EFFECTS OF DIETARY SUPPLEMENTATION OF FLAXSEED AND METABOLIZABLE PROTEIN ON REPRODUCTION IN MARES AND EWES

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MASTER OF SCIENCE

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ABSTRACT

Two experiments evaluated the effects of dietary supplementation on maternal reproductive parameters. In Experiment 1, 16 mares were assigned to dietary treatments: control, flaxseed, or linseed meal for 16 wk. Blood samples were analyzed for progesterone (P_4), serum chemistry panel values, and fatty acids. Flaxseed supplementation increased ($P < 0.01$) alpha-linolenic acid concentration, but treatment had no other effects. In Experiment 2, 45 multiparous ewes were allotted to dietary levels of metabolizable protein (MP): 60% (MP60), 80% (MP80), or 100% (MP100) from d 100 to d 130 of gestation. At d 130 ewes were slaughtered and tissues harvested. There was a day x fetal number interaction for P_4, estradiol 17β, and thyroxine (T_4) along with a treatment x fetal number interaction for T_4. There was a day effect for cortisol and triiodothryonine. Results indicate dietary supplementation alters maternal parameters including hormones and fatty acids.

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LIST OF ABBREVIATIONS

µg .....Microgram

µL ..... Microliter

µm .....Micrometers

AA.....Arachidonic acid

ALA .....Alpha linolenic acid

ALB..... Albumin

ALKP ..... Alkaline phosphatase

ALT.....Alanine aminotransferase

AST .....Aspartate aminotransferase

AUC .....Area under the curve

B .....Beginning

BCS ..... Body condition score

BF..... Backfat thickness

BUN .....Blood urea nitrogen

BW .....Body weight

BWT..... Body wall thickness

C .....Celsius

CA .....Calcium

CH .....Corpus hemorrhagicum

CIDR .....Controlled internal drug release

CK .....Creatine kinase

CL .....Corpus luteum

CON ..... Control

CP ..... Crude protein

CREA ..... Creatinine

CV ..... Coefficient of variation

d ..... Days

DE ..... Digestible energy

DHA ..... Docosahexaenoic acid

dL ..... Decilitre

DM ..... Dry matter

DP ..... Digestible protein

DPA ..... Docosepentaenoic acid

E ..... Ending

E_2 ..... Estrogen

EBW ..... Eviscerated body weight

EPA ..... Eicosapentaenoic acid

F ..... Final

FLAX ..... Flaxseed

FLX ..... Flaxseed

FSH ..... Follicle stimulating hormone

FSM ..... Flaxseed meal

FSO ..... Flaxseed oil

g ..... Grams

GGT ..... Gamma-glutamyltransferase

GLC.....Gas liquid chromatography

GLOB..... Globulin

GLU ..... Glucose

GnRH ..... Gonadotropin-releasing hormone

h.....Hours

H_2 .....Hydrogen

IgG .....Immunoglobulin G

kg.....kilograms

LA .....Linoleic acid

LDH .....Lactate dehydrogenase

LEA.....Loin eye area

LH .....Luteinizing hormone

LM.....Longissimus muscle

LSM ..... Linseed meal

m .....Meter

max.....Maximum

Mcal .....Megacalorie

mg ..... Milligram

min .....Minimum

mL ..... Milliliter

mm .....Millimeters

MP..... Metabolizable protein

n..... number of samples

ng.....Nanogram

NRC ..... National Research Council

P .....Pre-treatment

P_4.....Progesterone

pg..... Picogram

PGE_2.....Prostaglandin E_2

$PGF_{2\alpha}$ .....Prostaglandin F2alpha

ppm .....Parts per million

PUFA .....Polyunsaturated fatty acid

RDP.....Ruminally degradable protein

RIA.....Radioimmunoassay

RUP..... Rumen undegradable protein

SCP .....Serum chemistry panel

SD ..... Standard deviation

SDG.....Secoisolariciresinol diglycoside

SEM .....Standard error of the mean

T_3..... Triiodothyronine

T_4.....Thyroxine

TBIL..... Total bilirubin

TNF-α.....Tumor necrosis factor-α

TP.....Total protein

CHAPTER I. LITERATURE REVIEW

Introduction

Efficient animal production is heavily reliant on good reproductive management. A variety of circumstances and events influence these successful production practices. Environment, management practices, nutritional balance, reproductive technologies, nutritional supplementation, and breeding soundness of the animals are all factors which can positively or negatively affect reproductive efficiency and offspring outcome. While producers can do very little to control the environment of animals that live outdoors, they do have the ability to alter nutritional aspects. A balanced production setting encompassing adequate nutritional diets as well as correct reproductive management can help to optimize production. This literature review will focus on three main topics, starting with a review of the female reproductive system, followed by fatty acid and metabolizable protein (**MP**) supplementation and the way these may influence reproduction.

Reproductive Cycle

Classifications

Understanding the female reproductive cycle is imperative to a successful breeding program. The estrous cycle is defined as reproductive events beginning with estrus, also termed "heat", and continuing until the following estrus. The estrous cycle consists of two major phases, the follicular phase and the luteal phase. These two phases are dominated by different ovarian structures and hormones which work together to ensure a successful ovulation and possible pregnancy. The specifics of each phase of the estrous cycle are detailed below.

The follicular phase encompasses proestrus and estrus. Proestrus is a time of preparation for mating when there is formation of the follicle and a rise in estradiol ($\mathbf{E_2}$) secretion. The

second part of the follicular phase, estrus, is a time of sexual receptivity and peak E_2 secretions. Common indicators of estrus in livestock may include: standing to be mounted, flagging of the tail, winking of the vulva, and urination. The primary ovarian structure during this phase is the follicle and the dominant hormone is E_2 which is secreted from follicles on the ovary. The follicular phase concludes with ovulation (release of the egg from the follicle).

The second phase, the luteal phase, includes metestrus and diestrus. Metestrus is a time when formation of the corpus hemorrhagicum (**CH**) (the structure present on the ovary where ovulation occurred) begins. The CH then becomes what is known as the corpus luteum (**CL**). Formation of the CL is accompanied by a rise in the hormone progesterone (**P_4**), secreted from the CL, and a drop in E_2. The second part of luteal phase, diestrus, is the period where a fully functional CL is present and P_4 is highest (Senger, 2003). The primary structure during the follicular phase is the CL and the dominant hormone is P_4. If pregnancy is not established, the CL will be lysed and the dam will return to the follicular phase and the cycle will restart.

When examining the estrous cycle in more depth, variations are noted among our domestic livestock species in reproductive classification, length of the estrous cycle, duration of estrus, and gestation length. When considering our four main livestock species (cow, ewe, mare, and sow) we note they are classified into two main reproductive classifications: polyestrous (having multiple estrous cycles distributed evenly throughout the year; includes cow and sow) or seasonally polyestrous (exhibiting multiple estrous cycles during a particular season of the year; includes ewe and mare). Length of the estrous cycle remains fairly consistent with cows, sows, and mares exhibiting an average 21 day estrous cycle and ewes demonstrating a slightly shorter cycle averaging 17 days (Stabenfeldt and Edqvit, 1993). The duration of estrus among the cow, ewe, sow, and mare shows more variation with means of 15 h, 30 h, 50 h, and 7 d, respectively

(Senger, 2003). Gestation length also varies greatly among these species with sows having the shortest gestation length (113 d), followed by the ewe (147 d), cow (280 d), and with mares exhibiting the longest gestation length (345 d). Although these species show variation between their cycles, the mechanism by which the cycle is regulated is very similar.

Key Estrous Cycle Hormones

The endocrine system plays an integral role in coordination of reproduction among our livestock species. Hormonal communication between the hypothalamus and anterior pituitary, both found in the brain, and the ovaries regulates the female reproductive system. These organs and the hormones secreted in the body work together through positive and negative feedback systems to regulate the estrous cycle. Therefore, altering a single hormone can consequently affect other hormones within the feedback system, causing alterations in the cycle.

As stated earlier, mares and ewes are seasonally polyestrous breeders. This seasonality is regulated by photoperiod (length of daylight). The mechanism which regulates seasonality will be discussed in depth later in the review, but for now, we will briefly discuss the hormone which helps to regulate seasonality, melatonin. Melatonin is secreted from the pineal gland which is found in the brain (Senger, 2003). Once secreted, melatonin can be either inhibitory or stimulatory to the other organs associated with reproduction in seasonally polyestrous species. Hence, melatonin is the signaling factor which tells seasonally polyestrous females when to start and stop cycling.

Gonadotropin-releasing hormone (**GnRH**) is produced by a network of neurons in the hypothalamus and is released in low amplitude basal pulses during most of the estrous cycle except prior to ovulation when an acceleration in the frequency of GnRH release is noted (Kalra et al., 1997). Gonadotropin-releasing hormone from the hypothalamus is secreted into the

hypothalamo-hypophyseal portal system and is the primary brain signal for the release of the gonadotropin hormones luteinizing hormone (**LH**) and follicle stimulating hormone (**FSH**) from the anterior pituitary (Schally et al., 1971; Kalra et al., 1997; Walton et al., 2011).

The two gonadotropin hormones, FSH and LH, work together to regulate ovarian activity (Turner, 1938). Follicle stimulating hormone is the key regulator of follicular recruitment and assists in maturation of ovarian follicles (Driancourt, 2001). The cells around the follicle produce E_2 which is responsible for estrus in our livestock species. Estradiol secreted from the follicle plays an important role in the behavioral changes during estrus and relaxation of the cervix in preparation for breeding.

Luteinizing hormone is important as it assists in rupture of the follicle, leading to discharge of the egg (Turner, 1938). Concentrations of LH remain low during the mid-luteal phase of the estrous cycle and rise before the onset of estrus to a peak usually at ovulation. This surge of LH is the trigger for rupture of the follicle. Following rupture of the follicle, the CH and then the CL are formed. The CL secretes P_4 which is vital in preparing the uterine endometrium for a fertilized egg. As P_4 increases, LH decreases to minimal concentrations by the mid-luteal phase when P_4 is greatest (Karsch, 1987). If fertilization is accomplished, the CL remains and continues to produce P_4 which is important for maintenance of pregnancy. High levels of P_4 will send a negative feedback to the hypothalamus inhibiting release of GnRH and consequently decreased release of FSH and LH, hence leading to cessation of cycling.

Prostaglandin $F_{2\alpha}$ (**$PGF_{2\alpha}$**) is produced by cells of the uterine endometrium (Senger, 2003). Oxytocin can stimulate the release of $PGF_{2\alpha}$ and it is thought that these two hormones work in a positive feedback loop to lead to luteolysis of the CL. In order for the uterus to secrete $PGF_{2\alpha}$ it must have adequate reserves of arachidonic acid (**AA**) which is the fatty acid $PGF_{2\alpha}$ is

synthesized from. This pathway will be discussed further in the fatty acid portion of this literature review.

Feedback System

The previously mentioned hormones work together to maintain regular cyclicity in our livestock species. (Figure 1.1) Positive and negative feedback systems control release of GnRH which in turn controls release of the other hormones from the anterior pituitary. During proestrus and into estrus when E_2 from the follicle is highest, E_2 signals the hypothalamus to release a surge of GnRH. This GnRH is delivered to the anterior pituitary where it binds and triggers synthesis and secretion of FSH and LH (Bliss et al., 2010). These two gonadotropins continue to stimulate production of E_2 which peaks just prior to ovulation. As the follicles continue to develop, they continue to produce E_2 as well as inhibin which suppresses FSH secretion. Once a threshold level of estrogen is reached, there is a significant increase in hypothalamic GnRH, culminating in a preovulatory surge of LH (Bliss et al., 2010). Following this surge, LH levels drop and remain fairly low throughout the other phases of the estrous cycle. Livestock species will ovulate shortly after the surge of LH.

Following ovulation, in metestrus, the CH is formed and P_4 levels begin to rise which is vital in preparing the uterine endometrium for a fertilized egg. This increase in P_4 provides negative feedback on the hypothalamus leading to a decrease in secretion of GnRH and resulting in a decrease in LH and FSH (Karsch, 1987). The decrease in hormone levels results in insufficient follicular development to produce high enough levels of E_2 to continue the estrous cycle. Diestrus is reached with a plateau of P_4 and a fully functioning CL. If fertilization is accomplished the CL will remain and continue to produce P_4; therefore, the inhibition will remain and cycling will cease. However, if pregnancy is not detected, pulsatile secretions of

$PGF_{2\alpha}$ will commence from the lining of the uterus. The presence of $PGF_{2\alpha}$ will result in lysing of the CL and a drop in circulating levels of P_4, removing inhibition from the hypothalamus and resuming the cycle.

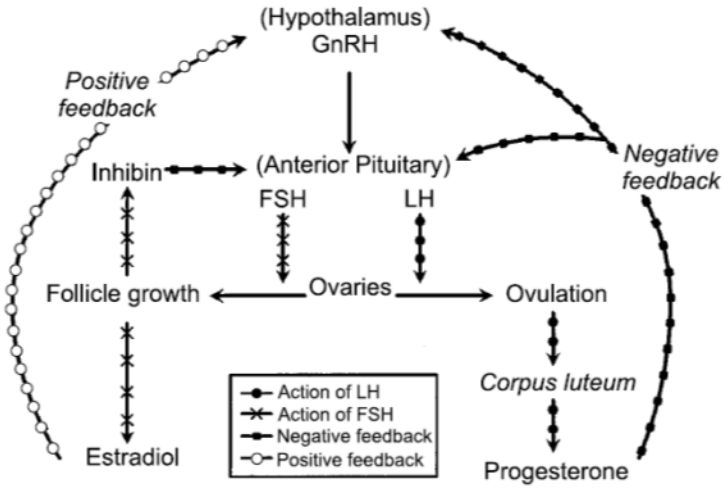

Figure 1.1. Female reproductive hormone feedback pathways (Coffey et al., 1997).

Photoperiod

This discussion of photoperiod will focus on the ewe (short day breeders) and mare (long day breeders) which are seasonally polyestrous livestock animals. Photoperiod is the most crucial environmental factor which affects the reproductive cycle in seasonal breeders as it controls release of the hormone, melatonin, from the pineal gland.

The pineal gland, a small gland located in the brain, is responsible for synthesis and secretion of melatonin. The process begins with the retina, where light is received. Information is then transmitted from the retina via the optic nerves to the suprachiasmatic nuclea located in the anterior hypothalamus (Senger, 2003). This output then travels to the superior cervical ganglion and finally on to the pineal gland. An increase in excitation of neurons caused by an increase in light, leads to increased inhibition of the pineal gland and thus decreased production of

melatonin. The opposite occurs with decreased light; inhibition on the pineal gland is removed and production of melatonin is resumed. In short, melatonin is of low concentration in the daylight and higher concentration during darkness (Waller et al., 1988).

Melatonin plays a critical role in hypothalamic-pituitary gonadal activity (Dickson, 1993). Melatonin release influences the release of GnRH from the hypothalamus, therefore influencing cyclicity in seasonally polyestrous animals. The way in which mares and ewes respond to melatonin levels is different. Mares are classified as long-day breeders, meaning as day length increases during the spring the mare will begin to cycle and during the short days of fall and winter mares will become anestrus (a period of sexual quiescence). In mares, the increased length of daylight inhibits melatonin release from the pineal gland. The decreased melatonin removes inhibition from the hypothalamus allowing for increased release of GnRH. Ewes are classified as short-day breeders, with short days of fall marking the beginning on their cycling and longer days of summer being a time of anestrus. In the ewe, increased melatonin stimulates increased GnRH release (Senger, 2003).

Manipulation of the Estrous Cycle

Manipulation of the estrous cycle serves as a production tool to maximize efficiency in breeding programs. Two common practices utilized to manipulate the estrous cycle are the use of artificial lighting and synthetic hormones. Although these two manipulation practices serve to meet the best needs of each production setting, many extrinisic factors such as poor nutrition and stress may also affect the estrous cycle. Nutritional impacts will be briefly discussed in this portion of the literature review while a more in-depth evaluation of the effects of nutritional manipulation will be provided in the fatty acid and MP sections of this review. These extrinsic

factors may negatively affect the maternal unit leading to economic loss as a result of increased costs associated with rebreeding or loss of offspring.

Manipulation of natural lighting schemes can lead to changes in regular estrous cycles for seasonal breeders. In long-day breeders an increase in lighting is required. Mares who were in a time of anestrus and then exposed to 16 hours of daylight, a combination of natural and artificial lighting, for at least 60 days, resulted in reduction in the number of days to first ovulation, increased number of mares that ovulated, and the total number of ovulations was greater (Malinowski et al., 1985). In addition, mares exposed to longer daylength during the long days of winter had increases in the number of follicles greater than 10 mm and 20 mm, average diameter of follicles, and diameter of the largest follicle (Sharp and Ginther, 1975). In the same study by Sharp and Ginther (1975), all treated mares exhibited one or more signs of estrus and two of the seven mares ovulated during the project while none of the control mares demonstrated signs of estrus. For short-day breeders, a decrease in lighting is necessary for cyclicity to resume. Also, in sheep, the use of melatonin either orally or implanted has helped to advance the breeding time (Stabenfeldt and Edqvist, 1993; Waller et al., 1988).

Cycling can be manipulated in our livestock species through use of synthetic progestins or administration of $PGF_{2\alpha}$. Methods which use P_4 to manipulate the cycle focus on the ability of these synthetic P_4 sources to mimic the action of natural P_4 produced by the CL (Abecia et al., 2012). Progesterone treatments have been utilized in mares to better manage the transitional period and help advance the first ovulation of the year as well as to block signs of estrus in performance animals. In mares, daily injections of at least 100 mg of P_4 blocked estrus and ovulation (Loy and Swan, 1966). Progesterone administered in gilts on d 15 of the estrous cycle

was successful in suppressing heat and ovulation and those on the high dose of 100 mg returned to a normal appearing heat on d 6 or d 7 after conclusion of injections (Ulberg et al., 1951).

Furthermore, progestins have been used to shorten the anestrus interval in our livestock species, therefore helping to alleviate some of the reproductive failure in the industry. Controlled internal drug release (**CIDR**) (coated with synthetic progesterone) treatment in cows has been shown to induce ovulation and initiate normal estrous cycles earlier than control cows (Perry et al., 2004). Transitional follicles in mares which were progesterone-primed had a higher response to human chorionic gonadotropin (synthetic LH) and 93.1% of the treated mares ovulated within 48 hours of treatment, whereas only 58.7% of non-primed mares ovulated (Cuervo-Arango and Clark, 2010). Use of synthetic P_4 has become a staple in many production settings because of its ability to successfully suppress heat and ovulation as well as shorten the anestrus cycle. Another common synthetic hormone used for manipulating the estrous cycle is $PGF_{2\alpha}$. This hormone has become popular due to its luteolytic properties (Miller et al., 1976; Abecia et al., 2012). Injection of $PGF_{2\alpha}$ has been shown to shorten the inter-ovulatory interval and interval between injection and estrus or ovulation in mares (Miller et al., 1976; Ginther, 2007). In addition, cattle treated with $PGF_{2\alpha}$ during diestrus showed a drop in P_4 by 50% 12 hours following administration, estradiol vastly increased by 24 hours, estrus began at 72 hours after $PGF_{2\alpha}$ treatment, and cows ovulated within 95 hours of administration (Hafs et al., 1974). However, the timing of $PGF_{2\alpha}$ administration is crucial to its mode of action, as it must be given in the presence of an active CL (Abecia et al., 2012). Cattle administered $PGF_{2\alpha}$ on day three of the estrous cycle showed no signs of luteolysis (Hafs et al., 1974). In addition, an experiment by Douglas and Ginther (1975) found that newly formed CL in mares, noted as those from d 1 to d 4 post ovulation, are not susceptible to the luteolytic effects of $PGF_{2\alpha}$, whereas older CL, those from d 4 to d 13 post

ovulation, are susceptible to the effects of $PGF_{2\alpha}$. Due to these findings it has become a common practice in production settings to administer this synthetic hormone in a two shot series for synchronization of a herd. The second shot given 14 days following the initial shot serves to lyse the CL in any mares which were in the early time frame of CL formation and not sensitive to $PGF_{2\alpha}$ at the time of the first injection.

It is well documented that plane of nutrition can negatively affect fetal outcome and embryonic loss. In a study on mares by Van Niekerk and Van Niekerk (1998), it was shown that mares receiving a low quality protein diet had an early embryonic loss rate of 35.7% compared to 7.3% for mares receiving a higher quality protein diet. This same trend of increased embryonic loss with decreased protein was noted in rats, where complete removal of protein from the diet induced an 86-100% embryonic loss (Leathem, 1966). Furthermore, it has been shown that mares fed to lose body condition pre-partum and then maintained at a low body condition had a lower pregnancy rate compared to mares kept at an ideal body condition (Hennecke et al., 1984).

Stress is another extrinsic factor which should be considered when discussing alteration of the estrous cycle. Van Niekerk and Morgenthal (1982) reported that stress associated with factors like severe pain and infectious disease resulted in a 30-50% decrease in circulating progesterone levels in pregnant mares. This vast decrease in progesterone levels may lead to luteal insufficiency resulting in embryonic loss.

As discussed in the previous sections, the estrous cycle is complex and reliant on all mechanisms to work together for successful ovulation to occur. A comprehensive understanding of the estrous cycle including species variations, key hormones, duration and manipulation of estrous are essential when designing and implementing good reproductive management

procedures. Quality nutrition is also essential to maximize efficiency and minimize embryonic loss. The next part of this review will focus on fatty acids and the effects supplementation with fatty acids can have on reproduction.

Fatty Acid Supplementation

Omega-3 and omega-6 fatty acids are termed essential fatty acids, meaning they cannot be synthesized within the body and must come from the diet (Calder and Grimble, 2002; Coletta et al., 2010). These essential fatty acids are necessary for important physiologic functions such as energy storage, cell membrane function, cell signaling, regulation of inflammation, and cell proliferation (Bilby et al., 2006; Coletta et al., 2010; Fabian and Kimler, 2013). Understanding how nutritional supplementation changes plasma concentrations of omega-3 and omega-6 fatty acids is vital, as these are precursors for prostaglandins, thromboxanes, and leukotrienes which play important roles in inflammatory and reproductive processes. This literature review will focus primarily on nutritional supplementation with omega-3 fatty acids and its effects on reproductive status, health benefits, and organ mass.

Omega-3 fatty acids are commonly supplemented either using a marine derived source or through a plant source (Figure 1.2). The most common sources of marine based omega-3 fatty acids come from seafood, fish oils, and algae (Coletta et al., 2010). Cold water oily fish such as salmon, tuna, and mackerel have been shown to possess the most biologically potent omega-3 fatty acids, eicosapentaenoic acid (**EPA**) (C20:5n-3) and docosahexaenoic acid (**DHA**) (C22:6n-3). These fatty acids can be incorporated directly into the cell membranes after ingestion and absorption (Fabian and Kimler, 2013). Supplementation of horses with marine derived sources has been shown to elevate the concentrations of EPA and DHA in plasma (James et al., 2000; King et al., 2008).

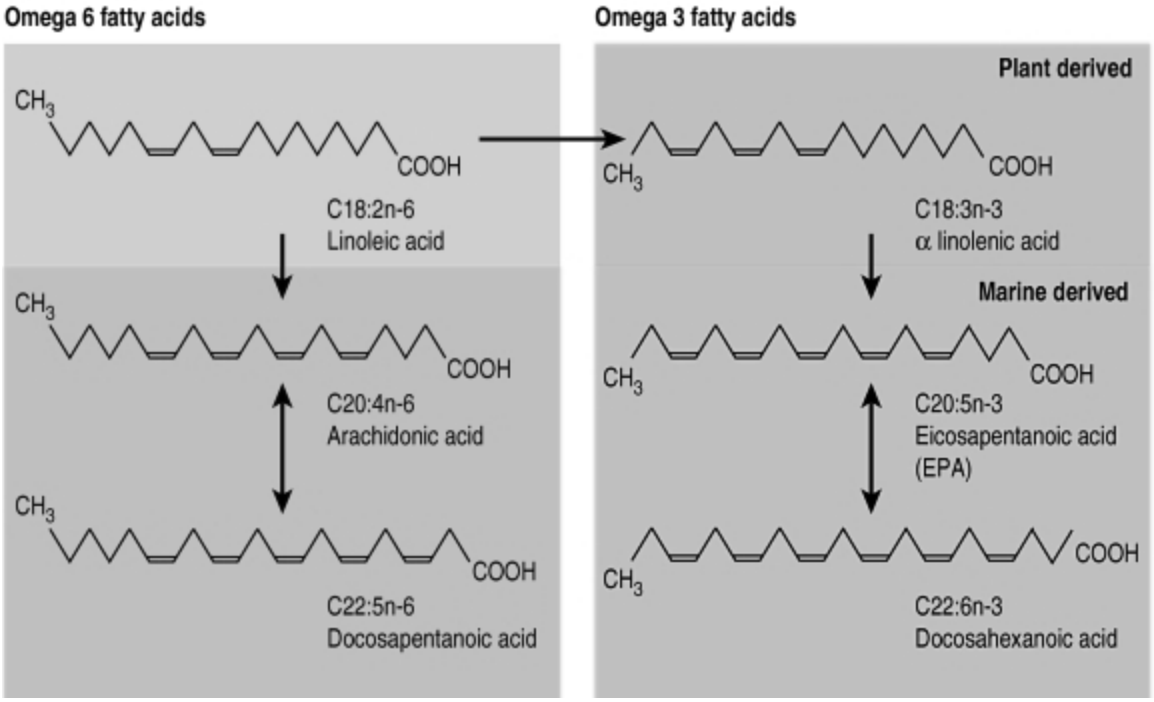

Figure 1.2. Structures of the two classes of polyunsaturated fatty acids (Din et al., 2004).

Common plant sources of omega-3 fatty acids which supply high levels of alpha-linolenic acid (**ALA**) (C18:3n-3) include: flaxseed, walnuts, and canola oil (James et al., 2000; Fabian and Kimler, 2013). Inclusion of flaxseed into the diet leads to increases in the concentration of total PUFA in plasma with significant increases in ALA and EPA; however DHA was not altered by flaxseed supplementation (Hansen et al., 2002; Farmer et al., 2007). A similar result of increasing EPA but not DHA was also observed in pregnant rats supplemented with high levels of flaxseed (Wiesenfeld et al., 2003).

Changes in plasma fatty acid levels may prove to be important to improved physiology. For instance, ALA may affect reproduction as omega-3 fatty acids have been shown to be important as structural fats and precursors for prostaglandins (Singh et al., 2011). Furthermore, omega-3 fatty acids lead to the production of eicosanoids which are important in animal health as anti-inflammatory agents. In addition, omega-3 fatty acid supplementation may lead to

alterations in organs which could affect overall physiology of the animal. As will be discussed in the following sections, supplementation with different types and levels of fatty acids elicits varying changes to plasma fatty acid concentrations and can have both positive and negative effects on animal physiology.

Reproductive Effects

Hormones

As discussed previously, reproduction is dependent upon hormonal balance and any change in reproductive hormones may alter reproductive efficiency. Follicle numbers, suppression of $PGF_{2\alpha}$, and maintenance of the CL are very important steps for establishment and maintenance of pregnancy. Flaxseed supplementation has been shown to affect all of these steps by exhibiting either estrogenic or anti-estrogenic effects.

Flax serves as a source of lignans, principally secoisolariciresinol diglycoside (**SDG**), which are members of a class of compounds termed phytoestrogens. These are diphenolic compounds similar in structure to endogenous sex steroid hormones (Duncan et al., 2003; Frische et al., 2003), and are so named due to their ability to produce responses via estrogen receptors (Collins et al., 1997). When SDG is consumed, it is broken down by intestinal microflora and enzymes into enterodiol and enterolactone (Axelson et al., 1982; Begum et al., 2004). Enterodiol and enterolactone are structurally similar to E_2 and exhibit estrogen-like or anti-estrogen-like properties depending on dose, duration of administration, and stage of development (Collins et al., 2003; O'Neil et al., 2009). These new compounds are absorbed into the bloodstream and proceed to interact with estrogen receptors.

Ovarian and endometrial synthesis of $PGF_{2\alpha}$ has been shown to be reduced by dietary fatty acids. Wiesenfeld et al. (2003) found feeding pregnant rats 40% flaxseed or 26% flaxseed

meal (**FSM**) led to a significant decrease in serum levels of AA compared to controls. This reduction in AA is likely the result of a shift in the diet to include a larger ratio of omega-3 fatty acids and a change in the ratio of omega-6:omega-3 fatty acids. It is well known that linoleic acid, an omega-6 fatty acid, is metabolized to AA which serves as a precursor for the pro-inflammatory prostaglandins (James et al., 2000). Furthermore, an increase in ALA as a result of inclusion of flaxseed in the diet may lead to an increase in EPA which has been shown to act as an inhibitor of AA conversion to prostaglandin E_2 (**PGE_2**). The mechanism for this is due to competition in the enzymatic pathway at the point of cyclooxygenase converting AA to prostaglandin H_2 which is then further converted to PGE_2 and $PGF_{2\alpha}$ (James et al., 2000). As was discussed in the reproductive section of this literature review, $PGF_{2\alpha}$ is a substance which lyses the CL, allowing the female to return to estrus. Also, during early pregnancy in pigs, estradiol from embryonic origin is involved in the shift of $PGF_{2\alpha}$ secretion from an endocrine to exocrine pathway (Bazer et al., 1986). These changes in the level or pathway of $PGF_{2\alpha}$ may help to explain the reduced embryonic mortality seen in animals supplemented with omega-3 fatty acids (Petit and Twagiramunga, 2006). This information is relevant from a production standpoint as fatty acid supplementation may serve as a way for producers to reduce embryonic loss.

Supplementation with flaxseed may also affect progesterone concentrations. Lessard et al. (2003) noted an increase in progesterone in dairy cattle fed flaxseed. These findings were supported by Petit and Twagiramungu (2006) who reported increases in blood progesterone concentrations and also reported a larger CL in supplemented cattle which could be one contributing factor to this hormonal increase. In contrast to the previously noted studies, no difference in plasma concentrations of progesterone was detected in dairy cattle by Bilby et al. (2006). Furthermore, treatment with flaxseed, flaxseed oil (**FSO**), or FSM had no effect on

circulating progesterone, prolactin, or estradiol concentrations in sows (Farmer et al., 2010). In contrast, Tou et al. (1999) documented certain levels of flaxseed supplementation markedly increased serum estradiol levels in rats. These contradicting results are most likely the result of differing species, experimental unit numbers in the trials, and varying sources, duration, and levels of flaxseed supplied.

Estrous Cycle/Puberty

As discussed previously, the reproductive cycle of our livestock species can be altered through various pathways including both natural and artificial means. Any alterations in the dam's estrous cycle or initiation/delay of puberty can either have positive or negative consequences for the producer. Flaxseed has been shown to both negatively and positively affect these two parameters of reproductive interest. In rats, flaxseed has produced a dose-related cessation, irregularity, or lengthening of the estrous cycle (Orcheson et al., 1998). The percent of rats being either acyclic or irregular increased with increasing levels of flaxseed (Orcheson et al., 1998). Female offspring exposed to 10% flaxseed during pregnancy and lactation had lengthened estrous cycles due to prolonged time in the estrus phase. However 16.7% of the 5% flaxseed supplemented rats in this same study were acyclic because of persistent diestrus. In women supplemented with flaxseed, the luteal phase of estrous was significantly longer than the follicular phase and the ratio of progesterone:estrogen was higher (Phipps et al., 1993). This may provide a better environment for the embryo and improved embryo survival leading to increased conception rates. In addition, no anovulatory cycles were noted in flaxseed supplemented females, while three were noted in controls, indicating a more normal estrous cycle in the flaxseed supplemented females (Phipps et al., 1993).

In addition to altering estrous cycles, flax supplementation has shown a dose dependent effect on the onset of puberty in rats (Tou et al., 1999). In female rat offspring, exposure to 10% flaxseed during pregnancy and lactation resulted in offspring reaching puberty significantly earlier and at a lighter body weight than those on control diets. In this same study, those exposed to 5% flaxseed had later puberty onset at the same weight as the control diet group (Tou et al., 1998). The results of this study indicate that level of flaxseed supplementation can have both positive and negative effects on the estrous cycle.

Supplementation with flaxseed has resulted in varying outcomes on the estrous cycle. More research is needed to pinpoint exact dosage and length of exposure necessary to positively influence the estrous cycle or onset of puberty.

Follicular Dynamics

Changes to size of the follicle or CL may affect conception rate. Therefore it is important to understand the effect of flaxseed supplementation on follicular dynamics and CL size. Studies have shown cows which were induced to ovulate with follicles < 11.5 mm had a smaller CL, secreted less progesterone, had decreased pregnancy rates, and increased embryonic mortality (Vasconcelos et al., 2001; Perry et al., 2005). Studies examining supplementation with flaxseed showed no effect on follicular diameter, the number of class 1, 2, and 3 follicles, or number of corpora lutea in the rat and dairy cow (Petit et al., 2001; Collins et al., 2003). In contrast to these findings, flax supplementation was shown to increase the mean diameter of the ovulatory follicle in dairy cattle (Robinson et al., 2002; Ambrose et al., 2006). However, if the increased follicle sizes were still within a normal range, there may not be a benefit to this increased follicle size. This project would need to be carried out further to look at overall pregnancy rates and embryonic survival to determine if the increase in follicular size was truly beneficial. If a

supplementation level is found which results in healthier follicles and CLs the result may be a decrease in pregnancy loss or increase in conception rate, which would both positively affect producers.

Pregnancy

Maintenance of pregnancy once it is established and gestation length are important considerations, as embryonic loss and premature offspring can have a huge economic impact on the producer. In a study by Ambrose et al. (2006), dairy cattle supplemented with flaxseed tended to have higher ($P < 0.07$) conception rates and lower pregnancy loss. In addition, Petit et al. (2001) reported flaxseed supplementation in dairy cattle resulted in modulated progesterone concentrations and lowered pregnancy loss. In contrast, Bork et al. (2010) found no change to pregnancy rates with low levels (3.35%) of flaxseed supplementation in dairy cattle. Pregnancy in rats was established and maintained with high supplementation levels up to 40% flax and 26% FSM and this did not negatively affect estrogen balance (Collins et al., 2003). A few differences between these experiments were Bork et al. (2010) was feeding an isocaloric diet with lower levels of flaxseed than Petit et al. (2001). These differences may account for some of the variations noted in pregnancy outcome between the two studies. In addition, results from the above study by Collins et al. (2003) indicate that supplementing at very high levels was not detrimental to pregnancy outcome.

There are contradictory results on the effects of flaxseed supplementation on gestation length. In rats supplemented with ground flaxseed or FSM, gestation length was not affected (Collins et al., 2003). However, in women, supplementation with omega-3 fatty acids (marine source) caused a significant increase in gestational age at delivery (Coletta et al., 2010). This increase in gestation length may not be advantageous if the fetus is not comprised at the earlier

gestation length. However, if supplementation is able to help offset some pre-term labors and allow more time for fetal development, this would be very beneficial. More research is needed looking at flaxseed supplementation and changes to gestation length.

The effect of supplementation with flaxseed on fetal development, rebreeding, lactation, and neonate outcome are important for economic success. Bork et al. (2010) reported no difference in the flaxseed treated group for days to first postpartum or second postpartum AI or days open. Collins et al. (2003) showed feeding high levels of flaxseed to rats had no effect on fertility, body weight gain, litter size, or fetal development. Wakefield et al. (2008) found contradicting results noting high maternal dietary omega-3 fatty acid exposure reduced normal embryo development in the mouse. If supplementation affects embryonic development these changes may be compounded as the offspring ages.

The results on offspring survival and performance with omega-3 supplementation are varying. Farmer et al. (2010) found flax supplementation to have no effect on birth weight, total number born, number of stillborns, or milk components in sows. However, percentage neonatal mortality on d 2 and d 21 postpartum was less for flax, FSM, and FSO compared to control (Farmer et al., 2010). Petit et al. (2004) found dairy cattle supplemented with flaxseed had a higher milk yield than controls. This does not mean there were any beneficial changes made to the composition, just that the amount was increased. This increase may be beneficial to support offspring growth; however, this was not evaluated.

Results from the above experiments show both positive and negative outcomes of supplementing with flaxseed when evaluating pregnancy. As noted previously, these discrepancies may be due to species difference and/or level, type, and length of supplementation.

Health Benefits

Preliminary findings in projects supplementing flaxseed have elicited positive health benefits. Flaxseed has been shown to have health benefits associated with decreased inflammation and reduced skin allergies. Some of the key mediators of inflammation are $PGF_{2\alpha}$ and n-6 eicosanoids which are derived from AA (James et al., 2000). As we examine how supplementation with fatty acids may alter inflammation it is important to note that the membrane of most cells contains a large amount of AA along with other fatty acids (Calder and Grimble, 2002). Therefore altering AA may impact the ability of cells to produce eicosanoids and may alter inflammatory response. Another route by which inflammation may be affected is through the modulation of cytokines. Tumor necrosis factor- α (**TNF-α**) is a potent inflammatory cytokine released in response to a stimulus (James et al., 2000). Karcher et al. (2014) saw a decrease in TNF-α expression in fish oil treated calves. This decrease in TNF-α expression may indicate a decreased inflammatory response. In support of a decreased inflammatory response, O'Neill et al. (2002) noted a significant decrease in the area of allergic reaction for horses with a dermatological ailment which were supplemented with flaxseed. However, this was a pilot study and more research is needed to truly understand this mechanism.

Flaxseed supplementation has been shown to alter reproductive structures and tumor growth which is most likely due to its estrogenic/anti-estrogenic properties. In a study by Tou and Thompson (1999), lifetime or gestation and lactation exposure to 5 or 10% flaxseed elicited mammary gland structure changes that could reduce mammary cancer risk. In the case of breast cancer, flaxseed, enterodiol and enterolactone have been shown to counteract estrogen induced tumor growth and angiogenesis in rats (Jungestrom et al., 2007). These changes to mammary tissue structure may affect milk production which in turn may affect offspring growth.

Organ Function

Flaxseed supplementation has been shown to alter organ mass including the liver and kidneys in cattle and rats (O'Neil et al., 2007; Sprando et al., 2000). A change to organ mass, however, does not necessarily indicate that function of the organ has been altered. One way to evaluate function is through examination of various enzymes and proteins which are found in the blood. In rats fed flaxseed or FSM, there was no dietary effect on blood urea nitrogen (**BUN**), albumin (**ALB**), aspartate aminotransferase (**AST**), or alkaline phosphatase (**ALKP**) (Wiesenfeld et al., 2003). Hansen et al. (2002) found flaxseed supplementation in horses had no effect on AST, gamma-glutamyltransferase (**GGT**), or creatine kinase. The serum enzymes AST, ALB, GGT, and ALP serve as indicates of liver function, while ALB and BUN are indicators of protein and renal function (Duncan et al., 1994). These results indicate no major effects on kidney or liver function (Duncan et al., 1994). However, in the study by Wiesenfeld et al. (2003), rats supplemented with 40% flaxseed had a significant increase in alanine aminotransferase (**ALT**) and significantly reduced levels of serum protein and creatinine. Alanine aminotransferase activity in many species is very low, so this alone should not be used as an indicator of liver function. Protein and creatinine differences could be due to variation in the diets provided to the animals as well as individual animal differences.

Other studies have shown that rat female offspring exposed to flaxseed during pregnancy or lactation exhibited increases in uterine and ovarian weights. Male rats supplemented with 5% flaxseed from gestation through postnatal d 132, had reductions in relative prostate weight and cell proliferation but showed no difference in sex hormone levels (Sprando et al., 2000). Male rats exposed to 10% flaxseed also had greater accessory sex gland and prostate weights (Tou et al., 1998). Interestingly, in the study by Sprando et al. (2000), exposure to 20% flaxseed

produced a lower prostate weight compared to controls while the 40% showed no statistical significance from control.

Changes in visceral organ weight suggest that function and efficiency may be altered with flaxseed supplementation. Changes to reproductive organs may have lasting effects on reproductive performance of these animals by altering hormone production or environment for the germ cells or fetus. Further research is needed to understand discrepancies in the literature which may be due to age, level, and type.

Many of the effects of supplementation with flaxseed could be beneficial in a production setting. However, results of the examined studies indicate the exact effects of flaxseed supplementation are still under investigation with a tremendous amount of contradicting results. The outcomes from supplementation seem to be dependent on species, dosage, length of exposure, and source of flaxseed. More research is clearly needed to optimize the level and source of supplementation for the producer.

Metabolizable Protein

As discussed previously, proper nutrition plays important roles in growth, reproduction, and lactation. Protein is one nutrient which has received vast consideration as it is essential to all body tissues and protein requirements vary with stage of production. Growth, pregnancy, and lactation all lead to increased protein requirements due to increased output necessary for tissue and bone growth, fetal maturation, and milk production. From a business standpoint, finding an optimum protein and energy level for each animal production group is important to maximize productivity and minimize loss.

Protein in most livestock diets is expressed as crude protein (**CP**) which is comprised of protein and non-protein nitrogen. However, CP values do not account for value to the rumen

microbes or rumen undegraded protein. Feed protein is considered to be either rumen degradable protein (**RDP**) which is protein required to meet the requirements of the rumen microorganisms and can be turned into microbial protein, or rumen undegradable protein (**RUP**) which is protein that escapes from the rumen without breakdown. Therefore what leaves the rumen is known as metabolizable protein (**MP**), composed of microbial protein and RUP.

One problem encountered in animal production is insufficient intake of protein or energy (or both). This places the animal in a negative energy balance where energy necessary for physiological functions, including growth, maintenance, reproduction, or lactation, are greater than the intake from feed (Dunn and Moss, 1992). When negative energy balance occurs, a loss of body condition usually follows. This has been noted in multiple species including: rats, swine, and sheep (Guilbert and Goss, 1932; Hammell et al., 1976; Drouillard et al., 1991). In heifers fed a diet only meeting 81% of crude protein requirements, but meeting all other nutrient needs, heifers gained slower and weighed less within 14 d of calving. Body weights and condition scores were also lower within 24 h of calving compared to heifers receiving adequate protein (Anthony et al., 1986). This same trend of adverse dietary restriction effects was noted in lambs receiving MP restriction which decreased final body weight (**BW**) and resulted in a loss of body protein, fat, and water (Drouillard et al., 1991). Furthermore, the absolute weights of lamb's liver, stomach complex, and intestines were reduced in response to the treatment when compared to their control counterparts (Drouillard et al., 1991). These negative effects on body composition may affect function as well as production.

Functional integrity of the endocrine system is reliant on adequate nutrition which is imperative for synthesis and release of hormones (Leathem, 1966). One reason for this relationship is gonadotropins released from the pituitary including FSH and LH are protein in

nature (Leathem, 1966). Therefore reduction in protein intake may negatively affect reproductive status through manipulation of these gonadotropins.

There has been conflicting results on the effects of protein restriction on hormone concentrations. Differences noted between studies may be accounted for by differences in type, level, or length of protein restriction, and reproductive status and age of the animals. Murray et al. (1979) noted no difference in serum P_4 in gilts consuming a diet low in CP. In contrast, others have noted mean P_4 concentrations which tended to be greater in heifers consuming a low protein diet (Jordan and Swanson, 1979; Anthony et al., 1986). Jordan and Swanson (1979) also found P_4 to be negatively correlated to LH; however, Knutson and Allrich (1988) reported no difference in LH concentrations in dairy cattle fed restricted diets. The LH results may differ due to the time during the estrous cycle that sampling occurred. If blood samples were taken in the luteal phase LH levels would be much lower while P_4 concentrations would be elevated. However if sampling occurred in the follicular phase P_4 concentrations would be low and LH would increase close to ovulation. Without ultrasound examination of each animal, it is difficult to know exactly what part of the cycle the animal is in and if all animals in each treatment group are at similar days in their estrous cycle. Another explanation for the differences is that Knutson and Allrich (1988) were looking at restriction which involved both protein and energy not strictly protein restriction.

Nolan et al. (1988) also noted no difference between CP deficient groups and control groups for LH pulse frequency, pulse amplitude, and basal and mean LH concentrations; however, cows receiving adequate nutrients had increased LH pulse frequency as time in the postpartum period increased. In the same study, Nolan et al. (1988) noted restricted cows had a GnRH induced LH peak of lower magnitude than control cows. Injection of dairy cattle with

GnRH led to a greater release of LH in cows fed increased CP levels (Jordan and Swanson, 1979). This observation may indicate that the adequately fed cows were closer to resuming estrus following parturition. This may be important to producers as shortening the interval from parturition to estrus would result in a more efficient production setting and indicates that the maternal unit is recovering more quickly from the effects of parturition. In the same study there was no difference in anterior pituitary GnRH receptor number or concentration between treatment groups, indicating a change to the function of the receptors (Jordan and Swanson, 1979). Dietary restriction and duration may not have been enough to lead to extreme results in this study. Furthermore, this study was completed during the luteal phase when LH levels are low making it difficult to note differences in the hormone.

Results suggest that protein restriction had minimal effects on the protein hormones. We would expect to see P_4 and LH being negatively correlated because of the reproductive feedback system with the spike in LH leading to ovulation followed by formation of the CL, a main source of P_4. However, the change in LH pulse frequency as time in the postpartum period increased may be a result of duration of time spent in a low protein state and a reduction in the body protein stores. More research is needed to help explain the conflicting results as changes in key hormones may result in loss of reproductive efficiency.

Noting the above changes in the endocrine system it is expected that other parts of the reproductive system will also be changed. In females, global nutrient restriction has resulted in lowered ovarian and uterine weights, ovarian atrophy, a decrease in number of follicles, and an increase in associated anestrus (Leathem, 1966). These changes to the ovary may result in negative effects on follicular quality and subsequently decreased conception rates. In addition,

the more time a female spends in anestrus, the less time that is available in which that female may be bred and producing offspring.

Changes in hormone, ovarian, or uterine environments are likely to negatively affect the estrous cycle. Heifers consuming diets deficient in CP had reduced pre and post-partum weight gains, decreased number of animals showing estrus by 110 d after calving, decreased first service conception rates, and increased interval from parturition to first postpartum estrus (Sasser et al., 1988). Other noted adverse effects on the estrous cycle include reduced ovulation rates in gilts (Murray et al., 1979) and cessation of estrus, long and irregular cycles, and lack of fertility in rats (Guilbert and Goss, 1932). In contrast, Knutson and Allrich (1988) showed a restriction to 80% of National Research Council (**NRC**) requirements for protein and energy did not influence the duration of estrus or behavioral estrus traits in dairy heifers. However, there was no measure of ovarian function, and thus these results do not ensure that function of the reproductive organs was not affected by the restriction.

Deficiency in protein supplied to the maternal unit has also resulted in varying effects on parturition and offspring. Beef heifers fed low level protein diets during the last trimester had weak labor, increased incidence of dystocia, increased perinatal mortality, reduced postnatal growth of calves, and prolonged postpartum anestrus (Kroker and Cummins, 1979). In sows, no difference was noted in the number of pigs per litter in protein restricted gilts; however, birth weights were lower and mortality higher in piglets from gilts consuming a low protein diet (Hammell et al., 1979). Pigs from low protein sows were lighter through 45 d of age and consumed more feed per unit of gain (Hammell et al., 1979). Bond and Wiltbank (1970) also noted a similar trend with calves from low protein dams gaining slower than calves from dams on moderate or high protein diets. In contrast to these results Anthony et al. (1986) saw no effect

on calf birth weight, calf body measurements, calf vigor score, or severity of calving difficulties in cows consuming diets supplying various amounts of protein. However, Anthony et al. (1986) did not follow the offspring past initial measurements to see if calf performance was altered. Changes to offspring size and growth rates may be attributed to changes in milk production and composition, as well as length and level of protein restriction.

Another important area to consider when looking at effects of protein restriction is lactation. In discussing lactation, it is important to consider milk composition and alterations to mammary tissue which may both be altered by dietary protein supply. Milk has a significant protein component and in addition, immunoglobulins such as immunoglobin G (**IgG**) are protein in nature. Colostrum supplies IgG which is essential in providing offspring with antibodies to improve immunity. A study by Elliott et al. (1971) examined various levels of dietary protein and the effects on milk and colostrum protein content in sows. Results from this study showed dietary protein levels to have minimal effects on milk and colostrum protein levels. As a follow up to this study King et al. (1993) also looked at varying maternal dietary levels and noted in early lactation the protein component of sow milk was not significantly different. However, when the milk composition was examined in late lactation, the higher protein level diet resulted in higher protein content in the milk. This difference is likely due to the fact the maternal unit had less body stores of protein to utilize for milk production as lactation progressed, therefore resulting in decreased protein content in the milk late in lactation when adequate protein was not supplied in the diet. It is also important to note that both of these trials reported marked changes in the overall composition of the milk including changes to concentrations of fats and solids (Elliott et al., 1971; King et al., 1993).

Alterations in mammary tissue may lead to changes in milk production which ultimately can alter offspring performance. Kusina et al. (1999a) found no effects of dietary protein level on mammary parenchymal tissue or concentration of total amount of DNA, RNA, or protein of the tissue. However, multiple studies have noted an effect of dietary protein level on milk production with increased protein levels eliciting increased milk production and feed restriction decreasing milk production in heifers, dairy cattle, and sows (Bond and Wiltbank, 1970; King et al., 1993; Lapierre et al., 1995; Kusina et al., 1999b). As a result of increased milk yield, an increase in total weight gain per piglet was also noted (King et al., 1993; Kusina et al., 1999b). Although changing dietary protein did not show measurable differences in mammary tissue, function of the tissue may still be altered as a result of the dietary insult. This may be attributed to the high level of energy as opposed to the low protein in the study by Bond and Wiltbank (1970), but since the same effect on milk production was seen by Kusina et al. (1999b) there seems to be a reoccurring trend of low protein negatively affecting milk production. Another explanation for the change to milk production may be an alteration in blood flow.

In sows, Guan et al. (2004) found increasing dietary protein levels lead to increases in most arterial plasma amino acid concentrations as well as many of the arteriovenous differences of plasma amino acids across the mammary gland. This increase may be beneficial to the milk quality and composition of milk supplied to the offspring.

As shown above, nutritional intake not only affects the dam, but also may adversely affect the offspring. From a production standpoint the more time an animal spends “open” or not bred, the fewer offspring that maternal unit will be able to raise. Changes to milk production may affect offspring growth, in turn leading to decreased offspring performance. This may have a huge economic impact on producers as more time and money will be spent growing those

animals. Therefore, it is vital for the maternal unit to be supplied with the nutrients necessary for optimal growth, reproduction, and lactation.

Conclusion

For the livestock producer, a solid understanding of reproduction as well as some nutritional influences is important. As was discussed in this review, understanding and manipulating the estrous cycle, as well as supplementing the diet, can have both beneficial and detrimental effects on the maternal unit. Further research that helps clarify the current discrepancies in the literature will be beneficial. Therefore, the next two chapters will evaluate the effects supplementation with flaxseed and metabolizable protein has on both maternal reproductive and health parameters in the mare and ewe.

CHAPTER 2. EFFECTS OF FLAXSEED SUPPLEMENTATION ON MARE PROGESTERONE AND BLOOD PARAMETERS

Abstract

To examine the effect of flaxseed and linseed meal supplementation on mare reproductive and health parameters, 16 quarter horse mares ages two to five years old were allotted randomly to one of three treatments. All diets were designed to be isocaloric and were fed at a level suggested to meet National Research Council (**NRC**, 2007) requirements of a maintenance horse. The control (**CON**) diet composed of a basic sweet feed mixture of corn and oats was modified to provide 0.1% of horse BW in whole flaxseed (**FLX**) or 0.06% of horse BW in linseed meal (**LSM**). Horses were fed the concentrate meal at 0.4% of body weight with the remainder of intake coming from free choice grass hay. Following a two week adaption period horses were placed on their respective diets for 16 weeks. Jugular blood samples were collected twice weekly and analyzed for progesterone (**P_4**) concentration. In addition, number of estrous cycles was recorded and fatty acids and chemistry panels were analyzed to evaluate differences in dietary treatments. There was no effect of diet on P_4 concentration, number of estrous cycles, or chemistry panel values. Dietary treatment did influence alpha-linolenic acid concentration, with mares receiving the FLX treatment showing increased ($P < 0.01$) concentration when compared to both CON and LSM mares, which were not different. Results indicate FLX and LSM fed at the levels in this study have no effect on estrous cycle characteristics measured or serum chemistry panel values. However dietary treatments do affect plasma fatty acid concentrations.

Keywords: estrous cycle, flaxseed, horse, linseed meal, reproduction

Introduction

Dietary substances capable of altering hormone levels are of interest to horse owners for both performance and reproductive reasons. Estrus and pregnancy are hormone sensitive times; consequently changes in hormone levels can be either beneficial or detrimental depending on timing and hormonal alterations. There are synthetic sources or factors such as nutrition and stress that may lead to hormonal alterations and thus alter the reproductive capabilities of the maternal unit.

Nutritional supplementation is one area of research which is growing and being used to improve reproductive efficiency. Previous research has shown omega-3 fatty acids and arginine to improve reproduction in both stallions and mares (Kelley et al., 2013; Schmid-Lausigk and Aurich, 2014). One specific omega 3-fatty acid, alpha-linolenic acid (**ALA**), has elicited varying outcomes on estrous cycle characteristics and hormone concentrations.

Flaxseed is a rich source of ALA an essential omega-3 fatty acid that serves as a precursor for eicosapentaenoic acid formation. Eicosapentaenoic acid serves as a precursor for prostaglandin synthesis (Robinson et al., 2002). Flaxseed also serves as a source of lignans, principally secoisolariciresinol diglycoside (**SDG**) (Thompson et al., 1991). Lignans, including SDG, are phytoestrogens which produce responses via estrogen receptors (Collins et al., 2003). Secoisolariciresinol diglycoside is broken down by intestinal microflora and enzymes into enterodiol and enterolactone (Axelson et al., 1982; Begum et al., 2004). These new compounds are absorbed into the bloodstream and interact with estrogen receptors. Alterations in prostaglandin synthesis and increased interactions with estrogen receptors have the potential to affect reproductive parameters in mares.

Although little work has been done examining the effects of feeding flaxseed and estrous cycle parameters in horses; in rats, flaxseed has produced a dose-related cessation, irregularity, or lengthening of the estrous cycle. There may be multiple explanations for these changes to the cycle. This may be partially explained by the increase in serum estradiol (**E_2**) levels leading to more time spent in the follicular phase (Orcheson et al., 1998; Tou et al., 1998; Tou et al., 1999). Another explanation may be increased time spent in the luteal phase under progesterone (**P_4**) control. Increased time spent under P_4 control would be advantageous to performance horses in which behavioral aspects associated with estrus can be detrimental to the animal's performance. Contradicting information has been noted for effects of flaxseed supplementation on P_4 concentrations with both no change and increased P_4 concentrations seen in cattle (Lessard et al., 2003; Bilby et al., 2006). In horses, increased P_4 concentrations could be beneficial to reproductive efficiency as it would assist in providing a positive uterine environment for the growing fetus, thus hopefully decreasing the percentage of embryonic loss. In the equine industry embryonic loss is a major concern. Fertilization rates in mares are estimated to range from 71% to 96% with the majority of studies finding an embryonic loss of 8-18% (Ball, 1988; Meyers et al., 1991). For owners in the horse industry, the ability to use a feed supplement to either enhance performance and/or decrease embryonic loss would be of great economic benefit.

Due to conflicting research results on the effects flaxseed supplementation elicits in horses, the objective of this study was to acquire an understanding of flaxseed supplementation on mare hormone levels as well as blood parameters indicative of overall health. Our hypothesis was feeding flaxseed would elicit results similar to what has been seen in rats including changes to the estrous cycle and hormone concentrations.

Materials and Methods

Animal care and use was approved by the Institutional Animal Care and Use Committee at North Dakota State University, Fargo.

Animals and Diets

Sixteen non-pregnant quarter horse mares ranging in age from two to five years old and weighing between 323 kg and 472 kg were obtained from the North Dakota State University (**NDSU**) Research Extension Center in Dickinson, ND. Mares were transported in mid-April to the NDSU Equine Center in Fargo. At the Equine Center, mares were individually housed in 3.05 x 3.05-m stalls at night and turned out into a dry lot during the day with free choice grass hay (5.53% CP, 1.79 Mcal/kg DE) and water available. Mares were individually fed a grain meal each morning and evening. Mares had a two week adaption period at which time the control sweet feed diet was utilized. Following the adaption period mares were blocked by age and randomly assigned to one of three nutritional diets: control (**CON**) (14.80% CP, 3.51 Mcal/kg DE) (n = 5), flaxseed (**FLX**) (16.70% CP, 3.53 Mcal/kg DE) (n = 6), and linseed meal (**LSM**) (17.40% CP, 3.54 Mcal/kg DE) (n = 5). Composition of the diets is shown in Table 2.1. Fatty acid composition of the diets is shown in Table 2.2. Secoisolariciresional diglycoside content of the diets and hay are shown in Table 2.3.

Diets were designed to be isocaloric and meet National Research Council (**NRC**, 2007) requirements of an average maintenance horse. The CON diet composed of a basic sweet feed mixture of corn and oats was modified to provide 0.1% of horse BW in whole flaxseed (FLX) or 0.06% of horse BW in linseed meal (LSM). Horses were fed the concentrate at 0.4% of body weight with the remainder of intake coming from free choice grass hay. Horses were allowed 30

min to consume the concentrate and orts were weighed and recorded. Body weight was measured weekly and diets adjusted accordingly.

Table 2.1. Diet composition

	Treatment[1,2]		
Item, % of Concentrate	CON	FLX	LSM
Concentrate			
Corn	31.85	24.52	28.70
Oats	27.65	22.98	25.93
Beet Pulp	4.00	10.00	5.00
Flaxseed, Whole		25.00	
Linseed Meal			15.50
Sunflower Meal	11.00		
Sun Oil	8.00		8.00
Molasses	5.00	5.00	5.00
Balancer Pellet	12.50	12.50	12.50
Nutrient			
% CP	14.80	16.70	17.40
DE (Mcal/kg)	3.51	3.53	3.54

[1]Concentrate fed at 0.4% of BW daily
[2]Treatment: CON = control diet; FLX = diet supplemented with flaxseed at 0.1% of BW; LSM = diet supplemented with linseed meal at 0.06% of BW

Table 2.2. Diet fatty acid composition (percent)

	Component			
Item, % of Diet[1]	HAY	CON	FLX	LSM
C13:0	5.35	0.25	0	0.32
C14:0	9.71	0.24	0.25	0.31
C16:0	27.45	8.05	8.21	7.99
C18:0	15.13	3.73	4.25	3.88
C:18:1n9c	5.74	47.12	22.83	46.52
C18:2n6c	15.53	38.96	25.06	37.80
C18:3n3	21.08	1.05	39.40	2.65
C22:0	0	0.60	0	0.55

[1]C13:0- tridecanoic; C14:0- myristic; C16:0- palmitic; C18:0- stearic; C18:1n9c- oleic; C18:2n6c- linoleic; C18:3n3- alpha-linolenic; C22:0- behenic

Table 2.3. SDG content of the diet

Treatment[1]	SDG Content[2]
FLX	1.1 mg/g
LSM	1.1 mg/g
Control	0.0 mg/g
Hay	0.0 mg/g

[1]Treatment: CON = control diet; FLX = diet supplemented with flaxseed at 0.1% of BW; LSM = diet supplemented with linseed meal at 0.06% of BW
[2] Secoisolariciresinol diglycoside content in mg/g

Blood Collection and Analyses

Upon arrival and prior to estrous synchronization, a pre-treatment jugular blood sample was obtained from each mare to serve as a baseline for hormone and serum chemistry panel (**SCP**) values. Mares were then synchronized using dinoprost tromethamine (Lutalyse, Pfizer, New York, NY). Mares were given two intramuscular injections of Lutalyse 14 d apart (1 mg/45.5 kg BW). Treatment diets were initiated following synchronization and mares remained on their assigned diet for 105 d. Jugular blood samples were obtained twice weekly on Tuesdays and Fridays at 7 a.m. prior to feeding. Serum and plasma samples were stored for analysis of P_4, polyunsaturated fatty acids (**PUFA**), and SCP.

Progesterone was analyzed as previously described in detail by our lab (Galbreath et al., 2008). Briefly, a 50 uL sample of maternal serum was analyzed in duplicate. Progesterone concentrations were measured by chemiluminescence immunoassay using an Immulite 1000 system (Siemens, Los Angeles, CA) by which lesser, medium, and greater P_4 pools were assayed in duplicate. The intraassay and interassay CV were 12.50% and 10.56% respectively.

Arachidonic acid (**AA**), linoleic acid (**LA**), and ALA were measured to evaluate the changes in fatty acid profiles over time. Separation of fatty acid methyl esters was achieved by GLC (Model CP-3800, Varian Inc., Palo Alto, CA) with a 100 m x 0.25 mm (i.d.) x 0.2 um (film thickness) capillary column (SP-2560, Supelco, Bellefonte, PA) and H_2 gas as the carrier at 1.5

mL/min. Initial oven temperature was maintained at 120° C for 2 min and then ramped to 175° C at 6°C/min and ramped to 250°C at 5°C/min. Injector temperature was 260°C and flame ionization detector temperature was 300°C. Identification of peaks was accomplished using purified fatty acid standards (Sigma-Aldrich, St. Louis, MO; Nu-Chek Prep, Elysian, MN) (Lake et al., 2006).

Serum chemistry panels were analyzed using a VetTest Chemistry Analyzer (IDEXX, Westbrook, ME). Pre-treatment (**P**) and final (**F**) serum samples were analyzed for changes in protein and enzyme levels. Change (Δ) between P and F samples was calculated for each assay analyzed. Measurements included albumin (**ALB**), alkaline phosphatase (**ALKP**), aspartate aminotransferase (**AST**), blood urea nitrogen (**BUN**), calcium (**CA**), creatine kinase (**CK**), creatinine (**CREA**), gamma-glutamyltransferase (**GGT**), globulin (**GLOB**), glucose (**GLU**), lactate dehydrogenase (**LDH**), bilirubin (**TBIL**), and total protein (**TP**).

Statistics

Data were analyzed using PROC GLM and MIXED procedures of SAS 9.2 (SAS Inst. Inc., Cary, NC) with mare as the experimental unit. Nutritional treatment, age, effect of day, and all interactions were included as fixed effects. In the model, age was defined as two year olds (n = 8) and any mares three or over were classified as three year olds (n = 8). Means are reported as least square means and considered significant when $P \leq 0.05$.

One mare was removed from the study because of a skeletal injury unrelated to the trial. Her data was included in the analysis until the injury at d 92.

Results

All mares readily consumed the diets and there were no orts reported. There was no effect of treatment on mare final weight ($P = 0.92$), although all treatment groups lost weight over the

course of the 16 week trial period (-10.65 kg CON; -11.26 kg FLX, -5.12 kg LSM). As would be expected the two year old mares were significantly lighter at both the initial weigh-in ($P < 0.01$) and final weigh-in ($P < 0.01$) when compared to the older mares.

No significant difference ($P = 0.15$) in P_4 levels was noted between CON (0.95 ng/mL), FLX (1.06 ng/mL), or LSM (1.80 ng/mL) (Figure 2.1).

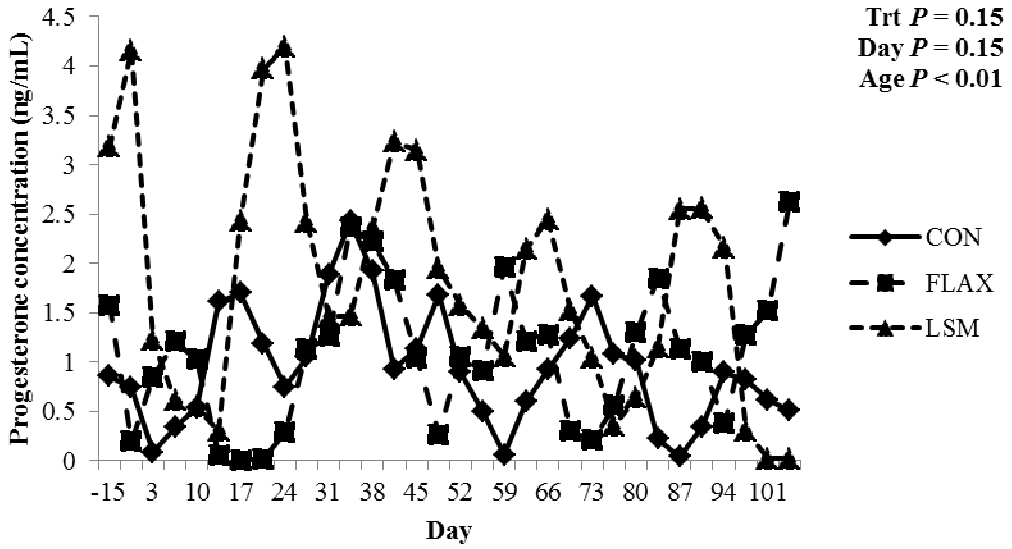

Figure 2.1. Effect of dietary treatment on P_4 (progesterone) concentration[1,2]

[1]Treatment: CON = control diet; FLX = diet supplemented with flaxseed at 0.1% of BW; LSM = diet supplemented with linseed meal at 0.06% of BW

[2]Dietary treatments fed at 0.4% of mare BW daily

Results for SCP values are shown in Table 2.4. There was no significant ($P \geq 0.06$) effect of treatment or age on ALB, AST, BUN, CA, CK, CREA, GGT, GLOB, GLU, LDH, or TP. Treatment differences were noted among groups for final TBIL (P = 0.05) with FLX showing higher levels of TBIL at the end of the trial. Furthermore, two year olds exhibited higher ending levels of TBIL ($P = 0.03$) and ALKP compared to three year olds. There was also a greater change in TBIL ($P = 0.03$) for three year olds compared to two year olds. No significant interactions were noted between treatment and age ($P \geq 0.09$). However, all assay values were

within the normal reference range provided by VetTest Chemistry Analyzer (IDEXX, Westbrook, ME) and therefore were not considered medically significant.

Table 2.4. Serum chemistry panel assay results for dietary treatment and age

	Treatment[1,2]				Age			P-Value		
[3,4]Assay	CON	FLX	LSM	SE	2	3	SE	Trt	Age	[5]Normal
P ALB (g/dL)	3.02	3.19	3.18	0.12	3.13	3.13	0.10	0.63	0.99	1.9-3.2
F ALB (g/dL)	3.01	3.13	3.18	0.08	3.13	3.08	0.07	0.41	0.63	-----
Δ ALB	0.67	-0.09	0.33	6.04	1.66	-1.05	4.95	0.10	0.71	-----
P ALKP (U/L)	178.88	182.53	164.95	27.41	205.72	145.19	22.37	0.88	0.08	10-326
*F ALKP (U/L)	156.52	160.00	167.39	11.60	186.35[a]	136.26[b]	9.51	0.81	< 0.01	-----
Δ ALKP	-5.21	-7.93	2.74	9.85	-4.58	-2.35	8.07	0.72	0.85	-----
P AST (U/L)	259.81	353.77	273.08	56.05	255.16	335.94	45.73	0.42	0.24	100-600
F AST (U/L)	261.87	275.67	267.15	13.15	277.47	258.99	10.77	0.75	0.26	-----
Δ AST	-4.11	-10.68	0.32	9.26	2.56	-12.21	7.59	0.67	0.20	-----
P BUN (mg/dL)	16.50	16.14	14.00	0.92	14.54	16.56	0.75	0.16	0.08	10-25
F BUN (mg/dL)	15.56	15.83	15.38	0.91	14.71	16.47	0.74	0.93	0.12	-----
Δ BUN	-0.69	2.28	9.19	5.60	8.07	-0.89	4.61	0.49	0.20	-----
P CA (mg/dL)	11.94	11.83	11.85	0.28	11.75	12.00	0.23	0.97	0.46	10.4-12.9
F CA (mg/dL)	11.80	11.97	11.80	0.14	11.86	11.86	0.12	0.60	1.00	-----
Δ CA	-0.49	1.16	-0.27	2.79	1.09	-0.82	2.28	0.89	0.57	-----
P CK (U/L)	155.83	215.46	217.50	51.17	219.42	173.11	42.27	0.70	0.46	10-350
F CK (U/L)	90.43	128.17	105.53	16.85	125.40	90.69	13.64	0.30	0.10	-----
Δ CK	-44.37	-22.42	-38.68	15.17	-34.53	-35.78	12.47	0.56	0.95	-----
P CREA (mg/dL)	1.71	1.77	1.69	0.07	1.69	1.76	0.06	0.64	0.45	0.8-2.2
F CREA (mg/dL)	1.87	1.95	1.93	0.09	1.86	1.98	0.07	0.83	0.29	-----
Δ CREA	8.69	10.63	15.89	3.21	10.11	13.36	2.64	0.32	0.41	-----
P GGT (U/L)	20.59	20.76	18.67	3.67	22.68	17.33	2.99	0.90	0.23	0-87
F GGT (U/L)	25.65	19.33	22.74	1.98	23.88	21.27	1.62	0.12	0.28	-----
Δ GGT	24.20	2.89	18.72	12.34	8.66	21.89	10.14	0.45	0.38	-----
P GLOB (g/dL)	3.17	3.04	2.92	0.11	2.93	3.16	0.09	0.38	0.10	2.4-4.7
F GLOB (g/dL)	2.95	3.00	2.88	0.11	2.89	2.99	0.09	0.68	0.43	-----
Δ GLOB	-8.43	-1.70	-0.75	4.72	-1.88	-5.38	3.88	0.55	0.54	-----
P GLU (mg/dL)	96.27	97.85	99.99	1.66	97.08	98.99	1.35	0.37	0.34	64-150
F GLU (mg/dL)	87.54	87.67	88.49	2.33	88.47	87.32	1.91	0.95	0.68	-----
Δ GLU	-9.04	-10.09	-11.51	3.04	-8.64	-11.79	2.49	0.86	0.40	-----
P LDH (U/L)	705.19	841.52	1009.93	111.81	752.58	951.84	91.23	0.24	0.15	250-2070
F LDH (U/L)	516.62	603.17	658.25	65.01	593.41	591.95	53.26	0.39	0.99	-----
Δ LDH	-26.73	-22.38	-31.69	10.92	-19.11	-34.76	8.95	0.82	0.25	-----
P TBIL (mg/dL)	0.91	1.09	0.79	0.11	0.90	0.96	0.09	0.15	0.67	0-3.5
*F TBIL (mg/dL)	0.75[a,b]	0.92[a]	0.68[b]	0.07	0.88[a]	0.69[b]	0.05	0.05	0.03	-----
*Δ TBIL	-12.77	-14.87	-9.90	8.53	0.08[a]	-25.10[b]	6.99	0.91	0.03	-----
P TP (g/dL)	6.20	6.23	6.13	0.12	6.05	6.33	0.10	0.79	0.06	5.6-7.9
F TP (g/dL)	6.10	6.12	6.08	0.14	6.10	6.10	0.11	0.98	0.99	-----
Δ TP	-1.58	-1.44	-0.68	3.26	1.13	-3.60	2.67	0.98	0.24	-----

[1]Treatment: CON = control diet; FLX = diet supplemented with flaxseed at 0.1% of BW; LSM = diet supplemented with linseed meal at 0.06% of BW

[2]Dietary treatments fed at 0.4% of mare BW daily

[3]Assay abbreviations: albumin (**ALB**), alkaline phosphatase (**ALKP**), aspartate aminotransferase (**AST**), blood urea nitrogen (**BUN**), calcium (**CA**), creatine kinase (**CK**), creatinine (**CREA**), gamma-glutamyltransferase (**GGT**), globulin (**GLOB**), glucose (**GLU**), lactate dehydrogenase (**LDH**), bilirubin (**TBIL**), and total protein (**TP**)

[4]**P** = beginning of trial prior to treatment initiation and synchronization; **F** = final day of trial; Δ = change

[5]Normal range of assays using VetTest Chemistry Analyzer (IDEXX, Westbrook, ME)

*Although values were significantly different from each other the values fell within the normal range for the assay, therefore are not considered medically significant

There was no effect of treatment on AA ($P = 0.36$) or LA ($P = 0.25$) values as shown in Table 2.5. Alpha-linolenic acid was significantly higher ($P < 0.01$) in FLX mares (0.64 mg/g) compared to CON (0.19 mg/g) or LSM (0.16 mg/g) treated mares, which were not different. The change in AA (Figure 2.2), LA (Figure 2.3), and ALA (Figure 2.4) over time is shown below.

Table 2.5. Effects of treatment on plasma fatty acid concentration

	Treatment[1,2]				Age				
Item[3]	CON	FLX	LSM	SE	2	3	SE	Trt	Age
AA (mg/g)	0.23	0.21	0.20	0.14	0.23	0.20	0.11	0.36	0.10
LA (mg/g)	10.54	9.36	9.55	0.50	9.79	9.84	0.41	0.25	0.94
ALA (mg/g)	0.19[a]	0.64[b]	0.16[a]	0.37	0.32	0.34	0.03	<0.001	0.51

[1]Treatment: CON = control diet; FLX = diet supplemented with flaxseed at 0.1% of BW; LSM = diet supplemented with linseed meal at 0.06% of BW
[2]Dietary treatments fed at 0.4% of mare BW daily
[3]Fatty acid abbreviations: arachidonic acid (**AA**), linoleic acid (**LA**), alpha-linolenic acid (**ALA**)

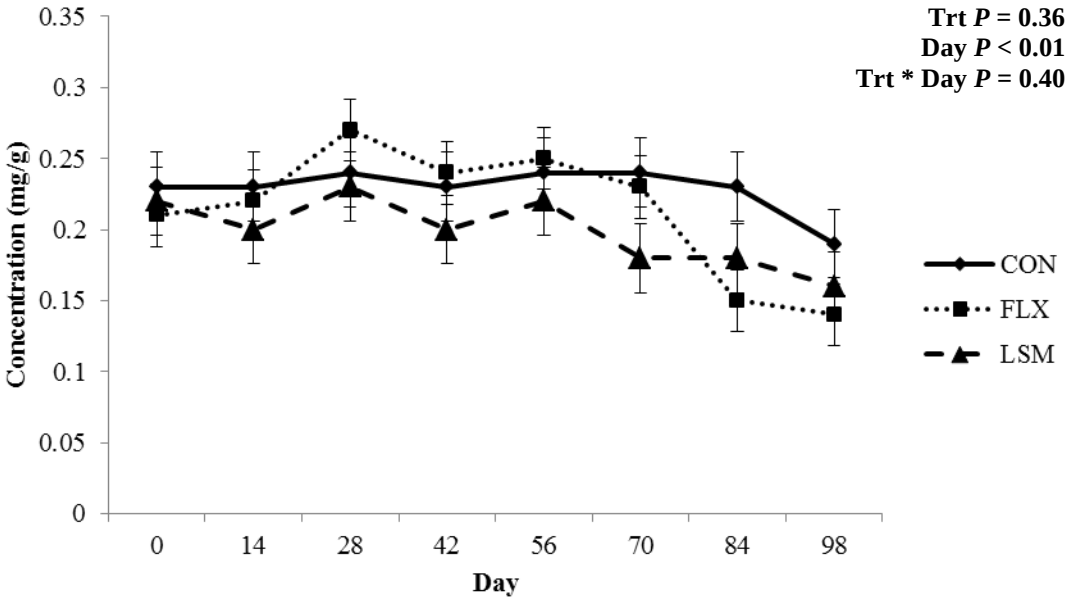

Figure 2.2. Effect of dietary treatment on AA (arachidonic acid) concentration[1,2]
[1]Treatment: CON = control diet; FLX = diet supplemented with flaxseed at 0.1% of BW; LSM = diet supplemented with linseed meal at 0.06% of BW
[2]Dietary treatments fed at 0.4% of mare BW daily

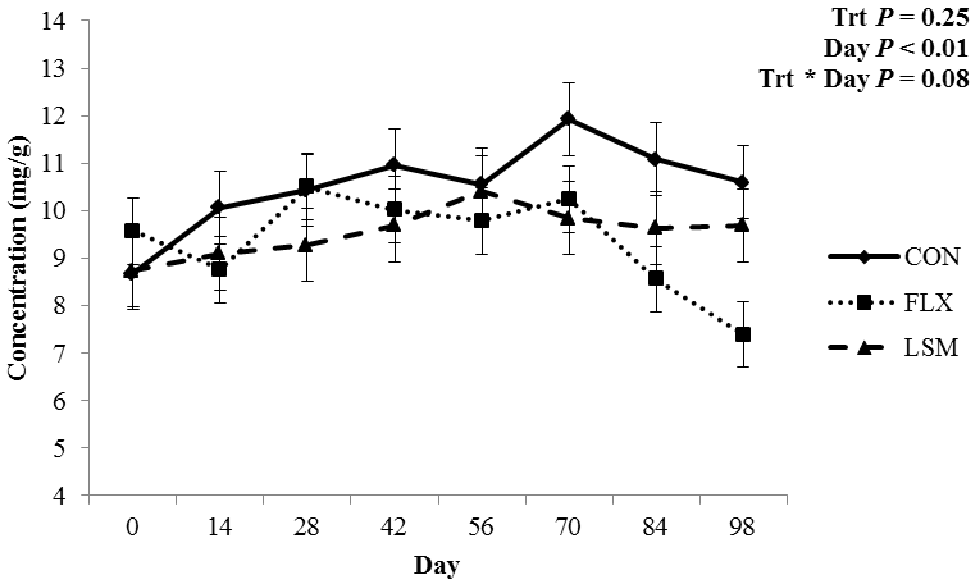

Figure 2.3. Effect of dietary treatment on LA (linoleic acid) concentration[1,2]
[1]Treatment: CON = control diet; FLX = diet supplemented with flaxseed at 0.1% of BW; LSM = diet supplemented with linseed meal at 0.06% of BW
[2]Dietary treatments fed at 0.4% of mare BW daily

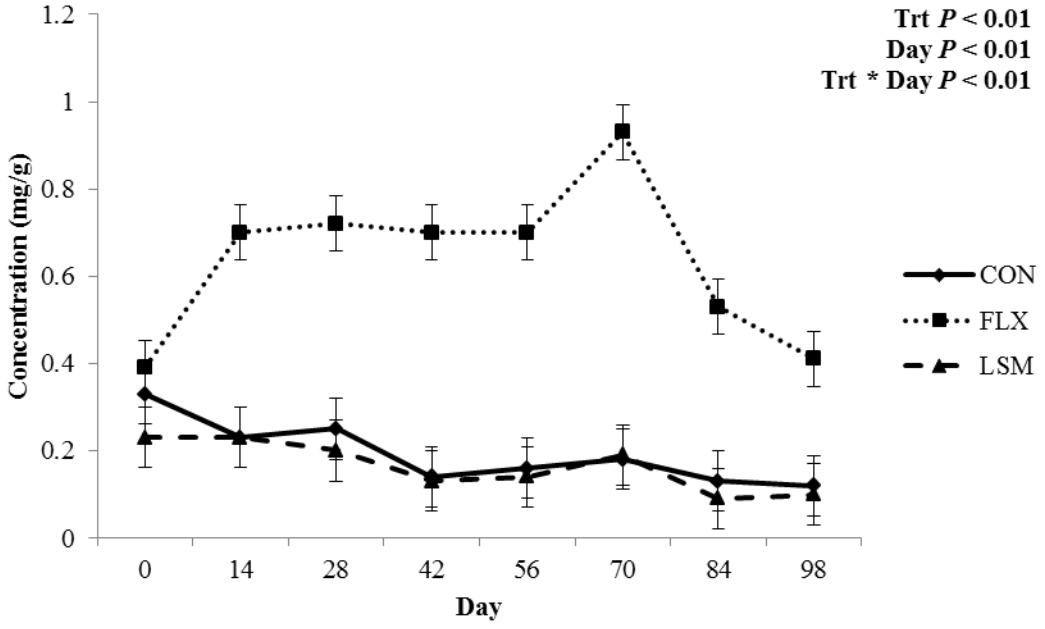

Figure 2.4. Effect of dietary treatment on ALA (alpha-linolenic acid) concentration[1,2]
[1]Treatment: CON = control diet; FLX = diet supplemented with flaxseed at 0.1% of BW; LSM = diet supplemented with linseed meal at 0.06% of BW
[2]Dietary treatments fed at 0.4% of mare BW daily

Discussion

Experiments have shown flaxseed to be a good source of ALA as well as SDG. Linseed meal is the result of grinding flaxseed and extracting the oil and supplies a much lower level of ALA while maintaining a similar concentration of SDG compared to unprocessed flaxseed. Thus, diets were designed to determine differences due to levels of ALA or SDG. There were no effects of treatment on LA or AA. As expected by diet design mares fed FLX had higher serum ALA concentrations compared to CON or LSM, which did not differ. Results from this study agree with the study in mares by Hess et al. (2012) which found no difference between control diets and flaxseed diets for LA or AA. Hess et al. (2012) also found levels of ALA which were higher in the flaxseed group compared to the control group (Hess et al., 2012). In addition, studies in other livestock species have noted similar increases in ALA concentration with flaxseed supplementation (Farmer et al., 2007; Bork et al., 2010). However, the results from this study are contradictory to a study by Wiesenfeld et al. (2003) who saw a decrease in AA and LA with supplementation of flaxseed. These differences are most likely due to the higher supplementation level of up to 58% linolenic acid supplied by the diet compared to 39.40% in the current study. This higher level of omega-3 fatty acids may cause a shift leading to lower levels of omega-6 fatty acids such as LA. This in turn, could lead to lower levels of AA as LA is metabolized to AA (James et al., 2000).

It is noted that both AA and LA had significant day effects ($P < 0.01$). The decrease in AA (an omega-6 fatty acid) from the beginning to the end of the trial may be explained by the shift in omega-6 fatty acids to omega-3 fatty acids thus changing the ratio of omega-6:omega-3 fatty acids in favor of omega-3 fatty acids. This shift to overall increased omega-3 fatty acids and reduced ratio of omega-6:omega-3 was seen after 24 d of supplementation with a marine based

protected fatty acid source in mares by King et al. (2008). Although contrary to our study, they saw no change in concentration of ALA and noted an increase in AA. However, the study by King et al. (2008) only fed the supplement for 28 days so the concentration of AA may have changed if supplementation had continued for a longer duration. The lack of change in ALA is expected as King et al. (2008) fed a marine based source of omega-3 fatty acids as opposed to the plant based source utilized in the current study.

When looking at the LA data it is noted that levels were not different at the beginning of the experiment, then became significantly ($P < 0.01$) different in the middle of the trial, and were back to not being significant by the end of the experiment. In addition, there tended to be a treatment by day interaction with mares receiving the FLX diet demonstrating much lower concentrations of LA at the end of the experiment. If the number of animals in the trial had been larger we may have seen this treatment effect become significant.

There was a treatment by day interaction for ALA with mares receiving the FLX diet maintaining much higher ($P < 0.01$) concentrations of ALA throughout the trial compared to mares on the other two treatments. Farmer et al. (2007) saw a similar trend of increasing ALA and lower values of AA by the end of their 155 d feeding trial in gilts receiving flax in various forms. It is interesting to note that by the end of this experiment all concentration levels for the fatty acids tended to be dropping. One possible reason we see a decrease in concentrations may be the shift in fat usage to a source of energy as nutritional demands may not have been met for all horses and over time this decreased nutrient allocation would result in breaking down body stores for energy usage.

Flaxseed and LSM diets supplied 1.1 mg/g SDG. This amount is similar to levels used in rats, which have resulted in significant changes to puberty and the estrous cycle (Tou et al.,

1998). Results from this project coincide with other studies which have found no significant differences in P_4 concentrations between flaxseed supplemented animals and CON groups (Bilby et al., 2006; Farmer et al., 2010). However, this is contradictory to Lessard et al. (2003) which noted increased P_4 levels in flaxseed supplemented dairy cows. These differences may be attributed to variation within project animals, level of supplementation, duration of supplementation, and flaxseed source provided.

Upon looking at dietary composition, diets were formulated for mature, average maintenance horses. As some of these were growing horses, nutrient intake may not have been sufficient for both growth and reproductive function in the younger mares. It has been shown in other livestock species that feed restriction resulting in low protein and energy intake can lead to cessation of cycling (Armstrong and Britt, 1987; Cassady et al., 2009). All mares on this project were synchronized prior to initiation of treatment; however it was noted at the completion of the trial when samples were being analyzed that three mares, all two year olds (1 CON, 2 FLX), were anestrus and did not cycle throughout the project. In addition, there were eight mares of all ages noted as exhibiting abnormal estrous cycles (3 CON, 3 FLX, 2 LSM). In these mares, cycling ceased and they returned to anestrus state at various times throughout the project. Five mares on the project had estrous cycles which continued throughout the project (1 CON, 1 FLX, 3 LSM). All mares should have reached puberty and age was distributed across treatment groups; the only horse which did not exhibit abnormal cycling or anestrus was the five year old in the sampling set. As there were only two five year olds in the project, there were not sufficient numbers to determine if the ability to maintain cyclicity was simply due to chance or the reflection of the diet more adequately meeting the needs of this mature mare. With only five horses showing normal cycling patterns, the number was too small to remove the abnormal

animals and still have a sufficient number for comparison. Thus, variation in cyclicity may have greatly obscured the P_4 results of this project.

In addition, when examining the diet composition, the grass hay quality was far lower than expected with only 5.53% CP and 1.79 Mcal of DE. Therefore, mares tended to receive low protein in comparison to requirements which may have further compounded the nutritional stress. Prior research in cattle has shown decreased protein intake to negatively affect P_4 concentrations (Knutson and Allrich, 1988). Gentry et al. (2002) demonstrated that decreasing mare body condition score (**BCS**) to 3-3.5 resulted in decreased P_4 concentrations and that these mares lacked significant follicular activity. Contrary to Gentry et al. (2002), Van Niekerk and Van Niekerk (1997) showed varying protein levels in mares resulted in no differences for serum progestagen or luteinzing hormone (**LH**). There were large variations in serum progestagen concentrations between individual mares in this study, which may explain why the authors did not see significant changes between the treatment groups.

Research in other livestock has also resulted in changes to the estrous cycle due to restricted protein levels. In growing gilts fed a low protein diet, an increased incidence of anestrus animals was noted (Jones and Maxwell, 1974). This same trend of increased anovulatory time was seen in horses fed a restricted energy diet as well as in mares with a low BCS (Gentry et al., 2002; Salazar-Oritz et al., 2011). These studies vary in the type of restriction which was being implemented (global, protein, or energy). More studies will need to be completed looking at these restrictions independently to determine whether each had a drastic effect on the estrous cycle or if it was a compounded issue of growth and low protein supplied. Furthermore, our current study only measured P_4 levels so the effect on the other important reproductive hormones, including LH and FSH was not measured. In future studies, measuring

additional reproductive hormones would give a more accurate overall picture of how the cycle is being manipulated.

Serum chemistry panels are predominately used as a health screening tool and use of these assays to evaluate effects of dietary supplementation is not a common practice. However, many of the assays in the SCP serve as indicators of organ and muscle function as well as protein synthesis and breakdown. All of these areas are of interest in a production setting and should be considered when changing or supplementing the diet. Since all values measured fell within the normal range of acceptable values they were not noted as medically significant, however if there were a larger sample size or in ill or compromised animals, these differences may become more evident.

In summary, results from the current study indicate low levels of flaxseed supplementation did not significantly affect P_4 levels. Although it is acknowledged that there were several factors that may have influenced results. Flaxseed supplementation at 0.1% BW daily does raise plasma ALA concentrations. This increase in ALA may be beneficial in terms of reducing inflammation and other positive health benefits related to omega-3 fatty acids. Due to confounding factors in the project it is difficult to evaluate our hypothesis. Although the current study did not show any effects of flaxseed supplementation on P_4 profiles or serum chemistry parameters, more studies need to be conducted in both growing and mature horses with higher levels of flaxseed supplementation to truly understand any effects it may have.

CHAPTER 3. EFFECTS OF LATE GESTATION METABOLIZABLE PROTEIN SUPPLEMENTATION ON EWE ORGAN AND BLOOD PARAMETERS

Abstract

To examine the effects of maternal metabolizable protein (**MP**) supplementation in late gestation on blood and organ parameters multiparous ewes (n = 45) were allotted randomly to one of three treatments, 60% (**MP60**), 80% (**MP80**), or 100% (**MP100**) of MP requirements fed from d 100 to d 130 of gestation. Blood samples were drawn before initiation of diets and prior to slaughter for chemistry panel analysis. Body measurements including loin eye area, back fat, and body wall thickness where obtained using ultrasound prior to treatment initiation and before slaughter to examine changes in body condition. At d 130 ewes were slaughtered and tissues harvested. Ewes carried singletons and twins therefore fetal number was included as a main effect. There was no effect of treatment or fetal number on loin eye area, back fat, body wall thickness, eviscerated body weight (**EBW**), or weights (g) of blood, perirenal fat, adrenals and thyroid ($P \geq 0.11$). Ewes on the MP80 treatment were heavier at final BW than MP60 ewes but neither were different then MP100 ewes. There was a treatment effect on heart weight with MP80 being heavier than MP60 and MP100 which were not different ($P \leq 0.01$). Kidney weight was also affected by treatment with MP60 being lighter compared to MP100 and MP80 which were not different ($P \leq 0.01$). Ewes carrying twins had increased liver, mammary, uterus, and gravid uterus weights ($P \leq 0.03$). Ewes with singletons had increased lung weights compared to ewes carrying twins ($P \leq 0.03$). When organ weight was examined as a proportion of EBW (g/kg) there was no difference in heart, perirenal fat, kidney, lung, or thyroid masses ($P \geq 0.06$). Ewes carrying twins had increased blood, liver, mammary, uterus, and gravid uterus weights as a proportion of EBW ($P \leq 0.02$). Initial chemistry panel results showed no differences in

parameters of interest. Treatment decreased aspartate aminotransferase and blood urea nitrogen ($P \leq 0.01$) in MP60 ewes compared to MP100 and MP80 ewes which did not differ. Change in gamma-glutamyltransferase was greater in ewes carrying twins ($P \leq 0.01$). Results indicate that fetal number and dietary MP supplementation during late gestation alters ewe organ weights.

Keywords: maternal, metabolizable protein, organ weights

Introduction

In examining nutritional planes of the dam, protein is important during pregnancy for development, survival, and growth of the fetus (Shields et al., 1985; Ocak et al., 2005) as well as maternal maintenance, lactation performance, and rebreeding success (Bond and Wiltbank, 1970; Anthony et al., 1986; Drouillard et al., 1991). In beef cows, Sasser et al. (1988) reported that diets fed to dams which were equal in energy but deficient in crude protein (**CP**) resulted in the deficient dams having decreased pre and post-partum weight gains, decreased first service conception rates, and increased interval from parturition to postpartum estrus. Inadequate protein supply in sows has been shown to affect maternal visceral organ weights while having less effect on carcass measurements such as backfat (Brendemuhl et al., 1989).

A large majority of research in protein supply has focused on the fetal unit and not necessarily an in-depth evaluation on overall effects on the maternal unit. Therefore, the objective of this study was to examine the effects of maternal metabolizable protein (**MP**) supply on visceral organ weights, serum chemistry panel (**SCP**) values, hormone concentrations, and ultrasound carcass characteristics. Our hypothesis was feeding an isocaloric diet with restricted metabolizable protein supply would negatively affect the maternal unit by reducing uterine and mammary gland development while increasing markers of inflammation and muscle wasting.

Furthermore, we expect twins to negatively impact the maternal unit, especially in protein deficient dams.

Materials and Methods

Animal care and use was approved by the Institutional Animal Care and Use Committee (#A0921) at North Dakota State University (NDSU), Fargo.

Animals and Diets

On approximately d 90 of gestation, 45 pregnant multiparous ewes were transported from the Hettinger Research Extension Center (Hettinger, ND, USA) to the Animal Nutrition and Physiology Center at NDSU (Fargo, ND, USA). Upon arrival ewes were individually housed in 0.91 x 1.2-m pens in a temperature controlled (12° C) and ventilated facility for the duration of the study. Lighting within the facility was automatically timed to mimic daylight patterns (12: 12 h light-dark cycle with lights on at 0700 and off at 1900).

Ewes were acclimated to low-quality hay (Table 3.1) and a supplement which met 100% of MP requirements (**MP100**) supplement, as determined by National Research Council (**NRC**) (2007); for 10 days prior to starting dietary treatment (Van Emon et al., 2014). Ewes were weighed on two consecutive days (d 99 and 100 of gestation) prior to initiation of treatment.

Table 3.1. Nutrient composition of fescue straw[1]

Item	
Diet, % DM	
DM, %	96.24
NEm, Mcal/kg	2.22
CP, % of DM	2.76
MP, % of DM	1.95
NDF, % of DM	80.17
ADF, % of DM	48.66
Ash, % of DM	6.00

[1]Ewes were fed fescue straw to limit MP intake

On d 100 ± 2 (SD) of gestation ewes were randomly assigned to one of three dietary treatments (Table 3.2) designed to be isocaloric and provide: 60% (**MP60**), 80% (**MP80**), or 100% (MP100) of metabolizable protein requirements on a DM basis during the last four weeks of gestation (NRC, 2007; Van Emon et al., 2014). Nutrient requirements were based on NRC (2007) recommendations for a 70 kg pregnant mature ewe carrying twins (Van Emon et al., 2014).

Table 3.2. Ingredient and nutrient composition of dietary supplements fed to ewes

	Treatment[1,2]		
Item	MP60	MP80	MP100
Ingredient, % DM			
Corn	18.50	15.00	5.00
DDGS[3]	7.00	20.00	30.00
Soyhulls	9.50		
Nutrient composition			
DM, %	95.51	95.89	95.90
NEm, Mcal/kg	2.00	2.22	2.14
CP, % of DM	13.45	20.53	25.03
MP, % of DM	8.41	13.01	16.31
NDF, % of DM	33.61	32.11	40.79
ADF, % of DM	15.71	8.33	11.61
Ash, % of DM	3.17	3.50	4.38

[1]Treatment: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80= diet designed to meet 80% of metabolizable protein requirements; MP60= diet designed to meet 60% of metabolizable protein requirements
[2]Diets formulated based on a 70 kg ewe carrying twins
[3]Dried distillers grains with solubles

Thirty-five percent of the total intake was fed as a supplement at 0700, ewes were given one hour to consume the supplement, and then low-quality forage (Table 3.1) was provided to supply the remaining 65% of total intake. The supplement was always completely consumed. Body weight was measured every 7 d throughout the treatment period and the amount of supplement and forage offered was adjusted for changes in body weight. Throughout the project ewes had free access to water and a trace mineralized salt block [Salt (min.) 95.5%, Salt (max.)

98.5%, Zinc (min.) 3,500 ppm, Iron (min.) 2,000 ppm, Manganese (min.) 1,800 ppm, Copper (min.) 280 ppm, Copper (max.) 420 ppm, Iodine (min.) 100 ppm, Cobalt (min.) 60 ppm; American Stockman, Overland Park, KS].

Sample Collection

Jugular blood samples (10 mL) were collected on d 100 of gestation prior to treatment initiation and then weekly on d 107, 114, 121, and 128. Final blood samples were taken on day 130 $\pm$ 1 of gestation prior to necropsy. All blood samples were placed on ice, held a minimum of 45 min, and then centrifuged at 1,500 × *g* for 30 min. Samples were stored at -20°C until further analysis.

Ewe Performance Measures

Ultrasound measurements (Aloka 500-SSV; Aloka Co. Ltd., Tokyo, Japan) were taken prior to treatment initiation (**P**) (d 89 $\pm$ 4 of gestation) and prior to necropsy (**F**) (d 124 $\pm$ 4 of gestation). Measurements were recorded for backfat thickness (**BF**), body wall thickness (**BWT**), and loin eye area (**LEA**) at the 12th rib. Backfat thickness was measured at a point three-quarters the length of the longissimus muscle (**LM**) from the backbone end, and the LM cross-section was traced to determine LEA. Change (Δ) was calculated for each variable of interest.

Necropsy Procedures

Necropsies were performed on d 130 $\pm$ 1 of gestation. Twenty four hours prior to necropsy animals were removed from feed and water. Immediately prior to slaughter a final BW was taken. Animals were stunned by captive bolt (Supercash Mark 2, Accles and Shelvoke Ltd., Sutton Coldfield, UK), exsanguinated, and detailed necropsies were performed. Mammary tissue was removed and weighed. Blood was collected and organs were harvested and weighed. Gravid uterine weight was recorded. Perirenal fat was removed from the kidneys and body wall and

weighed. Ewe eviscerated body weight (**EBW**) was considered to be the weight of the remaining carcass with head and pelt, but without all thoracic and abdominal internal organs.

Hormone Analysis

Progesterone (**P_4**) was analyzed as previously described (Galbreath et al., 2008). Briefly, a 50-µl sample of maternal serum was analyzed in duplicate. Progesterone concentrations were measured by chemiluminescence immunoassay using the Immulite 1000 (Siemens, Los Angeles, CA), where lesser-, medium-, and greater-P_4 pools were assayed in duplicate (1.72 ± 0.06, 3.64 ± 0.05, and 14.23 ± 0.22 ng/mL for lesser-, medium-, and greater-P_4 pools, respectively). The intra- and interassay CV were 3.79% and 7.41%, respectively.

Thyroxine (**T_4**) and triiodothyronine (**T_3**) concentrations were determined by chemiluminescence immunoassay using the Immulite 1000 (Siemens, Los Angeles, CA), utilizing components of commercial kits (Diagnostic Products Corp., Los Angeles, CA) as previously described (O'Neil et al., 2009). Within each assay, lesser-, medium-, and greater-T_3 and T_4 pools were assayed in duplicate (94.76 ± 2.93, 170.25 ± 2.87, and 337 ± 6.15 ng/dL and 2.40 ± 0.07, 7.86 ± 0.21, and 12.01 ± 0.27 µg/dL, mean ± SEM for lesser-, medium-, and greater-pools, for T_3 and T_4 respectively). Twenty-five-microliter and 15-µl serum samples were assayed in duplicate for T_3 and T_4, respectively. The intraassay CV was 6.19% and 5.60% for T_3 and T_4, respectively, and the interassay CV was 4.40% and 6.17% for T_3 and T_4, respectively.

Ewe serum samples were analyzed for cortisol concentration as previously described (Lekatz et al., 2010). Briefly, serum samples (10-µL) were assayed in duplicate by chemiluminescence immunoassay using the Immulite 1000 (Siemens, Los Angeles, CA). Within each assay, lesser-, medium-, and greater-cortisol pools were assayed in duplicate (4.29 ± 0.05,

12.92 ± 0.31, and 34.35 ± 0.56 µg/dL, mean ± SEM for lesser-, medium-, and greater-cortisol pools, respectively). The intra- and interassay CV were 9.33% and 4.20%, respectively.

Circulating concentrations of estradiol-17β were analyzed in all serum samples by RIA using methodology described by Perry and Perry (2008). Intra- and inter-assay CV values were 5.6% and 13.9% respectively.

Serum Chemistry Panel Analysis

Serum samples were analyzed using an automated analyzer (VetTest Chemistry Analyzer; Idexx Laboratories, Inc., Westbrook, ME). Variables measured included: calcium (**CA**), creatine kinase (**CK**), creatinine (**CREA**), lactate dehydrogenase (**LDH**), albumin (**ALB**), globulin (**GLOB**), total protein (**TP**), blood urea nitrogen (**BUN**), aspartate aminotransferase (**AST**), γ-glutamyltransferase (**GGT**), bilirubin (**TBIL**), glucose (**GLU**), and alkaline phosphatase (**ALP**).

Statistical Analysis

Ultrasound data, organ weights, and chemistry panel data (n = 44) were analyzed using the ordinary least squares [GLM procedure of SAS (SAS Inst. Inc., Cary, NC)] with treatment, fetal number, and the interactions between the variable in the model. Ewe body weight and endocrine data were analyzed with repeated measures ANOVA of the MIXED procedure of SAS and means separated with the PDIFF option of the LSMEANS statement. The model statement included day of gestation, treatment, fetal number, and all interactions. The covariance structures used were ante-dependence for P_4, cortisol, and E_2, unstructured for T_3, and autoregressive for T_4. In addition, the hormone data were further analyzed by calculating the area under the curve (AUC) with the use of SigmaPlot 8.0 (Systat Software, Inc. San Jose, CA) and were tested with GLM procedure of SAS. The model statement included treatment and fetal number. Means were

separated with the PDIFF option of the LSMEANS statement. Least square means and SEM are reported. Significance was noted when $P \leq 0.05$.

Results

There was no difference ($P \geq 0.23$) in initial BW between treatment groups (67.05 kg ± 1.11). Treatment did affect final BW with ewes receiving the MP80 diet being heavier ($P = 0.03$; 72.62 kg) than MP60 ewes (64.77 kg), but neither being different from MP100 ewes (69.08 kg). There was a treatment by day interaction ($P < 0.01$) for percentage change in BW with ewes on the MP60 diet exhibiting a negative change in body weight throughout the majority of the trial compared with MP80 or MP100 ewes, which had a positive change in BW and were not different (Figure 3.1).

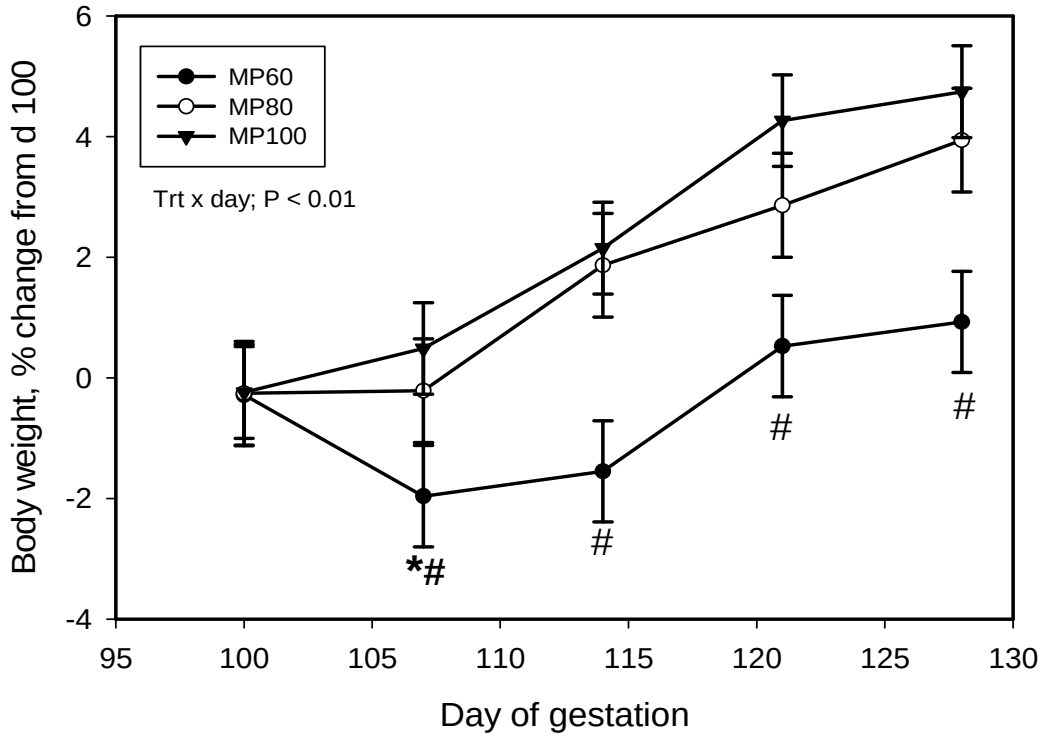

Figure 3.1. Treatment x day interaction for percent change in body weight

Results from the ultrasonography measurements are presented in Table 3.3. There was no difference among treatment groups for beginning ultrasound measures including: LEA, BF, or BWT ($P \geq 0.29$). Neither treatment nor fetal number affected final LEA, BF, or BWT ($P \geq 0.30$). Furthermore, the change for LEA, BF, and BWT were not different between treatment groups or affected by fetal number ($P \geq 0.29$)

Table 3.3. Effects of plane of nutrition and fetal number on ultrasound measurements

	Nutrition Treatment[1]				Fetal Number			P-value		
Item[2,3,4]	MP100	MP80	MP60	SE	1	2	SE	Trt	FN	Trt*FN
P LEA	10.52	10.68	10.18	0.43	10.58	10.34	0.35	0.71	0.63	0.50
P BF	0.50	0.48	0.50	0.03	0.51	0.47	0.24	0.92	0.29	0.85
P BWT	1.48	1.53	1.48	0.09	1.54	1.45	0.08	0.92	0.84	0.83
F LEA	9.81	10.11	9.15	0.48	9.94	9.44	0.39	0.36	0.38	0.24
F BF	0.30	0.30	0.28	0.02	0.31	0.28	0.02	0.65	0.30	0.34
F BWT	1.12	1.22	1.13	0.08	1.20	1.12	0.06	0.63	0.35	0.90
Δ LEA, %	-6.56	-5.36	-9.75	3.45	-5.65	-8.80	2.79	0.65	0.44	0.24
Δ BF, %	-37.21	-37.56	-37.56	3.96	-38.68	-41.01	3.21	0.29	0.61	0.28
Δ BWT, %	-23.37	-19.38	-22.87	3.21	-21.32	-22.42	2.60	0.67	0.77	0.19

[1]Treatment: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80= diet designed to meet 80% of metabolizable protein requirements; MP60= diet designed to meet 60% of metabolizable protein requirements
[2]P=beginning values taken prior to treatment initiation
[3]F=final measurements taken before necropsy
[4]Δ, % =change in measurements

Initial chemistry panel results (Table 3.4) showed no differences ($P \geq 0.08$) in parameters of interest except for PCREA and PTP. Initial CREA was lower ($P = 0.05$) for ewes carrying singletons compared to ewes carrying twins and PTP was higher ($P = 0.04$) in ewes carrying singletons compared to those carrying twins. It should be noted that although these values were different, all values for PCREA and PTP were within the normal assay range.

Treatment had an effect on change in ALB with ewes on the MP60 diet showing a greater ($P = 0.02$) change in ALB levels compared to MP80 and MP100 ewes which were not different. When evaluating change in AST, ewes on the MP60 diet experienced a loss in AST concentrations and this was different ($P < 0.01$) compared to the positive changes in MP80 and MP100 ewes which were not different.

Table 3.4. Serum chemistry panel assay results for dietary treatments[1,2] and fetal number

	Nutrition Treatment				Fetal Number			P-value		
Item[3,4,5]	MP100	MP80	MP60	SE	1	2	SE	Trt	FN	Normal[6]
P ALB (g/dL)	3.28	3.43	3.32	0.07	3.34	3.34	0.06	0.32	0.95	2.4-3.7
F ALB (g/dL)	3.16	3.16	2.96	0.07	3.13	3.06	0.06	0.06	0.38	-----
**Δ ALB, %	-3.26[a]	-4.47[a]	-11.18[b]	2.19	-5.63	-6.98	1.78	0.02	0.60	-----
P ALKP (U/L)	107.71	89.43	124.94	13.28	116.98	97.74	11.08	0.17	0.23	50-228
F ALKP (U/L)	129.50	86.09	113.50	14.24	114.55	104.84	11.88	0.11	0.58	-----
Δ ALKP, %	16.67	-4.44	-6.36	7.89	1.30	2.61	6.58	0.09	0.89	-----
P AST (U/L)	99.93	105.92	112.53	10.82	102.54	109.71	8.76	0.68	0.57	40-96
*F AST (U/L)	109.57	150.88	82.25	13.73	94.44	134.02	11.12	0.01	0.02	-----
Δ AST, %	12.68[a]	29.60[a]	-21.20[b]	7.26	-1.41[a]	15.46[b]	5.88	<0.01	0.05	-----
P BUN (mg/dL)	14.14	14.24	15.81	0.73	14.33	15.13	0.61	0.19	0.37	5-20
F BUN (mg/dL)	24.79[a]	18.08[b]	11.18[c]	2.00	17.44	18.59	1.67	<0.01	0.64	-----
Δ BUN, %	75.84[a]	29.67[b]	-26.71[c]	14.48	26.00	26.53	12.09	<0.01	0.98	-----
P CA (mg/dL)	9.76	10.14	10.13	0.13	9.96	10.06	0.11	0.08	0.54	9.1-10.8
**F CA (mg/dL)	8.98[a]	9.36[b]	9.80[c]	0.13	9.44	9.32	0.11	<0.01	0.40	-----
**Δ CA, %	-7.95[a]	-7.70[a]	-3.02[b]	1.25	-5.08	-7.37	1.04	0.01	0.14	-----
P CK (U/L)	225.93	236.03	105.66	53.29	177.93	200.49	44.48	0.15	0.73	8-100
F CK (U/L)	70.21	95.82	41.69	29.54	80.44	58.05	24.65	0.42	0.54	-----
Δ CK, %	-32.33	-42.64	-41.90	18.58	-29.50	-48.41	15.50	0.91	0.41	-----
**P CREA (mg/dL)	1.03	1.03	1.03	0.03	1.00[a]	1.06[b]	0.02	0.99	0.05	0.6-1.5
F CREA (mg/dL)	1.22	1.25	1.20	0.05	1.19	1.26	0.04	0.71	0.24	-----
Δ CREA, %	18.99	23.23	15.26	4.03	19.41	18.91	3.27	0.41	0.92	-----
P GGT (U/L)	84.14	86.73	88.80	4.61	86.56	86.56	3.84	0.77	1.00	33-55
F GGT (U/L)	75.71	81.04	84.04	3.81	84.41	76.12	3.18	0.30	0.08	-----
**Δ GGT, %	-9.75	-5.90	-3.98	2.56	-1.97[a]	-11.12[b]	2.13	0.27	<0.01	-----
P GLOB (g/dL)	3.84	3.86	3.76	0.08	3.90	3.74	0.06	0.64	0.09	3.2-4.1
**F GLOB (g/dL)	3.59	3.56	3.49	0.09	3.65[a]	3.44[b]	0.07	0.72	0.05	-----
Δ GLOB, %	-6.34	-7.01	-6.94	2.16	-5.88	-7.64	1.75	0.97	0.49	-----
P GLU (mg/dL)	51.43	58.78	56.41	2.52	55.47	55.61	2.10	0.13	0.96	50-80
F GLU (mg/dL)	37.14	38.96	40.59	2.33	42.30[a]	35.50[b]	1.94	0.57	0.02	-----
Δ GLU, %	-27.99	-30.20	-24.22	5.28	-23.30	-31.64	4.41	0.71	0.20	-----
P LDH (U/L)	1282.50	1243.71	1297.73	56.76	1322.02	1227.27	47.37	0.78	0.18	504-1049
*F LDH (U/L)	1152.29	1317.33	1010.64	74.53	1119.26	1200.91	60.36	0.03	0.35	-----
*Δ LDH, %	-10.03[a]	-1.00[a]	-20.57[b]	3.34	-14.97	-6.09	2.73	<0.01	0.03	-----
P TBIL (mg/dL)	0.22	0.28	0.27	0.04	0.23	0.28	0.03	0.51	0.26	0.1-0.4
F TBIL (mg/dL)	0.26	0.23	0.23	0.05	0.21	0.27	0.04	0.85	0.30	-----
Δ TBIL, %	52.38	-5.39	15.10	33.79	19.62	21.77	28.20	0.49	0.96	-----
**P TP (g/dL)	7.09	7.17	7.08	0.09	7.23[a]	7.01[b]	0.07	0.73	0.04	5.6-7.8
**F TP (g/dL)	6.74	6.70	6.45	0.10	6.79[a]	6.47[b]	0.09	0.13	0.02	-----
**Δ TP, %	-5.04[a]	-6.61[a,b]	-8.97[b]	0.10	-6.05	-7.69	0.82	0.02	0.17	-----

[1]Treatment: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80= diet designed to meet 80% of metabolizable protein requirements; MP60= diet designed to meet 60% of metabolizable protein requirements

[2]Diets formulated based on a 70 kg ewe carrying twins

[3]Assay abbreviations: albumin (**ALB**), alkaline phosphatase (**ALKP**), aspartate aminotransferase (**AST**), blood urea nitrogen (**BUN**), calcium (**CA**), creatine kinase (**CK**), creatinine (**CREA**), gamma-glutamyltransferase (**GGT**), globulin (**GLOB**), glucose (**GLU**), lactate dehydrogenase (**LDH**), bilirubin (**TBIL**), and total protein (**TP**)

[4]**P** = beginning of trial prior to treatment initiation and synchronization; **F** = final day of trial; Δ = change

[5]**FN** = fetal number

[6]Normal range of assays in sheep using VetTest Chemistry Analyzer (IDEXX, Westbrook, ME)

*Variables with Trt*FN interactions ($P \leq 0.05$); Results shown in Tables 3.5, 3.6, and 3.7

** Although values were significantly different from each other the values fell within the normal range for the assay, therefore are not considered medically significant

Final blood urea nitrogen concentrations increased ($P < 0.01$) with increasing nutritional plane. In addition there was a larger change ($P < 0.01$) from the beginning to the end of the experiment in BUN as MP levels increased. Final CA levels decreased ($P < 0.01$) with increasing level of nutrition, although the values were all within the normal range for the assay. Furthermore, the change in CA was smaller ($P = 0.01$) for MP60 ewes compared to the other two groups, which were not different. In addition there was a greater ($P = 0.02$) change in TP for MP60 ewes compared with MP100 ewes, with MP80 ewes not being significantly different from either of the other two nutritional groups.

Fetal number had an effect on AST, GGT, GLOB, GLU, and TP. Fetal number affected the change in AST with ewes carrying twins having a greater ($P = 0.02$) positive change in levels compared to ewes carrying singletons, which had a negative change in AST levels. Furthermore, GGT in ewes carrying twins was a greater ($P < 0.01$) change over time then ewes carrying singletons. Ewes carrying singletons had increased ($P \leq 0.05$) levels of GLOB and GLU at the end of the experiment as compared to ewes carrying twins. Ending TP was decreased ($P \leq 0.04$) in ewes carrying twins, although the values were within the normal range for this assay.

With the exception of FAST (Table 3.5), FLDH (Table 3.6), and change in LDH (Table 3.7) there was no interaction between nutrition and fetal number. Final AST was higher ($P = 0.01$) in MP80 ewes carrying twins compared with all other groups. Final values for LDH were highest ($P = 0.03$) in MP80 ewes carrying twins, which were not different from MP100 ewes carrying singletons. However, MP80 ewes carrying twins were different from all other groups and MP100 ewes carrying singletons were not statistically different from any groups. When looking at change in LDH MP80 ewes carrying twins were different ($P = 0.05$) from all other

groups. In addition MP60 ewes carrying singletons had a greater change in LDH then MP100 ewes carrying both singletons and twins.

Table 3.5. Treatment[1,2] x fetal number interaction for final aspartate aminotransferase

Treatment	Singles	Twins	SE	P-value
MP100	104.57[a]	114.57[a]	18.25	0.01
MP80	93.08[a]	208.67[b]	20.90	0.01
MP60	85.67[a]	78.83[a]	18.00	0.01

[1]Treatment: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80= diet designed to meet 80% of metabolizable protein requirements; MP60= diet designed to meet 60% of metabolizable protein requirements
[2]Diets formulated based on a 70 kg ewe carrying twins

Table 3.6. Treatment[1,2] x fetal number interaction for final lactate dehydrogenase

Treatment	Singles	Twins	SE	P-value
MP100	1193.00[a,b]	1111.57[a]	99.01	0.03
MP80	1095.33[a]	1539.33[b]	113.43	0.03
MP60	1069.44[a]	951.83[a]	146.64	0.03

[1]Treatment: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80= diet designed to meet 80% of metabolizable protein requirements; MP60= diet designed to meet 60% of metabolizable protein requirements
[2]Diets formulated based on a 70 kg ewe carrying twins

Table 3.7. Treatment[1,2] x fetal number interaction for change in lactate dehydrogenase

Treatment	Singles	Twins	SE	P-value
MP100	-10.21[a]	-9.85[a]	4.47	0.05
MP80	-12.89[a,b]	10.88[c]	5.12	0.05
MP60	-21.82[b]	-19.31[a,b]	4.39	0.05

[1]Treatment: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80= diet designed to meet 80% of metabolizable protein requirements; MP60= diet designed to meet 60% of metabolizable protein requirements
[2]Diets formulated based on a 70 kg ewe carrying twins

Maternal diet did not affect hormones by day ($P \geq 0.10$). From d 100 to d 130, there was a fetal number by day interaction ($P < 0.01$) in P_4 concentrations (Figure 3.2) with ewes carrying twins having increased P_4 concentrations over time compared to ewes carrying singletons. Both day and fetal number had significant ($P < 0.01$) effects on E_2 concentrations (Figure 3.3) with E_2 increasing over time and having a higher concentration in ewes carrying twins compared to ewes

carrying singletons. There was only a day effect noted for T_3 concentrations with T_3 concentrations decreasing as days of gestation increased (Figure 3.4). There was a day by fetal number interaction for T_4 ($P < 0.01$), which is shown in Figure 3.5. On d 100, all ewes had similar T_4 concentrations (Figure 3.5). With advancing gestation, T_4 levels decreased in both groups; however, ewes carrying singletons had a smaller decrease in T_4 concentrations compared to those carrying twins.

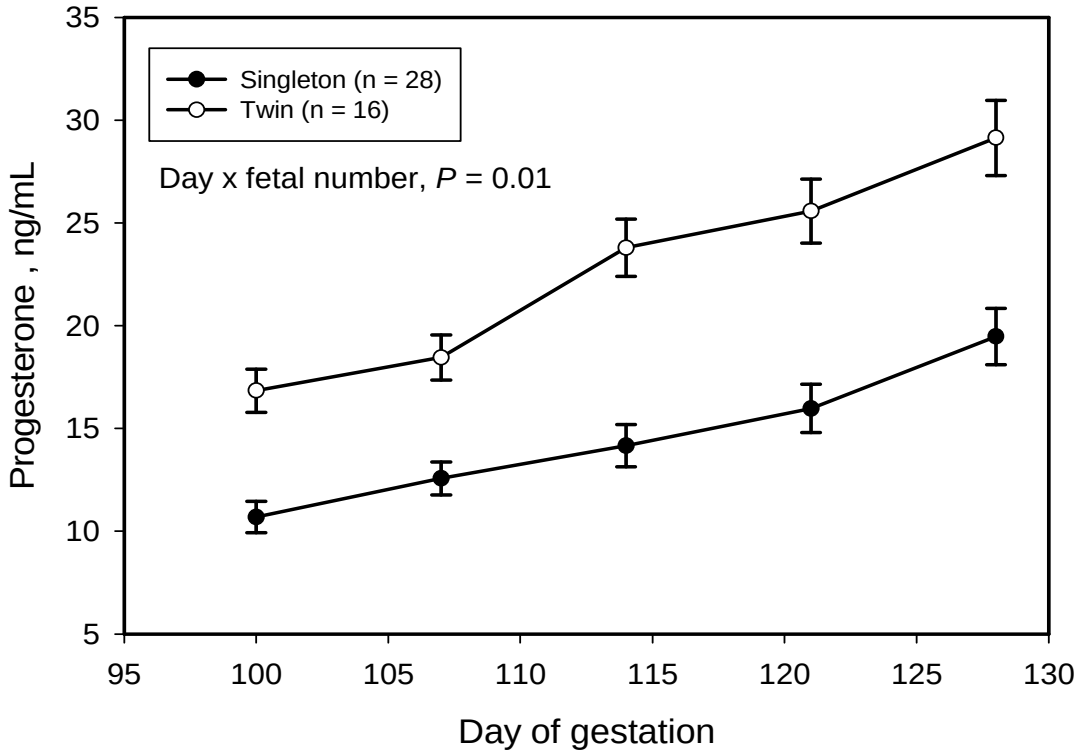

Figure 3.2. Day x fetal number interaction for concentration of progesterone

There was a treatment by fetal number interaction ($P = 0.02$) for T_4 AUC (Figure 3.5 inset). While treatment did not impact T_4 AUC in ewes carrying twins, ewes carrying singletons from the MP80 group had a greater AUC than MP60 ewes, with MP100 being least. Moreover, while fetal number did not impact T_4 AUC in the MP100 ewes, in both MP60 and MP80, ewes

carrying twins had greater T_4 than ewes carrying singletons. Day did have a significant effect ($P < 0.01$) on cortisol levels which decreased as gestation advanced (Figure 3.6).

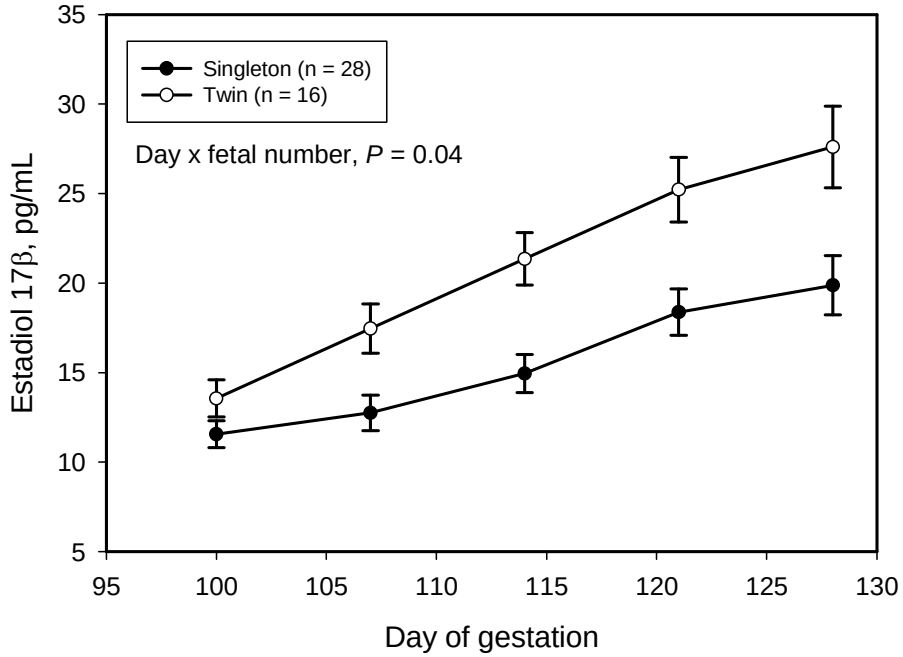

Figure 3.3. Day x fetal number interaction for concentration of estradiol

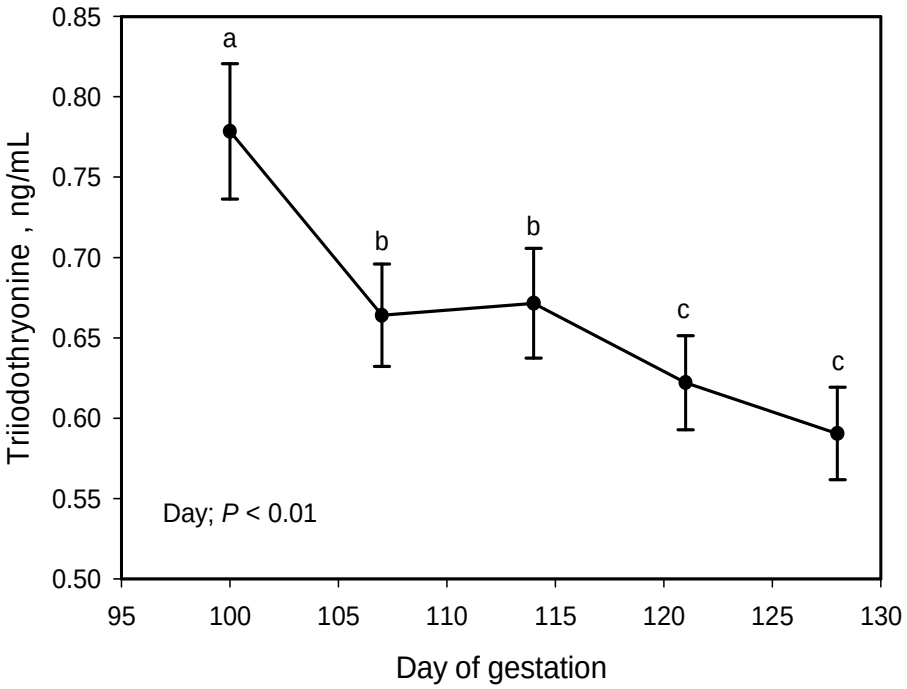

Figure 3.4. Day of gestation effects on triiodothyronine concentration

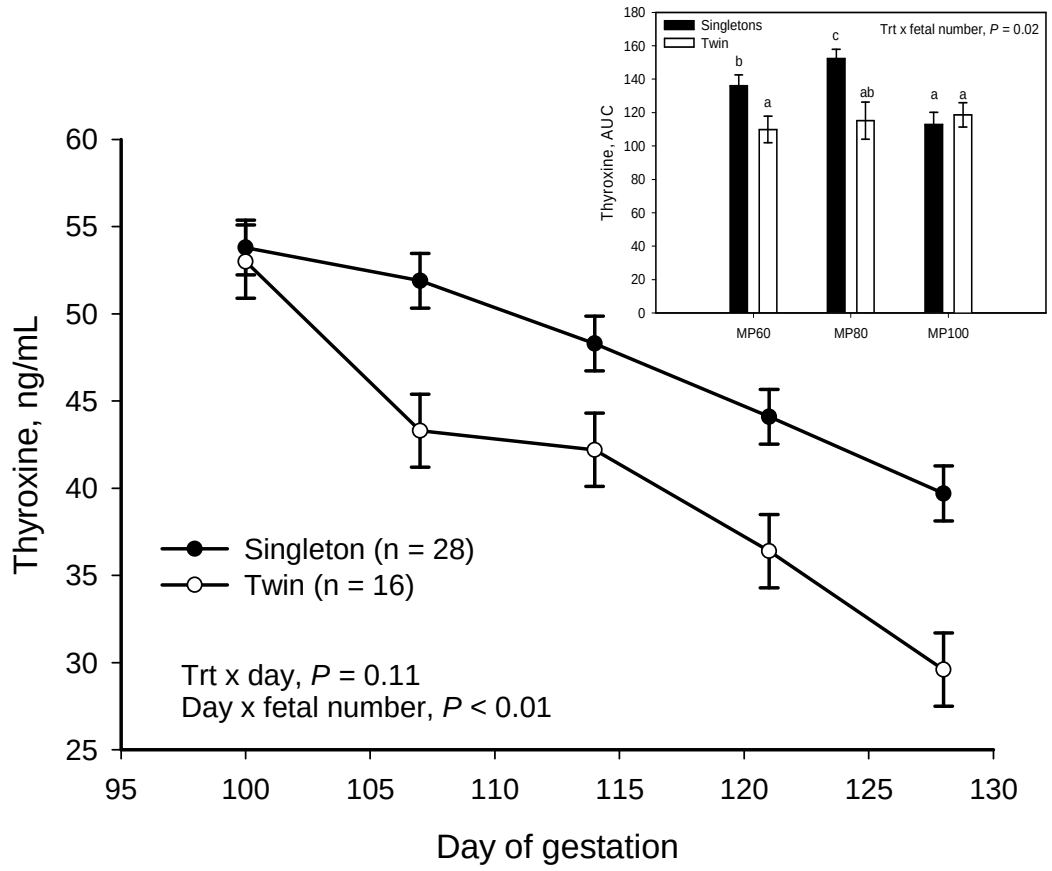

Figure 3.5. Day x fetal number and treatment x fetal number interactions for thyroxine concentration

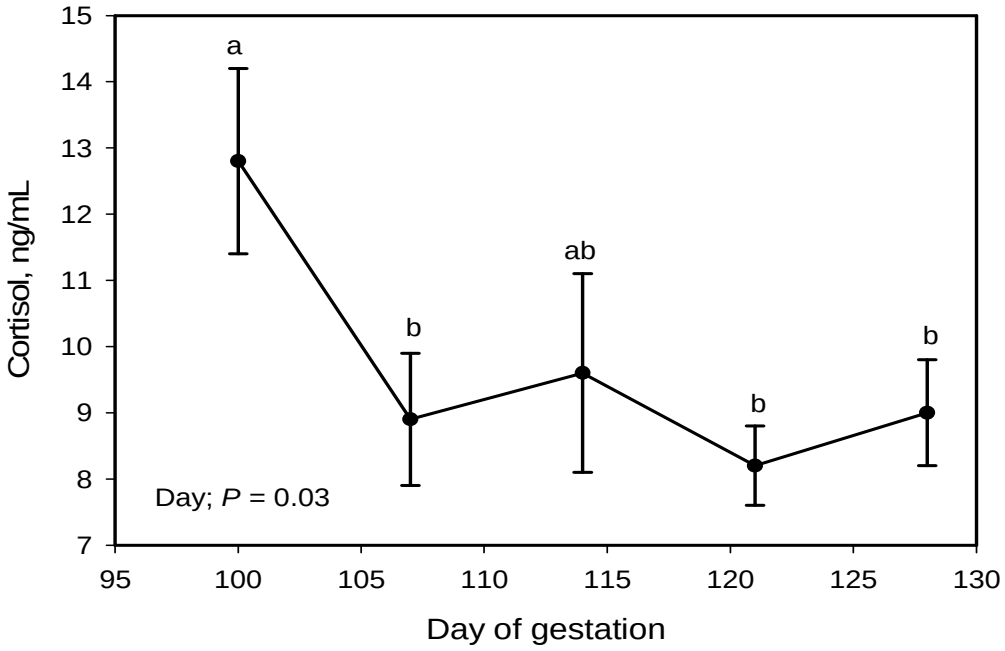

Figure 3.6. Day of gestation effects on cortisol concentration

Visceral organ weights are shown in Table 3.8. There was no treatment or fetal number effect on blood (g), perirenal fat (g or g/kg), thyroid (g or g/kg), or adrenal mass (g) ($P \geq 0.09$). There was a treatment effect on heart weight (g) with MP80 ewes possessing heavier ($P < 0.01$) hearts than MP60 and MP100 ewes, which were not different.

Table 3.8. Treatment effects on ewe organ weights

	Treatment[1,2]				Fetal Number			P-value	
Item[3,4]	MP100	MP80	MP60	SE	1	2	SE	Trt	FN
Initial Wt, kg	66.45	70.09	64.73	2.41	65.93	68.26	1.99	0.23	0.35
Final Wt, kg	69.08[a,b]	72.62[a]	64.77[b]	2.03	67.23	70.41	1.69	0.03	0.20
Evisc BW, kg	33.61	36.32	33.11	1.15	35.20	33.50	0.10	0.11	0.23
Blood, g	3417.92	3558.36	3225.51	209.00	3206.22	3594.97	169.07	0.54	0.12
g/kg	101.57	100.04	100.34	5.35	92.46[a]	108.84[b]	4.48	0.98	0.02
Heart, g	258.31[a]	308.56[b]	269.67[a]	10.82	282.01	275.68	8.78	<0.01	0.62
g/kg	7.78	8.33	8.40	0.25	8.06	8.28	0.21	0.18	0.48
Perirenal Fat, g	252.40	405.73	380.53	59.40	379.70	312.74	49.69	0.17	0.36
g/kg	7.06	10.99	11.17	1.42	10.25	9.24	1.19	0.09	0.56
Kidneys, g	128.95[a]	134.87[a]	116.46[b]	3.79	129.97	123.55	3.16	<0.01	0.17
g/kg	3.88	3.73	3.60	0.09	3.73	3.74	0.08	0.13	0.89
Liver, g	682.05	701.53	628.02	24.58	634.02[a]	707.05[b]	20.51	0.09	0.02
g/kg	20.49	19.44	19.42	0.61	18.17[a]	21.40[b]	0.51	0.37	<0.01
Lung, g	574.26	587.62	539.18	23.83	601.51[a]	532.54[b]	19.88	0.32	0.02
g/kg	17.20	16.20	16.57	0.54	17.18	16.14	0.45	0.42	0.12
Mammary, g	632.46	783.89	680.92	69.54	507.22[a]	890.97[b]	58.03	0.31	<0.01
g/kg	19.22	21.50	20.59	1.98	14.54[a]	26.33[b]	1.66	0.72	<0.01
Uterus, g	765.59	783.74	700.51	33.16	660.53[a]	839.36[b]	27.68	0.17	<0.01
g/kg	23.01	21.75	21.32	0.94	18.97[a]	25.09[b]	0.78	0.42	<0.01
Thyroid, g	4.37	4.07	4.25	0.40	4.38	4.08	0.33	0.87	0.54
g/kg	0.13	0.11	0.13	0.01	0.13	0.12	0.01	0.45	0.94
Gravid Uterus, g	9643.12	10126.06	9523.40	699.51	6971.25[a]	12557.13[b]	579.99	0.82	<0.01
g/kg	298.40	275.46	295.13	22.98	209.07[a]	370.26[b]	19.11	0.75	<0.01

[1]Treatment: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80= diet designed to meet 80% of metabolizable protein requirements; MP60= diet designed to meet 60% of metabolizable protein requirements
[2]Diets formulated based on a 70 kg ewe carrying twins
[3]g/kg = organ weight divided by eviscerated BW
[4]Interaction between fetal number and dietary treatment shown in another table

Kidney weight (g) was also affected by treatment with MP60 ewes having lighter ($P < 0.01$) kidneys compared to MP80 and MP100 ewes, which showed no difference. When organ mass was expressed per EBW, maternal diet did not impact any organ weight ($P \geq 0.09$). As shown in Table 3.8 ewes carrying twins had increased ($P \leq 0.02$) liver (g and g/kg), mammary (g and g/kg), uterus (g and g/kg), and gravid uterus (g and g/kg) weights compared to ewes carrying singletons. Ewes with singletons had increased ($P = 0.02$) lung weights (g) compared to ewes

carrying twins. Blood (g/kg) was increased (P = 0.02) in ewes carrying twins in comparison to ewes carrying singletons. Adrenal weight (g/kg) showed a significant interaction between treatment and fetal number (Table 3.9). In ewes carrying singletons, MP60 and MP80 had decreased adrenal weight (g/kg) compared to MP100. In ewes carrying twins, MP60 was greater than MP80. Moreover, in MP60 ewes carrying twins the adrenal glands were heavier (g/kg) than MP60 ewes carrying singletons (Table 3.9).

Table 3.9. Treatment[1,2] x fetal number interaction for adrenal gland weight per ewe weight

Treatment	Singles	Twins	SE	P-value
MP100	0.16[bc]	0.14[ab]	0.01	0.02
MP80	0.13[a]	0.12[a]	0.01	0.02
MP60	0.13[a]	0.16[bc]	0.01	0.02

[1]Treatment: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80= diet designed to meet 80% of metabolizable protein requirements; MP60= diet designed to meet 60% of metabolizable protein requirements
[2]Diets formulated based on a 70 kg ewe carrying twins

Discussion

We partially reject our hypothesis as no adverse changes were noted for uterine or mammary gland development. However, changes in the serum chemistry panel values indicate changes in markers of inflammation. The change in final BW with ewes receiving MP60 being lower than MP80 which were not different than MP100 agrees with other studies which have shown nutrient restriction to negatively affect final BW (Drouillard et al., 1991; Wester et al., 1995). Our data shows that level of MP during late gestation did not significantly alter any other maternal carcass characteristics including BF, LEA, and BWT, as shown by ultrasonography; however this may not serve as an indicator of function. Hammell et al. (1976) showed offspring from sows receiving inadequate protein levels were lighter at 21 and 45 days of age and gained less while consuming more feed. It should be noted this experiment evaluated an overall protein restriction and was not limited to metabolizable protein restriction. Another avenue to consider

when evaluating the overall effects of protein restriction is the possibility of the maternal unit offsetting some of the deficiencies if all other nutritional requirements are met. More research is needed to examine long term effects of MP restriction on both the maternal and fetal units.

The results of this project showed no major changes as a result of treatment on visceral organ weights as a proportion of the maternal weight, with the exception of the adrenal gland. The adrenal gland secretes several hormones including glucocorticoids, sometimes known as stress hormones. Especially in our MP60 ewes carrying twins, an increase in cortisol is expected as the maternal system is insulted with decreased protein supply as well as increased fetal number. The increase in cortisol in MP100 ewes carrying singletons is more difficult to explain as those ewes were receiving adequate nutrition.

Wester et al. (1995) reports similar results to the ones in this study with no sufficient differences noted in organ mass between treatment groups. These results are contradictory to Drouillard et al. (1991), which found weights of the liver to be reduced in the protein restricted lambs. The major difference between these studies is Drouillard et al. (1991) was looking at young lambs of both sexes which were not reproductively active. One explanation for the change in the young lambs is protein supply was being utilized for growth instead of organ mass.

We did see fetal number affecting blood, liver, mammary, uterus, and gravid uterus weights with ewes carrying twins having increases in these weights. These increases are logical and would be expected with increased fetal number due to increased demands from the fetal unit for size, waste expulsion, and milk output.

Diet has been shown to affect BUN based on protein supplied in the diet of various livestock species (Anthony et al., 1986; Kusina et al., 1999; Wallace et al., 2006). The decrease in serum BUN for low protein ewes in our current study was also noted in cattle by Anthony et

al. (1986). This decrease in BUN is expected as diets restricted in protein provide fewer precursors for nitrogen supply resulting from breakdown of protein.

The kidneys are the most important route for urea excretion. In ruminants, urea excreted into the rumen is degraded to ammonia, then used to synthesize amino acids and the excess is excreted. Aminotransferases are the enzymes that remove or add amino groups from amino acids. Aspartate is synthesized by a one-step transamination reaction catalyzed by AST using oxaloacetate as the precursor. Aspartate is a precursor of ornithine in the urea cycle. AST levels have been used as markers of tissue damage with increasing serum levels indicating an increased extent of damage. The decrease in AST and BUN for MP60 ewes may be positively correlated in a sense that with decreased availability of AST the efficiency of the urea cycle may be comprised leading to a decrease in urea excretion and lower levels of BUN.

In domestic animals GGT is mostly found in the kidneys, pancreas, liver, and intestine. Gamma-glutamyltransferase is highly active in the liver of sheep and serves as an indicator of liver disturbance in ruminants (Braun et al., 1983; Braun et al., 1986). Diets with restricted protein intake have been associated with decreased liver function (Alemu et al., 1977). Levels of GGT have also been found to be increased in mammary glands of pregnant animals (Pero et al., 2006). Elevated serum GGT serves as a marker of systemic inflammation and increased oxidative stress seen in ewes carrying twins. This may be one explanation for the greater change in GGT noted in ewes carrying twins.

In our current study, dietary treatment had no significant effect on E_2. This agrees with Anthony et al. (1986) who saw no change in concentration of E_2 for beef calves fed differing levels of crude protein. However, Anthony et al. (1986) found P_4 concentrations that tended (P = 0.07) to be greater for low protein heifers which is in contrast to our findings that MP restriction

had no effect on P_4 concentrations. Differences in the results may be attributed to the different types of protein restriction, length of restriction, and timing of restriction.

There was a day effect on cortisol with concentrations showing elevation at the beginning of the trial. This was likely a result of increased stress on the ewes at the beginning of the trial due to the transition to a new facility and increased handling. Other research projects have shown a similar increase in cortisol concentrations with increased stressors such as handling and transportation (Parrott et al., 1994; Broom et al., 1996).

Treatment or fetal number independently did not significantly affect T_3 or T_4 levels; however, T_4 had a significant treatment by fetal number interaction. In addition, T_4 levels were significantly affected by the interaction between day and fetal number. Other studies in both sheep and cattle have seen similar results of a linear decrease in T_4 with advancing days of gestation (Hung and Prakash, 1990; Ward et al., 2008). The tendency for singletons to have higher levels of T_4 may be due to portioning of metabolism to ensure survival of both fetuses in twins; however, more studies are necessary to investigate the differences in these thyroid hormones between dams carrying singletons and twins. The drop in T_4 for both ewes carrying singletons and twins late in gestation could have to do with metabolic changes associated with the initiation of lactation. Triiodothyronine concentrations dropped as day of gestation advanced. Again this is likely due to metabolic changes in preparation for parturition and lactation (Tiirats, 1997).

Although maternal MP dietary level did not affect ultrasound measurements or a large majority of the organ masses obtained, the changes observed in the serum chemistry panel concentrations indicate that function of these organs may be altered and are not completely understood. Furthermore, the minimal effect on the maternal unit does not adequately predict

adverse effects these dietary changes may have on offspring later in life. We must continue to be aware that the maternal and fetal units may respond differently to the same nutritional scheme. Moreover, postnatal development in twin born lambs may be more dependent on maternal responses to diet than singletons.

CHAPTER 4. OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

It is well known that proper maternal nutrition is vital for reproductive success as well as proper fetal growth and development (Bond and Wiltbank, 1970; Shields et al., 1985; Van Niekerk and Van Niekerk, 1998). Two very important areas of nutrition which should be evaluated are fatty acid supplementation and protein availability as they are important to all body tissues for physiological functions. Therefore, two experiments were conducted to examine the effects of supplementation with omega-3 fatty acids and dietary level of MP to determine effects to the maternal unit including hormone levels, serum markers of body disturbance, ultrasound measures, and fatty acid profiles.

In the mare flaxseed experiment (Chapter 2), we evaluated P_4 concentrations, SCP, and fatty acid concentrations. Results showed an increase in ALA concentration with flaxseed supplementation. There were no significant changes observed in P_4 concentrations or SCP data. As was discussed earlier there were limitations in this project which may have confounded results. The low protein level supplied to the mares as well as the lack of normal estrous cycles made it difficult to truly evaluate hormone levels and estrous cycle characteristics. If this project were to be replicated, it would be important to use ultrasound examinations prior to project initiation to ensure reproductive soundness, as well as determine the phase of the estrous cycle to help ensure blood samples were obtained during similar phases. In addition, all dietary analysis should be completed prior to treatment initiation.

During the second experiment, MP levels during late gestation were evaluated (Chapter 3). Ultrasound measurements showed no differences between treatment groups for body composition. There were minimal organ weights which were affected by treatment; however fetal number did affect all of the reproductive organs including: mammary, uterus, and gravid

uterus weight. Treatment alone did not affect any of the hormone concentrations measured in this study. One confounding issue in this project was the occurrence of both singletons and twins. If this project were to be repeated it would minimize variation to ensure all ewes were only carrying singletons. In addition, it would be beneficial to look at levels of MP which were restricted, control, and excess to see if there were any changes to the parameters which were measured in this study.

Although these two studies were very different and used two animal models, a few conclusions can still be made. First, although there are not noticeable changes to the maternal body, adverse changes may still be occurring to organs and function which can have detrimental effects to reproductive status. Second, although we did not see noticeable treatment effects on the hormones measured, it is important to note that the restriction in the MP experiment was fairly short. In addition, the results from the flaxseed experiment may have been obscured by the lack of consistent estrous activity in the mares. Therefore, if these dietary insults are affecting the hormonal profiles of the females, there may be adverse effects on reproductive performance. Third, although we did not observe an advantage of increased ALA on reproductive performance in the flaxseed project, feeding at a higher level may elicit a noticeable effect. More research is necessary to truly understand these changes and the affects longer supplementation or increased levels of supplementation may have on the reproductive performance of female livestock species.

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