSM

and 24 h E2 exposure. When LSM was fed for 1 d and E2 administered for 6 h, *KDR* mRNA expression was reduced ($P \leq 0.04$) compared to all other days.

When ewes were not exposed to E2, *KDR* mRNA expression was reduced ($P < 0.01$) in ewes fed LSM for 14 d compared to ewes fed LSM for 0, 1, or 7 d, which did not differ. For *FGFR2*, there was no effect of the duration of E2 exposure on day 0 and 7 of LSM feeding. However, on day 1 of LSM feeding, 24 h E2 exposure had increased ($P \leq 0.06$) FGFR2 mRNA expression compared to 0 and 6 h which did not differ. After 14 d of LSM feeding, 0 h E2 exposure had greater ($P < 0.01$) *FGFR2* mRNA expression compared to 6 and 24 h which did not differ. Feeding LSM for 1 d had greater ($P \leq 0.08$) *FGFR2* mRNA expression at 24 h E2 exposure compared to any other day.

Furthermore, 14 d LSM had greater ($P \leq 0.01$) *FGFR2* mRNA expression at 0 h E2 exposure compared to any other day or time. There was no effect ($P \geq 0.16$) of LSM or LSM by E2 interaction on *VEGF, FLT1, ANGPT 2, NOS3,* or *GUCY1B3* (Table 2.3). However, E2 did effect ($P \leq 0.02$) mRNA expression of *VEGF*, *FLT1*, *ANGPT2*, *NOS3*, and *GUCY1B3* (Table 2.3). When E2 was administered for 6 h, there was an increase ($P \leq 0.01$) in *VEGF* and *FLT1* mRNA expression compared to 0 and 24 h which did not differ. For *ANGPT2*, *NOS3*, and *GUCY1B3* mRNA expression, levels increased ($P < 0.01$) from 0 to 6 h of E2 and remained similar to 24.

Figure 2.1 A and B. Uterine mass and change in uterine mas of ovariectomized ewes implanted with estradiol-17β and fed linseed meal for 0, 1, 7, 14 days.

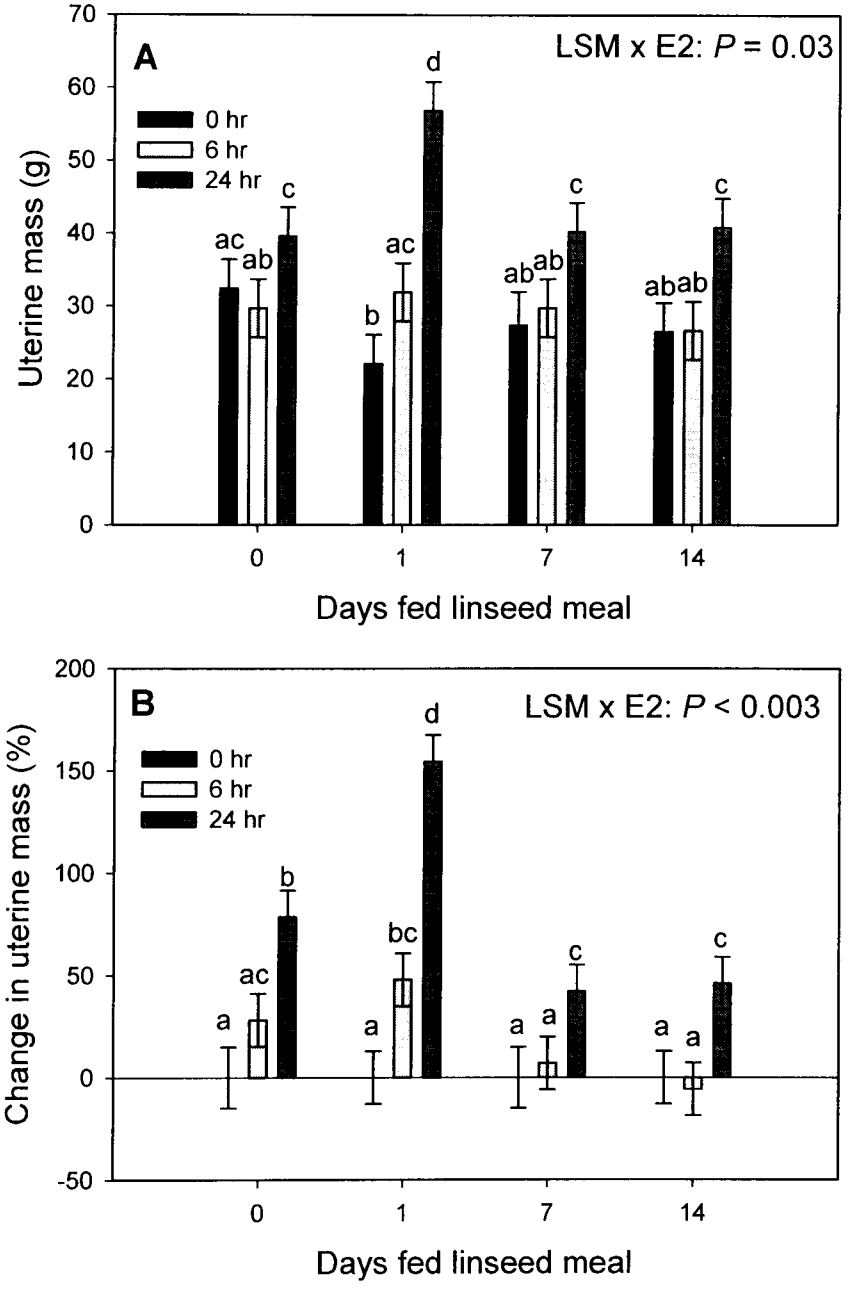

Figure 2.2 A and B. Uterine proliferation and change in uterine proliferation of ovariectomized ewes implanted with estradiol-17β and fed linseed meal for 0, 1, 7, 14 days.

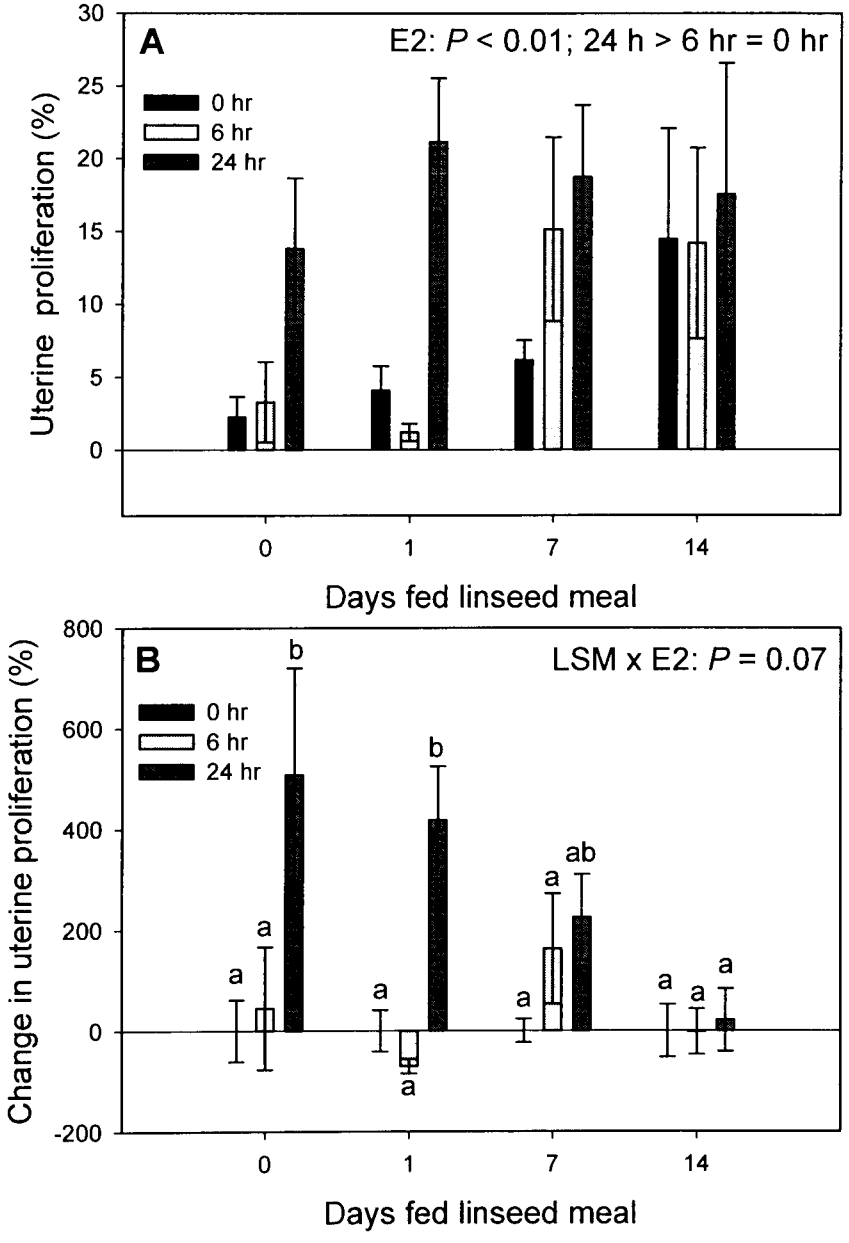

Table 2.2. Effects of feeding linseed meal and implanting ewes with estradiol 17-β on capillary area density, surface density, number density and area per capillary[1].

Item	Days fed LSM												SEM	*P*-values		
	0 d			1 d			7 d			14 d						
	Hours of E2 exposure			Hours of E2 exposure			Hours of E2 exposure			Hours of E2 exposure						
	0 h	6 h	24 h	0 h	6 h	24 h	0 h	6 h	24 h	0 h	6 h	24 h		LSM	E2	LSM×E2
Capillary area density, %	8.02	8.03	7.03	7.61	9.46	6.20	9.62	8.09	7.83	8.18	8.02	8.36	1.10	0.71	0.23	0.50
Capillary surface density	43.31	43.70	34.35	38.05	49.85	28.77	44.11	40.19	37.89	37.69	39.17	40.68	5.32	0.95	0.06	0.25
Capillary number density	1251	1420	883	1090	1444	773	1020	1223	760	978	1027	1020	203	0.66	0.02	0.78
Area per capillary, μm^2	73.12	62.99	83.04	72.11	83.29	92.93	109.51	72.89	110.4	89.20	79.13	82.67	16.48	0.29	0.25	0.74

[1]Treatments were days of linseed meal (LSM) feeding (0, 1, 7, 14 d) and length of estradiol-17β (E2) exposure (0, 6, 24 h).
[a,b]Means ± SEM within a row differ ($P < 0.05$).

Table 2.3. Effects of feeding linseed meal and implanting ewes with estradiol 17-β on caruncular expression of angiogenic factors vascular endothelial growth factor (*VEGF*), (*FLT1*; VEGF receptor-1), (*KDR*; VEGF receptor-2), basic fibroblast growth factor *(FGF2)*, fibroblast growth factor receptor 2 (*FGFR2*), angiopoietin 1 (*ANGPT1*), angiopoietin 2 (*ANGPT2*), angiopoietin receptor (Tie-2), endothelial nitric oxide synthase (NOS3), and soluble guanylate cyclase (*GUCY1B3*).

Item[1]	Days fed LSM															
	0 d			1 d			7 d			14 d						
	Hours exposed to E2			Hours exposed to E2			Hours exposed to E2			Hours exposed to E2						
	0 h	6 h	24 h	0 h	6 h	24 h	0 h	6 h	24 h	0 h	6 h	24 h	SEM	LSM	E2	LSMxE2
VEGF	0.088	2.104	0.069	0.072	3.736	0.137	0.153	3.104	0.102	0.119	0.771	0.160	0.919	0.51	<0.01	0.59
FLT1	0.030	0.103	0.024	0.026	0.125	0.046	0.041	0.113	0.037	0.045	0.052	0.032	0.178	0.75	<0.01	0.82
KDR	0.709^{ab}	0.893^{a}	0.314^{c}	0.640^{a}	0.246^{ce}	0.025^{f}	0.580^{b}	0.714^{b}	0.030^{df}	0.016^{d}	0.669^{ab}	0.011^{bd}	0.924	<0.01	<0.01	<0.01
FGF2	0.830	3.804	0.982	0.550	7.219	7.664	1.128	4.214	2.386	1.511^{e}	4.170	3.964	2.621	0.36	0.09	0.83
FGFR2	0.254^{ab}	0.121^{a}	0.157^{ab}	0.186^{ab}	0.234^{ab}	0.494^{cd}	0.396^{bc}	0.271^{abc}	0.225^{ab}	0.642^{d}	0.134^{ab}	0.257^{abc}	0.109	0.22	0.06	0.03
ANGPT1	0.029	0.058	0.032	0.037	0.078	0.114	0.044	0.073	0.035	0.022	0.079	0.051	0.034	0.51	0.23	0.81
ANGPT2	0.009	0.001	0.001	0.009	0.002	0.002	0.013	0.001	0.001	0.019	0.001	0.001	0.002	0.36	<0.01	0.22
Tie-2	0.315	0.272	0.109	0.157	0.474	0.331	0.228	0.484	0.185	0.244	0.240	0.182	0.113	0.56	0.07	0.42
NOS3	0.425	2.091	0.577	0.336	2.530	2.663	0.568	3.060	1.589	0.628	1.496	1.335	0.627	0.22	<0.01	0.39
GUCY1B3	0.737	3.2282	1.062	0.341	2.472	3.394	0.730	3.527	1.210	0.779	1.224	2.383	0.950	0.85	0.01	0.23

P-values

[a,b,c] Within a row, means with different superscripts differ ($P \leq 0.05$).

[1] Values are arbitrary units.

Discussion

Depending upon the variable measured, it appears that LSM may negate the actions of E2 on the uterus in our ovariectomized ewe model. Our laboratory has demonstrated that E2 can increase liver, duodenal, and jejunal mass, and after 14 d of LSM feeding, that growth is not only negated, but reversed (O'Neil et al., 2008; 2009) The uterus is specifically sensitive to E2, and many others have demonstrated the E2 can increase uterine mass (Johnson et al., 1997a).

We hypothesized that just as LSM negated the impacts of E2 in the liver and gastrointestinal tract, it would also have similar effects in the uterus. In this experiment, uterine mass increased with each d of LSM feeding when E2 was administered for 24 h. This agrees with previously published data (Johnson et al., 1997a; 2006) where uterine weight increased after E2 exposure in an ovariectomized ewe model. Interestingly, when ewes were exposed to E2 for 24 h, feeding LSM for 1 d increased the percentage change in uterine mass, however, this was negated when ewes were fed LSM for either 7 or 14 days.

While only E2 impacted the cellular proliferation in the uterine luminal epithelium, when expressed as a change in proliferation, we observed that by 14 d LSM feeding, the E2 stimulated increase in cellular proliferation at d 0 was diminished. Reynolds et al. (1998a) attributed the increase weight in estrogen sensitive tissue, i.e. the uterus, to an increase of both cellular proliferation and increased cellular size. The increase in proliferation with E2 indicates an estrogenic response which increased, until the potential of antiestrogenic effects of LSM fed over time.

In this study capillary number density and capillary surface density observed an increased at 6 h of E2 treatment but then decreased by 24 h of exposure. Reynolds et al.

(1998a) reported a similar increase in microvascular volume density due to E2 in ewes; however they reported a continued increase through 24 h. This increase was explained by vasodilation of the vasculature and dilation was maintained by potential growth via angiogenesis. The creation of the infrastructure needed to maintain vasodilation beyond 24 h was not observed in this study.

Vascular endothelial growth factor and *FLT1* did respond with an increase in mRNA expression from E2 exposure each day from 0 to 6 h but then decreased significantly from 6 to 24 h. Further there was no response in *FGF2*, *Tie-2* or *ANGPT1* from E2 h or d fed LSM. This is similar to data published by Johnson et al. (2006) where E2 exposure after 4 h increased expression of *VEGF* and *FGF2* mRNA (Johnson et al., 2006) in ewes. Furthermore, others have reported increase in *VEGF* mRNA expression from 4 h through 24 h after E2 treatment in ewes (Reynolds et al., 1998b).

In the current experiment, the only interactions of E2 and LSM on mRNA expression of angiogenic factors of caruncular tissue occurred for *KDR* and *FGFR2*. After feeding LSM for 14 d, the expression of *KDR* was drastically reduced compared to all other days when E2 was not administered. Interestingly, the expression was greatly suppressed by 24 h of E2 exposure regardless of d fed LSM. This suppression is in agreement with *KDR* expression in jejunum reported in ovariectomized ewes implanted with E2 (O'Neil et al., 2008). In contrast, the mRNA expression of *FGFR2* was greatest after 14 d of LSM feeding compared to other days when E2 was not administered. Moreover, after 24 h of E2 exposure, greatest mRNA expression of *FGFR2* was observed on d 1 of LSM feeding This is in disagreement with O'Neil et al. (2008), where length of LSM feeding or E2 exposure did not impact *FGFR2* expression in the jejunum. Johnson et al. (2006) reported E2

exposure for 2 h in ovariectomized ewes increased *FGFR2* mRNA of in intercaruncular endometrium, but not in caruncular tissue. Our laboratory has attributed this *FGFR2* response as local and extensive vascularization of the numerous gland system of intercaruncular endometrium.

The observation of the interaction was measured for 0, 6, and 24 h further observation beyond the 24 h time period may have resulted in an observable angiogenic response to LSM and E2 in uterine tissue. Reynolds et al. (1998a) reported increases in uterine vascularization in ovariectomized ewes by 72 h. The response was not only attributed to vascular dilation, but also to angiogenesis which was not observed until after 24 h exposure to E2. In conclusion, it appears that LSM can negate the proliferative effects of E2 on the uterus. Further studies are warranted to determine how LSM feeding in cycling and pregnant ruminant females could impact fertility and pregnancy.

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CHAPTER 3. GROWTH AND ATTAINMENT OF PUBERTY IN CALVES FROM COWS SUPPLEMENTED WITH LINSEED MEAL DURING LATE GESTATION AND EARLY LACTATION

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Abstract

Two studies were conducted to examine the effects of supplementing cows with linseed meal (LSM) during late gestation or early lactation on calf growth and development. In Experiment 1, (n = 72) multiparous cows received either LSM or a control (CON) supplement that were isocaloric and isonitrogenous, for the last 60 d of gestation. The offspring weights, average daily gain (ADG), heifer age at puberty; first conception percentage and steer carcass characteristics were recorded. In Experiment 2, (n = 198) multiparous cows received LSM or CON supplements during the first 60 d of lactation, and only heifer calves were investigated (n = 91). Heifer calf weight, ADG, and attainment of puberty were assessed. For both experiments, birth weight, weaning weight, and ADG were not affected ($P > 0.31$) by LSM supplementation. While final body weights were heavier ($P = 0.04$) for steer calves in Experiment 1, there was no effect ($P > 0.09$) of supplementation on carcass characteristics. In Experiment 1 and 2, attainment of puberty in heifer calves was not influenced ($P > 0.58$) by supplement type. Linseed meal supplementation during late gestation or early lactation does not appear to negatively impact calf growth, onset of puberty in heifer calves, or steer carcass quality, indicating

that LSM supplementation during late gestation or early lactation will not have negative effects on offspring growth or reproductive performance.

Key words: phytoestrogen, linseed meal, cattle

The material in this chapter was co-authored by B. R. Ilse, V. L. Anderson, D. S. Buchanan, J. D. Kirsch, K. G. Odde, G. P. Lardy, K. A. Vonnahme. B. R. Ilse completed the field sampling, prepared and analyzed the samples for laboratory analysis and conducted the statistical analysis. B. R. Ilse developed this manuscript and summarized results as the primary author. V. L. Anderson, D. S. Buchanan, J. D. Kirsch, K. G. Odde, G. P. Lardy, K. A. Vonnahme assisted in manuscript reviews and statistical interpretation of data.

Introduction

Linseed meal (LSM) is a byproduct of flax where the oil has been removed for industrial purposes. The protein rich LSM has been utilized in livestock diets. The flax hull, and thus LSM, contains the plant lignan, secoisolariciresinol diglycoside (SDG). Secoisolariciresinol diglycoside is converted into the mammalian lignans, enterodiol and enterolactone, by the colon bacteria in monogastric (Kuiper et al., 1998; Thompson et al., 1991; Chen et al., 2003) and by microorganisms of the rumen in ruminants (Gagnon et al., 2009; Petit et al., 2009; Zhou et al., 2009). Enterodiol and enterolactone can bind to estrogen receptor (ERα and ERβ) (Kuiper et al., 1998) and therefore can act in an estrogenic or antiestrogenic manner depending upon the duration of exposure or the tissue of interest (Zhou et al., 2009; Thompson and Ward, 2006; Clavel et al., 2006). Tou and coworkers (1998) reported when rat dams are fed a diet containing 10% flaxseed during gestation and lactation, their female offspring had a decreased age to puberty and

lengthened estrous cycle compared to controls. Furthermore, the female offspring which were born to or suckled the flaxseed supplemented dams during gestation and lactation had lower birth weights and increased uterine weights and the male offspring had reduced average daily gain and increased prostate weights compared to the basal diet (Tou et al., 1998).

Recently, our laboratory has reported that feeding LSM in the presence of estradiol-17β can inhibit cellular proliferation in the uterus (Ilse et al., pending) and the jejunum (O'Neil et al., 2008). Since estrogen concentrations are maximal during late gestation in beef cows (Smith et al., 1973), supplementation that could act to block the vasoactive properties of estrogen may not be ideal. Our hypothesis was that feeding LSM to the dam during critical developmental times of the offspring, namely late gestation and early lactation, would negatively impact the growth of the fetus or neonate, resulting in reduced growth performance, and earlier attainment of puberty, as observed in rats (Tou et al., 1998). Our specific objective was to compare LSM and a control supplement for beef cows during late gestation and early lactation on offspring growth performance, steer carcass composition, and attainment of puberty in heifer calves.

Materials and Methods

The North Dakota State University Animal Care and Use Committee approved all procedures and protocols prior to initiation of this research.

Animals and treatments

Experiment 1: Seventy-two multiparous, gestating Red Angus cross Simmental cows were housed at the Carrington Research Extension Center, North Dakota State University, Carrington, ND. Cows were allotted to 1 of 12 pens (6 hd/pen) at 192.5 ± 22.5

d of gestation. Cows were assigned, using cow weight as a blocking criterion, to receive either a sunflower meal (phytoestrogen-free) control (CON) or LSM supplement (Table 3.1). Pelleted supplements LSM or CON were offered (2.3 kg per hd/d) in a totally-mixed ration until parturition approximately 60 days (Table 3.2).

Secoisolariciresinol diglycoside content of LSM was 118.2 mg/g as fed. Totally mixed rations (Table 3.2) were formulated to provide recommended nutrients for 670 kg late gestation, mature beef cow (NRC, 1996).

Upon parturition, cows were comingled and cow-calf pairs managed similarly. Calves were weighed within 24 h after birth. Calves were weaned at 170 ± 10 d of age. Steer calves (n = 41; CON n = 21; LSM n = 20) were followed from birth through finishing period to harvest at average 397 ± 3.32 d of age.

Table 3.1. Experiment 1: Dry matter nutrient analysis of sunflower meal (CON) and linseed meal (LSM) pellet supplement fed to cows during late gestation.

Item	CON	LSM
Dry Matter, %	90.22	88.80
Crude protein, %	33.50	37.65
Crude fiber,%	17.92	15.01
NEm, Mcal/ kg	1.77	1.77
NEl, Mcal/ kg	1.70	1.70
Fat, %	3.82	3.21
Ca, %	0.42	0.41
P, %	1.07	0.97

At weaning and through the finishing period steers were managed similarly and fed as recommended by the National Research Council (NRC, 1996). Steer rations were primarily comprised of corn, field peas, barley, modified distiller's grain, corn silage, and wheat straw. The TMR was delivered once daily to appetite. Steers were implanted with terminal combination implant, trenbolone acetate and estradiol benzoate, (Synovex Choice,

Fort Dodge Animal Health, Fort Dodge, IA). Steer weights were obtained every 28 d from 198 ± 10.6 d age until harvest at 397 ± 3.32 d of age. Carcass data was recorded at the point of harvest by abattoir staff.

Table 3.2. Experiment 1: Maternal beef cow diet formulation during late gestation of cows supplemented a control or linseed meal pellet.

Item	CON	LSM
	% DM	
CON pellet	9.70	-
LSM pellet	-	9.70
Barley	27.6	27.6
Corn	29.9	29.9
Wheat Straw	32.8	32.8

[a] Sunflower meal (SFM) or Linseed meal (LSM)
[b] Supplements were offered at 2.2 kg/hd/d.
[c] Mineral was offered in ration to meet NRC (1996) requirements

Heifer calves (CON n = 14; LSM n = 16) were followed from birth until breeding at approximately 440 ± 2.72 days of age. Average daily gain performance was calculated based on live body weight difference between period weights divided by the number of days during the established period based on sex and management of the calves. On day 182 ± 10.4 d of age and every 14 days until 280 ± 10.4 d of age, heifers were weighed for calculation of ADG, and jugular blood samples collected and serum analyzed for progesterone (P_4) levels. Blood samples immediately were placed on ice until centrifugation, and serum stored at -20° C until analyses. A ration, comprised of corn silage, wheat straw, wheat middlings and modified distiller's grain, was delivered once daily as a TMR formulated based on (NRC, 1996) recommendations. Heifer performance observation continued with body weight recorded at 28 d intervals until natural breeding was initiated. Pregnancy rate of naturally serviced heifers was determined by ultrasonography at 502 ± 2.72 day of age.

Experiment 2: This study was conducted at the NDSU Beef Research Unit in Fargo, ND. Upon parturition (d 0), cow-calf pairs (n = 91) were assigned randomly to one of two lactation treatments: 1) a control supplement, consisting of dried distillers grain plus soluble and sunflower meal (CON; n = 6 pens) or 2) LSM pelleted supplement (n = 6 pens) consisted of corn grain and LSM. Supplements were formulated to be isonitrogenous and isocaloric and were offered prior to feeding of forage. Pelleted supplements were offered (2.2 kg per hd/d) in a totally mixed ration from day of parturition until d 60 of lactation (Table 3.3). Totally mixed ration was offered in fence line bunks once daily and alfalfa hay was fed ad libitum. Diets were formulated to provide nutrients for approximately 658 kg lactating mature cow (NRC, 1996). At completion of lactation supplementation period, cow- calf pairs were managed similarly.

Table 3.3. Experiment 2: Maternal beef cow diet formulation during early lactation of cows supplemented a control (CON) or linseed meal (LSM) pellet.

Item	CON	LSM
	% DM	
CON pellet	12.20	-
LSM pellet	-	13.7
Alfalfa hay	26.5	26.03
Corn silage	60.31	60.31

[a] Sunflower meal (SFM) or Linseed meal (LSM)

[b] Supplements were offered at 2.2 kg/hd/d.

[c] Supplements were formulated to be isocaloric (0.305 Mcal/kg) and isonitrogenous (12.9%).

[d] Mineral was offered in ration to meet NRC (1996) recommended requirements

Calves were weaned on d 207 ± 13 of age. Heifer calves (n = 91) were followed from birth to 315 d of age. At 229.2 ± 1.5 d of age and every 14 d until d 313.2 ± 1.5 of age, heifers were weighed, and a blood sample was collected from the jugular vein. Blood samples were immediately placed on ice, and serum removed after centrifugation.

Thereafter serum was stored at -20°C until P_4 was analyzed. Visual body condition score (BCS; 1-9 scale; Wagner et al., 1988) was taken on approximately d 229, 287, and 313. Heifers were managed similarly and fed based on nutritional requirements (NRC, 1996).

Analysis of serum and assays

Serum samples were analyzed for P_4 concentrations by competitive chemiluminescent immunoassay (Immulite 1000, Siemens, Los Angeles, CA), as previously described (Martin et al., 2007). Heifers were considered to have attained puberty when P_4 concentrations serum levels where higher than 1.0 ng/ml (Berardinelli et al., 1979). Intra- and inter-assay coefficients of variation were 11.5 and 5.3% for Experiment 1; and were 4.8 and 8.9% Experiment 2.

Statistical analysis

Experiment 1: Data were analyzed by Proc Mixed version of SAS (V.9.1; SAS Inst. Inc., Cary, N.C.). In experiment 1, pen was the experimental unit for effects of gestational on diet birth weight, WW, period weights, ADG and progesterone levels. The statistical model included the fixed effects of gestational diet of the cow and cow weight block. For Experiment 2: Data were analyzed on effects of lactational diet on birth weight, d 60 weight, weaning weight, adjusted weaning weight, final weight, BCS, and progesterone levels. Data were analyzed using the general linear model of SAS (V.9.1).

Results

Experiment 1:

Calf Growth Performance

Regardless of calf sex, calf birth weight and weaning weight were not significantly affected ($P \geq 0.37$) by cow diet (Table 4). Heifers post weaning body weights and overall

ADG were not affected ($P \geq 0.38$) by dam supplementation. Interestingly, by 397 d of age, steers born to LSM dams were lighter than steers from CON dams (548.2 vs. 584.7 ± 9.69; $P = 0.04$). However, ADG did not differ thought out the finishing period ($P = 0.54$).

Carcass performance

Steers hot carcass weight (HCW), back fat, ribeye area (REA), kidney pelvic heart fat (KPH) and final yield grade were not affected ($P > 0.13$) by cow supplementation treatment during gestation (Table3.4). However marbling score tended to be greater ($P = 0.08$) for the steers born from the dams supplemented with CON (507.4 ± 16.24) supplemented pellet treatment during gestation than the LSM (459.5 ± 16.24) treatment.

Heifer reproductive analysis

Age of puberty ($P = 0.58$) and mature body weight at breeding ($P > 0.58$) for heifer calves was not affected by supplementation (Table 4). Nine heifers attained puberty before 6 months of age (4 SFM and 5 LSM; $P > 0.10$).

Experiment 2:

Calf performance

There were no differences in birth weight between treatment groups before the beginning of maternal treatments (Table 3.5). There was no effect of treatment on d 60 weight ($P = 20$). There was a tendency ($P = 0.11$) for heifers from LSM cows to have a greater adjusted weaning weight compared to heifers from CON cows (Table 3.3). Heifer BCS was not affected by treatment ($P > 0.22$) but heifers from CON cows were heavier than heifers from LSM cows ($P = 0.04$) by 313 d of age when BCS observation terminated.

Heifer reproductive analysis

Serum progesterone levels were not different between treatments ($P = 0.85$). Only 27 heifers (30%) attained puberty by 315 d of age (14 CON and 11 LSM; $P = 0.42$).

Table 3.4. Experiment 1: Calf performance born from cows supplemented with control (CON) or linseed meal (LSM) pellet during late gestation.

	Steers				Heifers			
Item	CON	LSM	SEM	*P*- value	CON	LSM	SEM	*P*- value
Live body weight, kg								
Birth weight	43.4	44.1	1.14	0.73	41.2	42.0	1.53	0.74
Weaning weight (d 170)	261.3	255.3	4.15	0.37	251.5	240.8	9.31	0.45
Initial weight[bc]	286.5	280.5	4.12	0.36	259.6	248.4	8.32	0.38
Mid weight[bc]	450.2	435.2	7.66	0.23	295.3	284.3	8.09	0.38
Final weight[bc]	584.7	548.2	9.69	0.04	332.9	321.7	8.43	0.38
Gain, kg								
Initial ADG	0.9	0.9	0.04	0.97	0.6	0.5	0.12	0.83
Mid ADG	1.2	1.2	0.05	0.64	1.2	1.1	0.10	0.68
Final ADG	1.6	1.1	0.21	0.15	0.6	0.6	0.08	0.97
Overall ADG	1.4	1.3	0.08	0.54	0.8	0.8	0.05	0.85
Reproduction								
Day of pubertal age[d]	-	-	-	-	187.0	181.2	7.01	0.58
Breeding weight, kg (d 440)	-	-	-	-	478.8	471.0	11.86	0.66
Pregnancy, % (d 502)					100.0	90.1	5.64	0.26
Carcass								
Hot carcass, kg	349.0	345.8	3.18	0.53	-	-	-	-
Marbling[e]	507.4	459.5	16.24	0.09	-	-	-	-
Back fat, cm	1.0	1.0	0.13	0.77	-	-	-	-
Ribeye area, cm	32.3	33.0	0.38	0.13	-	-	-	-
KPH	2.5	2.5	0.05	0.59	-	-	-	-
Yield grade	2.6	2.3	0.16	0.34	-	-	-	-

[a] Control pellet (CON) was comprised of sunflower meal.
[b]Steer initial, mid, final weight were recorded on d 198, 298, 397 respectively.
[c]Heifer initial, mid, and final weight were recorded on d 182, 226, 280 respectively.
[d]Day of pubertal age was determined when P_4 blood serum was > 1.0 ng/ml.
[e]Marbling score: Small^0 = 400.

Table 3.5. Experiment 2: Effect of maternal supplement of control (CON) or linseed meal (LSM) pellet during early lactation on heifer growth performance.

	Treatment			
Item	CON[a]	LSM[b]	SEM	*P*- value
Weight, kg				
Birth	39.1	40.7	1.38	0.40
d 60	97.9	102.3	4.21	0.20
Weaning (d 207)	234.8	242.7	9.00	0.31
ADJ Weaning	242.5	253.8	7.20	0.11
Final (d 315)	286.1	292.4	6.40	0.04
BCS (1-9 scale)				
d 229	5.7	5.8	0.05	0.22
d 287	5.4	5.4	0.05	0.22
d 313	5.9	5.9	0.05	0.22

[a]Control pellet (CON) DDG and sunflower meal n = 49
[b]Linseed Meal pellet (LSM) n = 39
[c]Maternal pellet supplementation was fed at 2.2 kg/hd/d
[d]Diets were formulated as suggested by NRC, 1996 recommendations.

Discussion

We reject our hypothesis that feeding LSM to cows during late gestation or early lactation would impact growth or reproductive performance of beef calves. Recent research of metabolism of flaxseed in ruminants have indicated that mammalian lignans of enterolactone and enterodiol are present in ruminal fiuid, plasma and milk with levels of enterodiol being significantly lower than of enterolactone in titrated diets (Petit et al., 2008; Zhou et al., 2009; Gagnon et al., 2009). While we did not measure enterolactone or enterodiol in the current project, we assumed that exposure to these lignans would occur. We did not observe changes in growth or performance in offspring derived from dams that would have been exposed to the mammalian lignans during critical periods of development.

In the current study, LSM did not impact calf birth weight and weaning weight regardless of when LSM was supplemented. Tou et al. (1998) reported that maternal

supplementation of rats with 10% SDG rich flaxseed during lactation resulted in smaller pups at birth and reduced body weight at puberty. However, when LSM was supplemented to lactating cows, there was a tendency for heifers to have a greater adjusted weaning weight compared to heifers from CON cows.

Heifers born from cows supplemented during late gestation were similar to heifers born from control dams in post weaning body weights and overall ADG. Interestingly, by 397 d of age, steers born to LSM supplanted dams during late gestation weighed less than steers from CON dams. However, ADG did not differ thought out the finishing period. This is in agreement with data by Ward and coworkers (2001) who reported no difference in post natal gain in rat offspring born from dams supplemented with either flaxseed or purified SDG fed verses control animals.

Age at puberty was not significantly affected by treatment in heifers born from LSM supplemented dams during late gestation or early lactation trials. We were surprised by our puberty data as nine heifers in Experiment 1 attained puberty before 6 months of age, and in experiment 2 only 30% of the heifers attained puberty by the completion of the trial at 315 d of age. Previous studies (Martin et al., 2007), demonstrated heifers may start to cycle at 7 months of age, which can be determined my P_4 serum level analysis. The heifers that were born in Experiments 1 and 2 followed different reproductive maturity curves which were unpredictable based on similar ecoregion and conventional management for heifer development. Therefore the difference in age of reproductive maturity could be attributed to breed. Cattle breeds that have a greater mature weight weigh more at puberty and are older than heifers with a lighter mature weight (Martin et al., 1992). Additionally Martin et al. (1992) reported that breeds selected for high milk production, such as

Simmental breed used in Experiment 1, weigh less at puberty than similar moderate milking ability mature weight breeds, such as those used in Experiment 2, which may explain some of the differences between the two studies.

The heifers born from late gestation supplemented dams were followed through breeding and first conception. Mature body weight at breeding, conception rate, and pregnancy rates for heifers exposed to SDG in utero were not different compared to heifers born to dams supplemented with the CON treatment. This is in agreement with Ward (2001) who reported no difference in reproductive parameters in rat offspring born from flaxseed or purified SDG fed verses control animals. However, Tou et al. (1998) reported rat dams supplemented with 10% flaxseed during gestation and lactation gave birth to females who had decreased age to puberty, lengthened estrous cycles which resulted in persistent estrus this may result in greater lifetime reproductive productivity.

In Experiment 1, the HCW, backfat, REA, KPH and final yield grade from steers born from LSM supplemented cows, were not different from CON cows. However marbling score tended to be greater for the steers born from the CON supplemented pellet treatment during gestation than the LSM treatment Increase in body mass could indicate that the circulating levels of E2 increased the activity of angiogenic factors and vasculature resulting in the development of tissue mass.

In summary LSM supplemented in maternal diet during late gestation or early lactation did not have any negative effects on growth of calves, and did not influence onset of puberty in heifers. However, due to the physiologic impacts that LSM appears to have on several organ systems (O'Neil et al., 2009 and Ilse et al., pending) further research is warranted to determine effects of LSM on livestock during critical developmental stages.

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CHAPTER 4. GENERAL CONCLUSIONS

In conclusion to the investigation of linseed meal (LSM) supplementation to ruminants on estrogen sensitive tissue, growth and reproductive performance we concur with the review of the literature in that secoisolariciresinol diglycoside (SDG) rich LSM does have biological activity when E2 is present. However the extent of the estrogenic response or possibility of antiestrogenic characteristics of LSM supplementation cannot be determined by this thesis.

Further investigation is needed to determine the competitive binding or affinity of enterodiol and enterolactone to one or both of the E2 receptors ERα or ERβ which could be the determining factor of estrogenic or antiestrogenic effects of exogenous exposure of E2 mimicking compounds. Further Gagnon et al. (2009) reported that the species composition of the ruminal microbial ecosystem may play a role in the metabolism of SDG in the hull of the flaxseed or LSM determining the ratio of mammalian lignans enterolactone (EL) and enterodiol (ED) which competitively bind to ER resulting in potential estrogenic or antiestrogenic tissue response in ruminants. The potential estrogenic response would be of great interest over time especially during cyclic phases of estrous or pregnancy.

In regard to tissue development, Dunn et al. (2003) reported that supplementation of flaxseed to beef steers had no effect on IGF-1 concentrations, but when combined with an implant containing trenbolone acetate and estradiol-17β (TBA) resulted in an increase in IGF-1concentrations in circulation. However muscle biopsies indicated supplemental flax reduced IGF-1 mRNA independent of α-linolenic acid of the flax or implant strategy. O'Neil et al. (2009) also reported increased circulating IGF-1 levels due to LSM, but then levels decreased over the 14 day feeding period. Therefore Dunn et al. (2003) reported

possible antiestrogenic effect with the supplementation of flaxseed and exposure to a TBA implant. The reported average daily gain (ADG) of flaxseed supplemented steers was lower compared to implant alone. But when flaxseed was supplemented alone steers had greater ADG than the non-implanted or the non-flax supplemented negative control steers (Dunn et al., 2003). It appears that feeding flaxseed, in addition to steroidal growth implants, has a positive estrogenic tissue growth effect and then at an undefined threshold becomes a negative antiestrogenic effect on animal performance.

Other mechanisms of action in mammalian lignans effect during critical development periods of offspring may occur. Zhou et al. (2009) reported that supplementation of SDG to goats resulted in measurable SDG in serum, and that SDG supplementation also the resulted ruminal pH decrease and non-protein nitrogen and volatile fatty acids increased. With this evidence, performance of offspring fed SDG supplementation could be the result of greater partitioning of nutrients from the dam rather than estrogenic steroidal improvement to the offspring.

Further, heifers that reach reproductive maturity at an earlier age than later counterparts are more productive contributors of a beef cow herd (Lesmeister et al., 1973; Ciccioli et al., 2005). Steers that have a greater growth curve due to efficient partitioning of energy and utilize nutrients for muscle growth and intramuscular fat deposition have greater market value than less efficient counterparts.

To address the inconsistency reported and reviewed in the thesis of effects of linseed meal on growth and reproductive performance in ruminant's further research is needed on the biological micro flora population of the rumen. Specifically work should focus on ruminal metabolism of LSM and how particular species affect available

mammalian lignans, pH or direct nutrients to supplemented animal or offspring during critical developmental periods. Further research should include the long term effects of phytoestrogen exposure to ruminants over lifetime productivity and generational reproductive efficiency for potential epigenetic responses.

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