

IMPACTS OF FLAX ON FEMALE AND MALE REPRODUCTIVE TRAITS WHEN  
SUPPLEMENTED PRIOR TO BREEDING IN SHEEP

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**Title**

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## ABSTRACT

Two experiments determined the effects of flaxseed supplementation on reproductive parameters in sheep. In experiment one, 240 multiparous Rambouillet ewes were assigned to one of two treatments: basal ration alone or basal ration with a Flaxlic<sup>®</sup> Sheep Tub offered over 35 days. Serum was collected weekly for progesterone ( $P_4$ ). Flaxseed supplementation did not improve progesterone concentration or reproductive parameters ( $P \geq 0.26$ ). In experiment two, 120 Rambouillet ram lambs were assigned to one of two treatments: basal ration alone or basal ration with a Flaxlic<sup>®</sup> Sheep Tub offered over 112 days. Scrotal circumference measurements, serum for testosterone, and semen were collected on day 84 and 112. Day effects were found for select semen measurements, testosterone, weight, and scrotal circumference ( $P \leq 0.05$ ). There was no effect of treatment on testosterone concentration ( $P = 0.99$ ) or any semen quality characteristics ( $P \geq 0.33$ ). Overall, tub supplementation did not alter reproductive ability.

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## LIST OF ABBREVIATIONS

AA.....	Arachadonic Acid
ABP.....	Androgen Binding Protein
ADF .....	Acid detergent fiber
ADG .....	Average daily gain
ALA .....	Alpha Linolenic Acid; C18:3 $\omega$ -3
AOAC .....	Association of Analytical Communities
AOAC .....	Association of Analytical Communities
BCS .....	Body condition score
CL .....	Corpus luteum; corpora lutea
CON .....	Basal ration only group
CP .....	Crude protein
DM .....	Dry matter
DNA.....	Deoxyribonucleic acid
EPA.....	Eicosapentaenoic Acid
FA .....	Fatty Acids
FLX .....	Flaxlic <sup>®</sup> Sheep Tub supplemented group
FSH.....	Follicle stimulating hormone
GnRH.....	Gonadotrophin-releasing hormone
LA .....	Linoleic Acid; C18:2 $\omega$ -6
LH.....	Luteinizing hormone
P <sub>4</sub> .....	Progesterone
PG .....	Prostaglandin
PGE <sub>2</sub> .....	Prostaglandin E <sub>2</sub>
PGF <sub>2<math>\alpha</math></sub> .....	Prostaglandin F <sub>2<math>\alpha</math></sub>

PUFA .....Polyunsaturated Fatty Acids  
SC .....Scrotal circumference  
TAI.....Timed artificial insemination  
TDN .....Total digestible nutrients  
TMR .....Total mixed ration  
VFA.....Volatile Fatty Acids

## LIST OF SYMBOLS

- $\alpha$  .....alpha; referring to the alpha end, or carboxy end, of a fatty acid.
- $\omega$  .....omega; referring to the omega end, or methyl end, of a fatty acid.

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# 1. INTRODUCTION AND LITERATURE REVIEW

## 1.1. Introduction

One of the major issues in the sheep industry is reproductive efficiency. Many factors influence conception and fertility, and therefore reproductive efficiency. A few factors are genetics, season and nutrition. Breeding over time for specific traits can change reproductive capabilities, even influencing seasonality. A producer is also able to manage health and alter nutrition in a relatively short amount of time to arrive at an animal better suited for breeding.

Maintaining a proper weight is a challenge producers face between weaning and parturition. Proper nutrition is essential for maintaining weight during this time. Ewe body status is one of several external factors affecting embryo mortality (Vanroose et al., 2000). This can include both under and over-feeding, as well as deficiencies or excess of dietary components such as vitamins, minerals, or amino acids. Extra weight due to overfeeding can prevent a pregnancy, as adipose tissue may inhibit steroidal hormones. Adipose tissue in large quantities can negatively affect hormone pathways, particularly the effectiveness and binding of estrogen metabolites and production of luteinizing and follicle stimulating hormone (**LH, FSH**, respectively; Frisch, 1987).

Breed certainly affects reproductive capabilities, as many breeds have been bred for specific outcomes. For example, the Suffolk breed has been bred to produce large framed lambs with heavy muscling (Rosov and Gootwine, 2013). Blackface breeds like the Suffolk are often used as terminal sires in many white-faced operations. These breeds often have higher birthweights and subsequently less lambs compared to the more prolific breeds (Rosov and Gootwine, 2013). On the opposite end of the spectrum is the Polypay. The Polypay was specifically bred to be a highly prolific breed that has strong mothering characteristics (Hulet et

al., 1984). Prolificacy was not the only trait selected for. The researchers creating this breed also crossed sheep that had long breeding seasons, specifically the Rambouillet, Finn, and Dorset breeds (Hulet et al., 1984). In fact, at one year of age, the newly created Polypay already had fertility comparable to the Finn-Rambouillet cross. They also developed the ability for twice a year breeding that exceeded any of the breeds used to breed the Polypay, even Finn-Rambouillet and Dorset-Targhee crosses (Hulet et al., 1984).

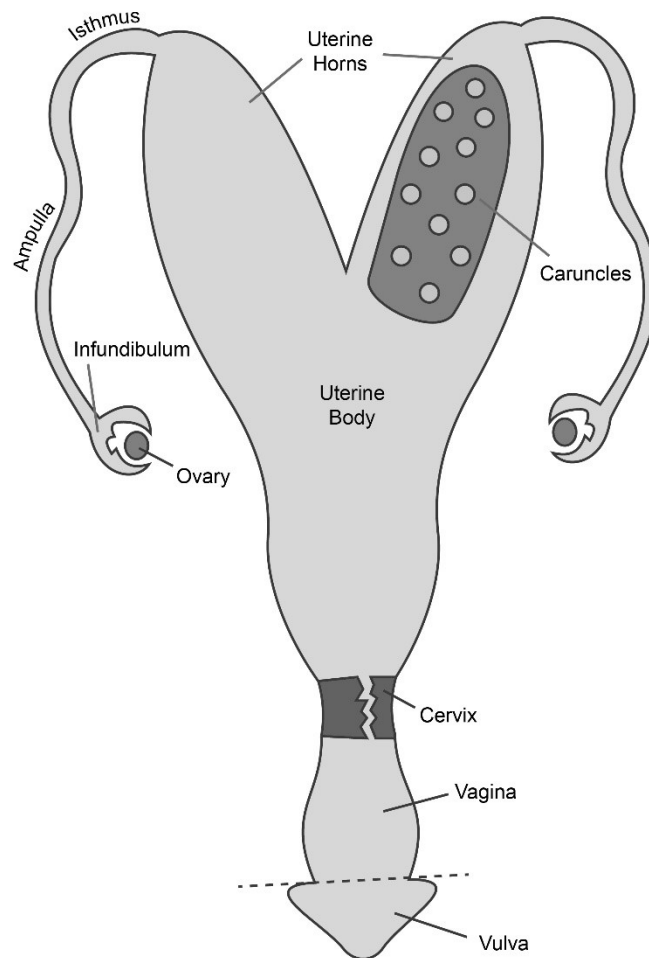
Season has much to do with a sheep's ability to reproduce. Sheep are known to be seasonally polyestrous and are considered the most fertile during the fall months (Martin et al., 2004; Entrican et al., 2006). Ewes experience multiple estrous cycles throughout the short-day period. As mentioned previously, some breeds have been bred to experience longer breeding seasons and have the ability to breed out of season (Hulet et al., 1984). To understand the ewe's estrous cycle and fertility, as well as the ram's reproductive efficiency and fertility, an understanding of reproductive endocrinology and anatomy is required.

## **1.2. Reproduction in Sheep**

### **1.2.1. Ewe Physiology- Parts and Functions**

Before diving into the endocrinology of the ewe, an overview of the organs and tissues involved in the female reproductive system is needed (Figure 1.1). The reproductive system is made up of many parts. The vulva is the entrance to the reproductive system. The vagina follows, which is the copulatory organ for the female. The cervix separates the vagina from the uterus. This structure develops a thick mucus plug during pregnancy, protecting the developing fetus from outside contaminants (Hashimoto, 1961). A major organ is the uterus, as it houses and nourishes a developing fetus, as well as transports semen for fertilization (Mattner and Braden, 1963; Baker and Degen, 1972). The site of fertilization is located within the oviduct. The oviduct

is comprised of three parts: the isthmus, ampulla, and infundibulum. The egg is released from the ovary, meeting the sperm in the oviduct.



**Figure 1.1.** Layout of the Ewe's Reproductive System.

The ovary is the female gonad. It is comprised of two main tissues: the cortex and the medulla (Sawyer et al., 2002). In ewes, the cortex is located on the outer-most edge of the ovary, and the medulla is at the center (Sawyer et al., 2002). The cortex is where the ova are housed, contained inside follicles. An ovum inside a follicle that has not started to mature is called a primordial follicle (De Felici, 2010). Follicles are stimulated to grow due to FSH. Follicle stimulating hormone is released from the anterior pituitary by gonadotrophin-releasing hormone (**GnRH**) released from the hypothalamus in a pulsatile manner when follicles are growing,



similar to a dripping faucet. Follicles inside the cortex go through waves of growth cycles and regressions (Driancourt, 2001). A large group of follicles will mature to primary follicles, while other follicles will regress, called atresia (McGee and Hsueh, 2000; Driancourt, 2001). This happens again when these primary follicles mature further to secondary follicles (Driancourt, 2001). Tertiary follicles, or dominant follicles, emerge. A ewe will likely have a few dominant follicles at this point. These dominant follicles will be present on the ovary during estrus (Driancourt, 2001).

The estrous cycle of the ewe occurs in two phases, subdivided into four stages. During the follicular phase, the follicle is the dominant structure present on the ovary. Stages within this phase are estrus and metestrus. During estrus, the follicles that have become dominant start producing large amounts of estrogen (Driancourt, 2001), causing the animal to display lordosis activity such as standing to be mounted and vocalization. Estrogen creates a contractile, active uterine environment meant to propel semen towards the ampullary-isthmus junction of the oviduct (Chang and Zhang, 2008). Estrus ends approximately when ovulation occurs. Ovulation occurs when a surge of GnRH from the hypothalamus is released, causing a sudden increase in the production of luteinizing hormone (**LH**) from the anterior pituitary (Driancourt, 2001). Luteinizing hormone binds to receptors on the surface of the ovulatory follicle, causing it to rupture (Driancourt, 2001). Metestrus is the stage following ovulation. During metestrus, estrogen levels begin to drop due to the destruction of the follicle. The dominant structure on the ovary is a corpus hemorrhagicum, the product of the ruptured follicle (Gümen and Wiltbank, 2005). The corpus hemorrhagicum slowly changes its physical properties and “heals” to become the corpus luteum (**CL**) or corpora lutea if there are multiple (Petculescu-Ciochină and Dumitrescu, 2012).

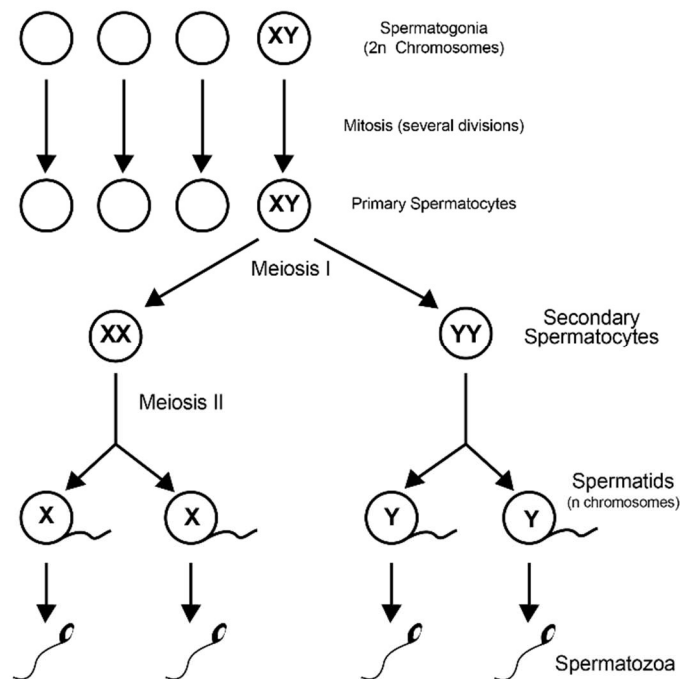
Upon emergence of the CL, the cycle moves into the luteal phase and into the diestrous stage. In this stage, the dominant structure on the ovary is the CL, which produces large amounts of progesterone ( $P_4$ ). If recognition of pregnancy does not occur within a given time period, the uterine endometrium will release a compound called prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ; Parent et al., 2003). This causes the CL to regress into a corpus albicans and cease to produce  $P_4$ . The last stage is called proestrus. Proestrus starts when  $PGF_{2\alpha}$  is released and the CL begins to regress (Thatcher et al., 1984; Parent et al., 2003). Follicles start to grow on the ovary again.  $P_4$  levels are dropping, and estrogen levels are rising (Thatcher et al., 1984). This marks the end of the luteal phase. If maternal recognition of pregnancy occurs, then the corpus luteum remains on the ovary, producing progesterone. Maternal recognition is a series of events that must occur for the body to recognize there is an embryo present, meaning that the ova released has been fertilized (Asselin et al., 1997; Parent et al., 2003). If recognition is successful, the uterus remains quiescent and  $PGF_{2\alpha}$  is inhibited, while  $PGE_2$  production is increased (Asselin et al., 1997). The continued release of  $P_4$  stimulates the production of histotroph, a fluid secreted by the uterine lining that nourishes and protects the developing embryo. Maintenance of the CL and its production of progesterone are important for establishing and maintaining pregnancy (Thatcher et al., 1997).

### **1.2.2. Ram Physiology- Parts and Functions**

The ram is equally as important as the ewe in the outcome of breeding. If quality sperm cells are delivered to the ova, the ewe increases her chances of a successful pregnancy. The main function of the male is the production of sperm, called spermatogenesis. Unlike females, GnRH in males is constantly produced in a steady, pulsatile rate. Due to this steady production, FSH and LH are also released at a steady rate. Males produce both testosterone and estrogen.

Testosterone is produced in the Leydig cells of the male, stimulated by LH. Testosterone can then be transformed into estrogen by the enzyme aromatase. Estrogen is taken in by Sertoli cells. A lack of estrogen leads to sterility (Gill-Sharma, 2018). Sertoli cells produce a compound called Androgen Binding Protein (ABP), which binds and stores surrounding testosterone in support of spermatogenesis (Gill-Sharma, 2018). Lack of testosterone during spermatogenesis prevents spermatid elongation and thereby poor quality in subsequent spermatozoa in the epididymis.

Spermatogenesis is the divisions of spermatogonia and subsequent differentiation of resulting spermatid to spermatozoa (Saunders, 2003). The goal of spermatogenesis is to have sperm available to inseminate the female at any time. Figure 1.2 exhibits how spermatogonia transform into spermatozoa. There is a continuous release of sperm cells into the seminiferous tubules in the testicle. The testicle is the male gonad, as it produces sperm cells, the male gamete. The release of sperm cells from Sertoli cells into the seminiferous tubules is called spermiation, much like the release of the egg from the follicle is called ovulation (Licht, 1973).



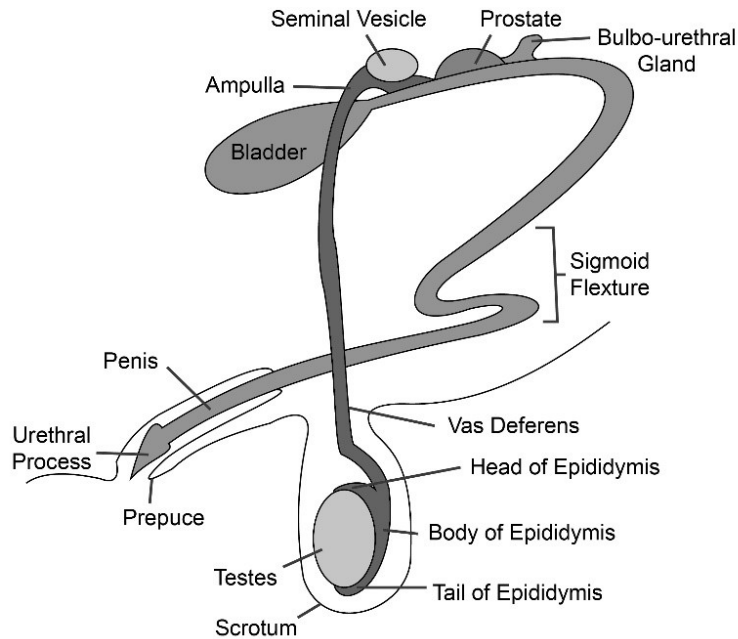
**Figure 1.2.** Layout of Spermatogenesis from a Spermatogonia cell to Mature Sperm. (Adapted from Bester, 2006)

The spermatogenesis cycle in the seminiferous epithelium is continuously occurring in different stages throughout the testicle. Completion of one round of spermatogenesis in one piece of seminiferous epithelium takes 10 days in the ram (Saunders, 2003). A full cycle of spermatogenesis takes about 47 to 49 days for the ram (Saunders, 2003).

The daily sperm production of an animal includes the number of spermatozoa produced per day by both testes. Scrotal circumference is an estimate of daily sperm production in rams. However, this physical measurement can be misleading. This is especially true in over-conditioned animals. However, scrotal circumference can generally predict semen volume and percentage of motile, normal cells (Wiemer and Ruttle, 1987). A study in goats by Agga et al. (2011) found scrotal circumference to be an accurate live body measurement to predict testicular and epididymal measurement, as well as weight in bucks.

Spermatozoa initially is released from the seminiferous tubules, as stated previously (Saunders, 2003). The spermatozoa then travel to the rete tubules in the mediastinum of the testicle, like a drain in the center of the testicle (Hees et al., 1989). In the ram, the spermatozoa move up into the efferent ducts, where they are released into the epididymis. The epididymis is made up of three parts: the head, body, and tail (Figure 1.3; Marengo and Amann, 1990). As the spermatozoa move through the epididymis, they progressively mature (Marengo and Amann, 1990). The head and body of the epididymis concentrate the spermatozoa, and function in the partial removal of the cytoplasmic droplet (Cooper, 2011). A cytoplasmic droplet is a drop of cytoplasm initially attached to the base of the sperm cell. During the process of maturation, this droplet moves down the length of the sperm cell, finally dropping off at the end of the tail (Cooper, 2011). The final maturation occurs at the tail of the epididymis, before being released at ejaculation (Cooper, 2011). By this time, the cytoplasmic droplet should be removed.

Spermatozoa fully mature inside the female, referred to as capacitation (Cooper, 2011). At ejaculation, the spermatozoa are mixed with nutrients and buffers from the accessory sex glands, becoming semen (Maxwell et al., 2007; Cooper, 2011).



**Figure 1.3.** Layout of the Ram’s Reproductive System

The anatomy of the sperm cell is made of three main parts: the acrosome, the nucleus, and the tail (Hardy et al., 1991; Johnson et al., 2011; Lindmann and Lesich, 2016). The nucleus is contained in the main body of the sperm cell. Like the ova, it contains half the number of chromosomes of a normal cell, though unlike the ova, the genetic material is condensed much more compactly (Johnson et al., 2011). The sperm cell’s nucleus merges with the egg to create a zygote (Rawe et al., 2008). The tail of the sperm cell is especially unique. The tail is a self-propelled flagellum containing an axoneme, aiding in forward movement (Lindemann and Lesich, 2016). The axoneme is made up of nine doublets and two central microtubule fibers that give the tail its structure and flexibility (Lindemann and Lesich, 2016). The acrosome covers the anterior two-thirds of the nucleus. It contains enzymes including acrosin and hyaluronidase that

bind to the egg in the acrosome reaction, and proteasomes that break down the sperm cell's tail and further aid fertilization (Hardy et al., 1991; Rawe et al., 2008).

### **1.3. Improving Reproductive Parameters**

#### **1.3.1. Breed**

Across the world, there is a large diversity of ruminant animals (Leroy et al., 2016). The more sheep there are in a country's population, the more diverse the breeds (Leroy et al., 2016). Many breeds are bred for a specific purpose, such as wool or meat production. Others are bred for their prolificacy, or their ability to give birth to and care for multiple offspring. There is as much variation within a breed as there is between breeds in terms of ram reproductive characteristics (Turner, 2009). One study compared Horned Dorset rams with Suffolks, where Horned Dorset rams produced semen with greater motility than Suffolk rams (Boland et al., 1985). Clearly, this implies that some breeds will have better reproductive characteristics than others. Improvement by breeding to obtain better reproductive traits is possible, demonstrated by Hulet et al. (1984), with the creation of the Polypay. The Polypay ewe exceeded the measured reproductive traits of the breeds it was comprised of (Hulet et al., 1984). This breed's history is a great example of the heritability of reproductive traits in sheep. Waldron and Thomas (1992a) studied the Rambouillet breed and found genetic correlations for reproductive traits. In their subsequent study, they found combining the selection of ovulation rate and litter size based on an index produces 123% as much response in litter size as selecting for litter size alone (Waldron and Thomas, 1992b). Selecting on an index for litter size, ovulation, and scrotal circumference combined further enhanced this response by 133% (Waldron and Thomas, 1992b). Focusing on both the female and male sides of reproduction is very important, as they both have an impact on the outcome of reproduction. Improvement of a breed's reproductive capabilities is reliant on

the ability of breeders to select above average stock, potentially based on the aforementioned index for litter size, ovulation and scrotal circumference (Waldron and Thomas, 1992b).

### **1.3.2. Season**

As discussed previously, sheep are seasonal breeders, or seasonally polyestrous. A breed with strong seasonality will not go into estrus when the days are long. As days start to get shorter, late summer to late fall for the northern hemisphere, these ewes start to cycle and come into heat. The controller of season is photoperiod. As photoperiod decreases, less light enters the retina in the eye of the ewe. The pineal gland is inhibited from producing melatonin during periods of long days and short nights (Vasantha, 2015; Szczepkowska et al., 2017). When greater than 13 hours of darkness is detected by the ganglionic synaptic fibers, inhibition of the pineal gland fails and starts producing melatonin (Vasantha, 2015). Increased melatonin secretion stimulates the hypothalamus to release GnRH in a pulsatile manner (Vasantha, 2015). Thus begins FSH and LH secretion from the pituitary gland, leading to the growth of follicles and start of the reproductive cycle (Towhidi et al., 2007).

Rams are also affected by season and photoperiod. When treated with melatonin to overcome the impact of photoperiod, rams were shown to have a higher fertility in April (out of season) than untreated rams (Figueiredo et al., 2015). Rams are affected by photoperiod not only in the production of sperm, but also at the cellular level. The Sertoli and Leydig cell structure changes, including the overall structure of the seminiferous epithelium (Gastel et al., 1995) This increases activity in these structures (Gastel et al., 1995). Thus, testosterone, produced by Leydig cells, is highest in autumn, even in Uruguay, where there are only mild seasonal changes (Gastel et al., 1995). This study is in agreement with Mickelsen et al. (1981b) who stated seminal quality

is highest in October and lowest in February for the Suffolk and Lincoln sheep breeds. Of course, this monthly pattern is reversed when sheep are located in the southern hemisphere.

As stated previously, scrotal circumference has long been an indicator of fertility and semen quality in rams. When exposed to three months of artificial short-day lengths, scrotal size reaches maximum size. Prolonged exposure to the same short-day length (8 hours light, 16 hours dark) over three years stabilizes testicular size and maintains function at near maximum levels (Langford et al., 1989). Without such treatment, scrotal circumference varies significantly throughout the year (Gastel et al., 1995). Average scrotal circumference is at its highest in October for the Suffolk and Lincoln breeds, also in correlation with improved sperm morphology (Mickelsen et al., 1981a). Fertility can be improved by keeping seasonality and photoperiod in mind when selecting stock. Breeds with strong seasonal tendencies should not be the first choice when breeding out of season. This seasonality could also be overruled by exposure to artificial light or hormones (Langford et al., 1989; Figueiredo et al., 2015).

### **1.3.3. Nutrition**

Proper feeding of ewes and rams prior to breeding is incredibly important. An increase in nutrition quality prior to breeding in ewes is referred to as flushing. Flushing helps facilitate follicular growth, providing more eggs for fertilization (Pearse et al., 1994). Flushing can help improve out of season breeding results as well, especially in conjunction with progesterone supplementation (Sheffield et al., 2018). Timing of flushing is also important. Ewes maintain the best fertility when fed from one week prior to ram turnout and at least two weeks during ram exposure (Venter and Greyling, 1994). The results of flushing also depend on the body condition of the ewes prior to flushing and the quality of the flushing ration provided (Venter and Greyling, 1994). A ewe with a lower body condition score, such as a 2 to 2.5 (1-5 scale; Kenyon



et al., 2014), will respond more effectively to a flushing protocol than a ewe that is already at a 4 in body condition (Venter and Greyling, 1994).

High protein feeds are not necessarily beneficial in a flushing ration. High protein supplementation has been linked to decreased efficiency of superovulation protocols compared to a low protein diet in sheep (Tur et al., 2017). However, supplementation with targeted amino acids, specifically arginine, has been shown to increase pregnancy rates in estrous synchronized ewes using CIDRs and PG-600 (Luther, 2009; Saevre et al., 2011, Asgari Safdar et al., 2017).

Feeding rations with higher energy and increased fat content is the preferred method for flushing. Flushing with high-energy rations has been shown to improve reproductive parameters in ewes (Daghighi Kia and Asgari Safdar, 2015; Asgari Safdar et al., 2017). When comparing a ration with fats added via flax oil to a control ration without fat, the added fat treatment returned the highest fertility rates, with 100% fertility in Afshari ewes (Asgari Safdar et al., 2017). The authors concluded the higher fat levels were correlated with a higher number of lambs born. Thus, Asgari Safdar et al. (2017) recommends a ration including increased fats, specifically flax oil, as a successful way to improve fertility, lambing number, and overall reproductive performance. Underfed, low body conditioned ewes may not have the energy stores available to produce leptin. Leptin is a physiological signal from adipose tissue to activate the GnRH – LH/FSH axis (Towhidi et al., 2007a; Towhidi et al., 2007b; Odle et al., 2017). There is a positive correlation between body energy stores and leptin level (Towhidi et al., 2007b). Though the season may be right for breeding, ewes without condition may not be able to produce GnRH in high enough levels to initiate an estrous cycle (Towhidi et al., 2007b; Odle et al., 2017).

Underfed ewes and rams will not perform to the best of their ability. Underfed rams exhibit DNA damage in their sperm cells (Guan et al., 2014). Not only will rams produce poorly,

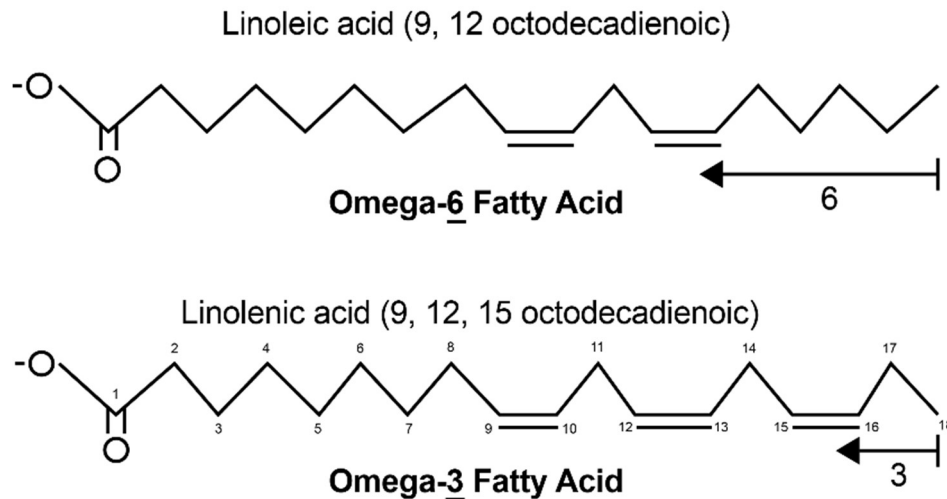
but also the sperm themselves will be abnormal. Underfeeding also reduces spermatogenic efficiency and individual spermatozoal quality (Guan et al., 2014). Feeding rams similarly to the flushing protocol of a ewe can improve reproductive outcomes as well. One study found improving nutrition hastens testicular growth in the spring in rams (Pérez-Clariget et al., 1998). This is especially important for producers wishing to breed out of season. However, the same study noted nutrition as only one part of the environmental cues for seasonal testicular redevelopment (Pérez-Clariget et al., 1998).

## **1.4. Fatty Acids and Reproduction**

### **1.4.1. Feeding Fatty Acids**

Sheep, like all animals, have certain requirements of nutrients for weight gain, wool growth, reproduction, digestion, and other bodily processes (NRC, 2007). A nutrient category often overlooked is fatty acids (FA). There are certain FAs sheep cannot make on their own, called essential FAs. Feeding these essential FAs in the correct amounts is important for maintaining the ewe or ram's condition and function.

Omega-3 ( $\omega$ -3) FAs are of particular interest as they are essential FAs (Morris, 2007). There is a balance in the body for omega-6 ( $\omega$ -6) and  $\omega$ -3 FAs. Typically, high concentrate livestock feeds are richer in  $\omega$ -6 FAs than  $\omega$ -3 FAs, so the ultimate goal to improve this is to increase the  $\omega$ -3 FAs in the diet (Lin et al., 2016). Feeding products with high  $\omega$ -3 FAs, including alpha-linolenic acid (ALA), will decrease the  $\omega$ -6/ $\omega$ -3 FA ratio (Lin et al., 2016). Figure 1.4. outlines the differences between  $\omega$ -3 and  $\omega$ -6 FAs.



**Figure 1.4.** Omega-3 and Omega-6 Fatty Acids. The naming and function of these essential fatty acids are based on where the double bond is located at the tail of the molecule, or the ‘omega’ end. The alpha end of the molecule contains the carboxyl group, with the omega side at the methyl end. An  $\omega$ -3 fatty acids’ double bond is located 3 carbons into the omega end, while  $\omega$ -6 fatty acids’ bond is 6 carbons in (Adapted from Jump et al., 2012).

Flaxseed is high in  $\omega$ -3, particularly ALA (Morris, 2007). Flaxseed had been used in a variety of studies to decrease the  $\omega$ -6 to  $\omega$ -3 ratio. Others have paired flaxseed with other products in the attempt to elicit an even greater response. Linpro<sup>®</sup> is a product that includes extruded field peas and flaxseed. Feeding this blend increases the volatile FAs (VFA) propionate and isobutyrate, while acetate decreases (Alvarado-Gilis et al., 2014). This change in VFAs improved feed efficiency. However, the same author found that this combination increases H<sub>2</sub>S production, 30% higher than when Linpro<sup>®</sup> was not added (Alvarado-Gilis et al., 2014). Ewe lambs fed flaxseed oil had higher concentrations of ALA in their tissues compared to the control (Duckett et al., 2016). Ewes fed flaxseed have also exhibited higher levels of ALA in milk products as well, inferring the ALA from flax can be absorbed from the diet to the bloodstream (Luna et al., 2008).

### **1.4.2. Omega-3 Fatty Acids and Reproduction**

Supplementing FAs, particularly essential FAs, can alter the physiology of the animal. For example, offering varying levels of polyunsaturated fatty acids (**PUFA**) can alter the total number and size of ovarian follicles, as well as improve ovulation rate, progesterone production by the corpus luteum, luteolysis timing and gestation length (Abayasekara and Wathes, 1999). Feeding oilseeds such as flaxseed and chia seed during estrous will increase the energy density of the diet and provide key FAs around the time of maternal recognition of pregnancy (Scholljegerdes et al., 2014).

One study found feeding supplemental fat to cows increases standing estruses (Scott et al., 1995). Another study found feeding the  $\omega$ -3 FAs will increase ovulation rate in rats (Trujillo and Broughton, 1995). As estrus occurs prior to ovulation in many livestock animals, these studies conclude similar results within the scope of supplemental fat and fatty acids.

The physiological changes are not only on the ewe side. In a study on Moghani rams, Jafaroghli et al. (2014) found the percentage of motile sperm and semen volume was increased by feeding 2.5% fish oil to increase  $\omega$ -3 FA content. Fish oil inclusion also improved the percentage of sperm with a normal acrosome (Jafaroghli et al., 2014). These different values improve the overall fertility in the ram. Jafaroghli et al. (2014) also reported fish oil increases docosahexaenoic acid in the sperm themselves. This suggests diet alters the composition of the sperm cells themselves, not only the production of normal sperm.

### **1.4.3. Feeding Flax to Increase Omega-3 Fatty Acids**

Flaxseed is an oilseed crop. North Dakota alone produced over 89% of flaxseed of the United States in 2017 (United States Department of Agriculture, 2018). Over past decades, flaxseed has been gaining popularity as a feedstuff for major livestock species. Flaxseed is made

up of about 41% fat; 57% of the total FAs in flaxseed are ALA, and 16% are linoleic acid, a  $\omega$ -6 FA (Morris, 2007). Flax supplementation has the potential to increase both  $\omega$ -3 and  $\omega$ -6 FAs. Flaxseed was shown by Lessard et al. (2003) to increase serum  $\omega$ -3 FA concentration as well as decrease the  $\omega$ -6/ $\omega$ -3 FA ratio in the blood (Lessard et al., 2003).

#### ***1.4.3.1. Flax and Female Reproduction***

Flax has been shown in multiple studies to influence reproduction. When dairy cows are fed a diet including flax with 56.7% ALA or sunflower seed with 0.1% ALA to supplement over 410 g of ALA or less than 1 g of ALA, respectively, it was found flax fed cows had larger mean ovulatory follicle size (Ambrose et al., 2006). However, the number of follicles did not differ. The corpora lutea from flax-fed cows were not larger than sunflower fed cows, unlike the follicles from which they likely were formed (Ambrose et al., 2006). Perhaps in conjunction with the lack of difference in corpora lutea size, plasma progesterone concentration was, in turn, unaffected by a flax supplemented diet (Ambrose et al., 2006). Conception to timed artificial insemination (**TAI**) increases as well, and pregnancy losses were lower in flax fed dairy cows compared to sunflower fed cows (Ambrose et al., 2006). This decrease in pregnancy loss in cattle was also found by Silvestre et al. (2011).

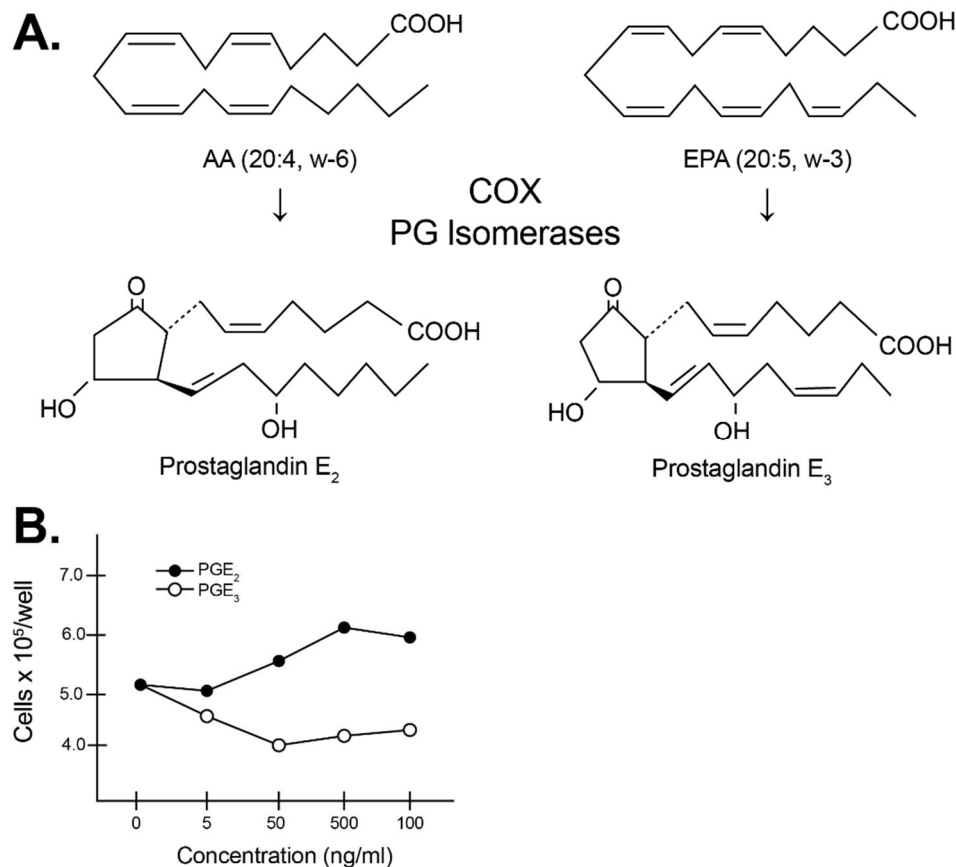
An interesting aspect of feeding FAs, especially  $\omega$ -3 and  $\omega$ -6 FAs, is the changes found in reproduction hormone production and concentration. Changes in the hormone profile may explain the physical reproductive responses to flaxseed. Flax has been shown to influence estrogen, progesterone, and various prostaglandins. Other hormones such as luteinizing hormone may also be affected, as such results as increased ovulations have been found in rats (Trujillo and Borughton, 1995).

Estrogen, described previously, is the controller of lordosis and estrus. Therefore, estrogen controls the follicular phase of the estrous cycle. It primes the ovary for ova release, as well as the uterus for semen transport. Bu and Lephart (2005) found if cycling or pregnant rats were given flaxseed, it regulated the neuroendocrine mechanism that controls core body temperature. This phenomenon may have beneficial effects for hot flashes in human subjects (Bu and Lephart, 2005). When given to women, flax significantly increases the less biologically active estrogen 2-hydroxyestrone, but flax inclusion does not increase 16 $\alpha$ -hydroxyestrone in urine (Brooks et al., 2004; Muti et al., 2000). Ratios of these estrogens are positively correlated with urinary lignan excretion (Brooks et al., 2004). Flax supplementation alters estrogen production to produce more of the less effective form, meaning the normal application of estrogen will not be as intense or perhaps non-functioning altogether.

Prostaglandin concentrations are altered similarly to estrogen when feeds with flax are fed. The main change is the shift of production from prostaglandin F<sub>2 $\alpha$</sub>  (**PGF<sub>2 $\alpha$</sub>** ) to prostaglandin E<sub>2</sub> (**PGE**). Embryo survival for embryo transfer improves when dams are supplemented with flax (Petit and Twagiramungu, 2006). The authors hypothesized survivability may be due to changes in prostaglandin concentration, such as a decrease of both PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  (Petit and Twagiramungu, 2006). In mice,  $\omega$ -3 supplementation decreases protein expression of PGE<sub>2</sub> receptor 2, while  $\omega$ -6 supplementation increases protein expression of these receptors on day four and five of pregnancy (Shahnazi et al., 2018). So potentially, not only does  $\omega$ -3 supplementation shift the prostaglandin production to PGE<sub>2</sub>, but it may also decrease the expression of the receptor for PGE<sub>2</sub>. Serum PGE<sub>2</sub> is significantly lower in flaxseed fed cows compared to both soybean and Megalac<sup>®</sup> supplemented cows (Lessard et al., 2003). Lowering the  $\omega$ -6/ $\omega$ -3 ratio increases trienoic prostaglandins and decrease dienoic prostaglandins (Fly and

Johnston, 1990). This may contribute to improved fertility, because trienoic acid has a lower biological activity (Fly and Johnston, 1990). Therefore, the compound would not be as effective as dienoic prostaglandins like  $\text{PGF}_{2\alpha}$  for luteolysis.

Prostaglandins are biologically active lipids and are derived from PUFAs (Herschman et al., 1995). Prostaglandins (**PG**) are not only involved in reproduction. They also play key roles in inflammation, immune response, and wound healing, associated with cell division (Herschman et al., 1995). Arachadonic acid (**AA**), an  $\omega$ -6 FA, is found in flaxseed. AA is converted into the precursor to all prostanoids, Prostaglandin  $\text{H}_2$  (Herschman, 1996). These are the dienoic PGs. Treinoic PGs, however, are derived from  $\omega$ -3 FAs, and have been shown to be biologically less active, as Fly and Johnston (1990) suggested. Figure 1.5 shows this activity via cell proliferation.



**Figure 1.5.** Description of Dienoic vs Trienoic Prostaglandin Activity. (A) Dienoic and Trienoic PGs and their derivatives AA and EPA, respectively. (B)  $\text{PGE}_2$  stimulates more cell proliferation compared to  $\text{PGE}_3$  when cells were treated at various concentrations. Proliferation was measured after 24 hours. (Adapted from Bagga et al., 2003).

Progesterone, described previously as the controller of pregnancy, is also changed by flaxseed inclusion in the diet. Serum progesterone concentration is significantly higher in flaxseed fed cows compared to controls (Lessard et al., 2003; Petit and Twagiramungu, 2006). However, this increase is not reflected in corpora lutea sizes, even though follicle size may be increased (Ambrose et al., 2006). In turn, conception improved (Ambrose et al., 2006) and embryo mortality tends to decrease as well (Petit and Twagiramungu, 2006). An increase in progesterone may explain a study by Santos et al. (2008), in which feeding  $\omega$ -3 FAs suppressed uterine prostaglandin release and improved maintenance of pregnancy. Similar to PGE<sub>2</sub>, not all studies conclude increased progesterone in serum, but instead report no change (Hutchinson et al., 2012). Therefore, flax feed type and the amount of  $\omega$ -3 FA fed may be connected to this response.

#### ***1.4.3.2. Flax and Male Reproduction***

Flax has been shown in multiple studies to influence seminal quality and male reproductive efficiency. Sperm concentration, motility, and morphology are key parts of male fertility. Moallem et al. (2015) reported improved motility and progressive motility of thawed semen from flax supplemented bulls, as well as increased ALA in the blood. When buffalo bulls were fed flaxseed oil, semen volume, mass movement, sperm motility, and sperm concentration were improved (Shah et al., 2016). Baiomy and Mottelib (2009) reported rams supplemented flax during the non-breeding season exhibited improved motility, volume, and morphology. Yan et al. (2013) reported sperm density, morphology, and motility improved when rats were fed an  $\omega$ -6: $\omega$ -3 ratio of 1:1.52, improved over ratios of 7.69: 1, 2.5:1, 1.17:1, and 1:2.85. The 1:1.52 ratio group also had higher litter sizes and birth weights when bred to female rats. This study demonstrates the importance of  $\omega$ -6/ $\omega$ -3 ratios. Flax supplementation to a breeding male



animal's ration certainly can improve semen characteristics. However, managing the  $\omega$ -6/ $\omega$ -3 ratios is perhaps the main controller of these changes, not just the addition of an  $\omega$ -3 FA rich feedstuff.

Serum testosterone concentration was significantly higher in fish-oil fed rams than controls of palm oil and sunflower oil (Esmaeili et al., 2014). Baiomy and Mottelib (2009) also reported an increase in testosterone in rams when fed flax during the long day period, despite being out of season. It was hypothesized an increase of testosterone was due to added cholesterol in the ration, instead of being due to an increase of  $\omega$ -3 FAs. Related to an increase of testosterone, libido was found to be greater in buffalo bulls (Shah et al., 2016) and boars (Estienne et al., 2008) when fed flaxseed. Libido and breeding behavior are regulated and controlled by testosterone concentrations in the blood (Katz and McDonald, 1997), and therefore agree with Baiomy and Mottelib (2009) and Esmaeili et al. (2014)'s findings of increased testosterone.

#### **1.4.4. Downfalls of Feeding Flax**

There are negative effects to be aware of when creating a ration of feedstuffs containing the targeted FAs. The two components of interest are lignans and isoflavones. One example of an isoflavone is in soybeans, called daidzein. Daidzein acts as an estrogen in the sexual differentiation of the female brain and alters the regulation of lordosis and estrous cycles in rats (Kouki et al., 2003). Essentially, the isoflavone replaces the naturally produced estrogen and elicit a different or decreased response than estrogen would. This was presented as beneficial to women. However, in livestock species a less biologically active estrogen or estrogen-like compound may impact display of estrus or even detrimentally alter the cycle itself. Many of the negatives of FA-altering feeds have to do with their lignans and isoflavones. These compounds

are converted into active form or act directly in the body. A phytoestrogen is a compound that acts as a weak estrogen agonist or antagonist, inhibiting estrogen from acting on the body.

Flaxseed contains a lignan called secoisolaricinesinol diglycoside (**SDG**; Brooks et al., 2004). Secoisolaricinesinol diglycoside is converted into an active phytoestrogen in the rumen environment. Secoisolaricinesinol diglycoside can range anywhere from 1 to 26 mg/g of seed. Flax supplementation has previously been shown to alter estrogen metabolism in favor of less biologically active estrogen metabolites (Brooks et al., 2004). In-vitro mammalian lignan production was found to be influenced by the percentage of flax included into the product (Nesbitt, 1997). Plasma lignan levels plateau at eight days of intake and maintain until 24 hours after intake in humans (Nesbitt, 1997). To truly see an effect of the phytoestrogen, as much as eight days may be required. The study in humans shows the effect may be gone after 24 hours; possibly implying flax must be continually fed throughout the targeted time period.

### **1.5. Conclusions**

The reproductive systems of ewes and rams are diverse and complicated. They are influenced by genetics and breed, season, nutrition, and all the intricacies of signaling pathways between various parts of the brain and reproductive organs. To improve reproduction and fertility in the sheep species, all parts of this puzzle must be used. Selection of premium genetics and choosing the appropriate breed for the setting is important. Breeding during the correct season for the breed or utilizing artificial hormones or lighting is next. Finally, improvement of reproduction can be attained by the use of a flushing protocol, while feeding the correct levels of nutrients for the growth of healthy follicles in ewes and for the production of healthy and motile sperm cells in rams. The correct level of nutrients is a debated topic. The following chapters will delve into  $\omega$ -3 FA supplementation in the hope of achieving the level of essential FAs required

for breeding ewes and rams. The  $\omega$ -3 FA supplementation will be in the form of flaxseed. Flaxseed is a very small particle feed, and thus is ineffective in an applied setting when fed in its natural form. Therefore, the following chapters will utilize a commercial Flaxlic<sup>®</sup> Sheep Tub to supplement flaxseed in a way that is more effective in a commercial setting and more utilizable to the sheep industry and its producers.

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## 2. IMPACTS OF FLAX ON FEMALE REPRODUCTIVE TRAITS WHEN SUPPLEMENTED PRIOR TO BREEDING IN SHEEP

### 2.1 Abstract

Fertility can be improved prior to breeding by improving nutritional management, especially in range sheep operations. Feeding fatty acids (FA), including omega-3 ( $\omega$ -3) FAs, have been shown to have positive effects on pregnancy parameters. The objective of this study was to determine the effects of feeding supplemental flaxseed via a Flaxlic® Sheep Tub during the flushing period on reproductive performance of spring lambing multiparous ewes. Multiparous Rambouillet ewes ( $n = 240$ ; aged 2 to 7 years) weighing an average of  $70.8 \pm 10.4$  kg were randomly assigned to 24 pens (10 ewes/pen) and fed a flushing diet for 35 d. Rams were assigned to receive a Flaxlic® Sheep Tub (FLX;  $n = 12$ ) or not (CON;  $n = 12$ ). Tubs were weighed on d 0, 3, 7, 10, 14, 17, 24, 29, and 35. Tubs weighing less than 5 kg were replaced with new tubs. Weight data for all ewes was taken on d 0, 7, 14, 21, 28, and 35, with two-day weights on d -1 and 0 and d 34 and 35. Serum samples were taken for serum progesterone concentration analysis on d 0, 7, 14, 21, 28, and 35 from 60 ewes per treatment (5 ewes/pen). On d 36, ewes and rams were comingled for breeding for 35 d. Birthweight, birth type, and sex of lambs were recorded at lambing for first cycle (149 to 166 days post ram turnout) and the overall lambing season. Initial and final weights were not different between treatment groups ( $P = 0.47$  and  $0.23$ , respectively). No treatment x day interactions ( $P = 0.82$ ) or treatment effects ( $P = 0.83$ ) were observed for serum progesterone concentration. A day effect was observed for serum progesterone concentration, which was higher on d 28 and higher still on d 35 ( $P < 0.001$ ). No differences were observed between treatments for 1<sup>st</sup> cycle ( $P \geq 0.26$ ) or overall ( $P \geq 0.61$ ) pregnancy rate, prolificacy rate, or lambing rate. These results are in agreement with studies in

cows where supplemental flax did not have an effect on progesterone concentration. However, the results are in contrast with studies in cows reporting increased conception and increased embryo survivability when supplemental flax was provided.

## 2.2. Introduction

Fertility can be improved prior to breeding by improving nutritional management, especially in range sheep operations. This can be done by improving the diet through a flushing protocol. Improved management strategies prior to breeding can lead to better ewe body weight and condition score maintenance, as well as improved conception and pregnancy rates.

The addition of flaxseed to a flushing protocol has the potential to further enhance the effects of flushing (Thatcher et al., 2006; Santos et al., 2008; Silvestre et al., 2011). Flaxseed supplements two important FAs: Alpha-linolenic acid (**ALA**; C18:3  $\omega$ -3), an omega-3 ( $\omega$ -3) FA, and linoleic acid (**LA**; C18:2  $\omega$ -6), an omega-6 ( $\omega$ -6) FA. Of the total fats in flax oil, 57% is ALA and 16% percent is LA (Morris, 2007).

Much research has been conducted feeding supplemental fats high in polyunsaturated fatty acids (**PUFA**). Feeding essential FAs can also have positive effects on follicular growth, embryo quality, and pregnancy rates (Thatcher et al., 2006; Santos et al., 2008; Silvestre et al., 2011). When  $\omega$ -3 FAs are supplemented, a shift in prostaglandin (**PG**) production can occur. Pregnancy rate may also be increased due to enhanced progesterone (**P<sub>4</sub>**) production by the corpus luteum (**CL**) and decreased embryo mortality (Santos et al., 2008; Silvestre et al., 2011; Petit and Twagiramungu, 2006). The addition of flaxseed has been linked to increased ovulation rate in various species (Scholljegerdes et al., 2011; Abayasekara and Wathes, 1999; Trujillo and Broughton, 1995).

Research on  $\omega$ -3 FA supplementation for ewe reproductive improvement is lacking. Luna et al. (2008) fed flax to ewes, however the study was focused on increasing  $\omega$ -3 FAs in the milk. Alpha-linolenic acid was detectable in the milk of the flax-fed ewes, which infers  $\omega$ -3 FAs can be transferred from the diet to the blood. Omega-3 FAs are available from flaxseed to be used by the body. Flaxseed supplemented in the right amount has the potential to lower PG concentrations, shift PG production to less active PGs, increase P<sub>4</sub> production, increase ovulation rate, decrease embryo mortality, increase follicular growth, and increase CL stability. Our hypothesis was the supplementation of flaxseed would increase ALA in the blood and therefore improve progesterone production and reproductive performance while preventing embryo death. The objective of the present study was to supplement flaxseed in an applied setting using Flaxlic® Sheep Tubs during a 35-d period prior to breeding to improve progesterone production, lambing rate, pregnancy rate, and prolificacy rate.

### **2.3. Materials and Methods**

All procedures were approved by the Animal Care and Use Committee of North Dakota State University (NDSU; protocol #A17071). This study was conducted at the NDSU Hettinger Research Extension Center in Hettinger, ND.

#### **2.3.1. Experimental Design**

Multiparous Rambouillet ewes ( $n = 240$ ) aged 2 to 7 years with a mean body weight of  $70.8 \pm 10.4$  kg were randomly assigned to 24 pens in groups of 10 ewes/pen. Twelve pens were given a Flaxlic® Sheep Tub (FLX; New Generation Feeds, Belle Fouché, SD;  $n = 12$ ). The other 12 control pens (CON;  $n = 12$ ) did not receive a flax tub, but instead supplemented mineral by adding to the basal ration with a commercial mineral premix described in Table 2.1. Pens were fed a diet of chopped hay for 35 days. The diet was balanced for a 70 kg ewe receiving a flushing

ration prior to breeding (Table 2.1.; NRC, 2007). The quantity of hay offered was altered throughout the study to account for weight changes of the ewes to maintain crude protein and energy supply for flushing. Feed samples were taken at the beginning and end of the 35-d feeding trial period. Samples were sent to Midwest Laboratories (Omaha, NE) for nutrient analysis. Dry matter (calculated from moisture measurement, method 930.15; Association of Analytical Communities [AOAC] Int., 1990), acid detergent fiber (ADF; ANKOM Tech. Method; Spanghero et al., 2003), crude protein (CP; method 990.03; AOAC Int., 2006), total digestible nutrients (TDN; Weiss et al., 1992), minerals, (method 985.01 modified; AOAC Int., 2006) and  $\omega$ -6 and  $\omega$ -3 FA were analyzed (method 996.06; AOAC Int., 2012). The ingredients for the Flaxlic<sup>®</sup> Sheep Tub by inclusion level are beet molasses, ground flaxseed (21%), flaxseed oil (6.4%), soybeans (45%), and select vitamins and minerals (Table 2.1). Ewe 2-day weights and body condition score (BCS; 1-5 scale; Kenyon et al., 2014) were taken on d -1 and 0 and d 34 and 35, with ewe body weight recorded weekly to monitor ewe health.

**Table 2.1.** Feedstuff Nutrient Composition of the Basal Ration for CON and FLX Treatments<sup>1</sup>

Nutrient, % DM <sup>2</sup>	Chopped Hay <sup>3</sup>	Flaxlic <sup>®</sup> Sheep Tub <sup>3</sup>	Sheep Mineral <sup>3</sup>
DM (% as fed)	87.45	-	99.0
ADF	37.1	2.5	0
CP	13.6	12	0
TDN	60.2	-	0
S	3.6	-	0.19
P	5.5	1.0	8.0
K	1.86	2.5	2.1
Mg	0.26	-	2.75
Ca	0.74	1.0-1.5	16.5
Na	0	-	11.0
Fe	711 ppm	-	131 ppm
Mn	67.5 ppm	0.12	52.60 ppm
Cu	5.3 ppm	0	5.50 ppm
Zn	25.0 ppm	1200 ppm	20.30 ppm
$\omega$ -3 Fatty Acids <sup>3</sup>	0.65 g·100g <sup>-1</sup>	0.07 g·100g <sup>-1</sup>	-
$\omega$ -6 Fatty Acids <sup>3</sup>	0.48 g·100g <sup>-1</sup>	0.025 g·100g <sup>-1</sup>	-

<sup>1</sup>CON = basal ration of chopped hay plus sheep mineral; FLX = basal ration of chopped hay plus Flaxlic<sup>®</sup> Sheep Tub. <sup>2</sup>Most measurements reported on a dry matter basis; fatty acid analysis reported on an as fed basis. <sup>3</sup>Chopped hay = basal ration; (-) indicates item was not measured.

Flaxlic<sup>®</sup> Sheep Tubs were offered ad libitum during week one. Ewe intake exceeded the recommended feeding rate due to the nature of a feedlot setting. The recommended feeding rate was 56.70-113.40 g · head<sup>-1</sup> · d<sup>-1</sup>. Therefore, for the remainder of the trial, ewes had access to the tubs from 8 PM to 8 AM. Flaxlic<sup>®</sup> Sheep tubs were weighed on d 0, 3, 7, 10, 14, 17, 24, 29, and 35 to monitor intake.

Blood was collected from five ewes from each pen for a total of 120 ewes to evaluate circulating P<sub>4</sub> to determine cyclic activity. Blood was collected via jugular venipuncture (21-gauge 3.81 cm Vacuette blood drawing needle) into 10 ml serum tubes (BD Vacutainer Serum) on d 0, 7, 14, 21, 28, and 35 before weighing. Samples were centrifuged at about 10 °C for 10 min at 3300 x g. Progesterone samples were analyzed at North Dakota State University using the

Immulite Immunoassay system (IMMUNULITE 1000 Progesterone; LKPW1; Siemens Diagnostic, Los Angeles, CA). The limit of detection was 0.1 ng/ml.

On d 17, ten mature rams were placed alongside the ewes for fence line contact to stimulate estrous activity. On d 35, ewes were comingled, placed on native pasture, and rams were turned in for breeding. The rams were fitted with marking harnesses with black crayons. On d 7, 14, 21, 28, and 35 post ram turnout, breeding marks were recorded and marking harnesses were checked for crayon wear and replaced, if necessary. Crayons were replaced with red crayons on d 14. After the last recording day, the breeding harnesses were removed. Ewes were moved to a new pasture in early October.

On October 10, ewes were moved to a dry lot and fed 1.81 kg/head chopped hay, 0.45 kg/head barley haylage, and 1 kg/head barley once every two days until parturition began in February. Pregnancy status was determined via ultrasound on October 10<sup>th</sup>, 53 d post ram turnout and again on November 14, 88 d post ram turn-out (ALOKA 500; convex transducer). Ewes were moved to the Hettinger Research Extension Center lambing barn at approximately d 130 of gestation.

Ewes with lambs were moved into a separate pen (0.9 m x 1.5 m lambing pen) within two hours of lambing for bonding and observation. After two hours, data were collected on lambing type (singles, twins, or triplets), birthweight, and lamb gender. Lambs were tagged and ear notched for identification at weaning. After lambs were confirmed to be healthy and suckling, the ewe and her lambs were moved into a larger pen with other ewes with lambs (7.6 m x 3.7 m lambing pen). Lamb grower pellet was available ad libitum via creep feeders for the lambs (Southwest Grain Market Lamb Supplement). Docking occurred between 7 and 14 days after

birth. Males were not castrated. Ewes and lambs were moved to outside pens approximately one hour after docking. Weaning weights were taken at approximately 60 to 75 d post-lambing.

### **2.3.2. Statistical Analyses**

Pen served as experimental unit ( $n = 12$ ). Hay intake, ewe weights, and BCS were analyzed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). First cycle and overall pregnancy, prolificacy, and lambing rates were analyzed using the GENMOD procedure of SAS (SAS Inst. Inc., Cary, NC) in a completely random design. The models for overall and 1<sup>st</sup> cycle pregnancy rates were binomial designs and included pen, treatment, and age. The models of 1<sup>st</sup> cycle and overall prolificacy and lambing rates were a multinomial design included pen, treatment, and age. Pregnancy rate was defined as the percentage of ewes pregnant per ewe exposed in the first 16 days and overall lambing. Prolificacy rate was defined as the number of lambs born per ewe lambing in the first 16 days and overall lambing. Lambing rate was defined as the number of lambs born per ewe exposed in the first 16 days and overall lambing. Serum progesterone concentrations were analyzed using the MIXED procedure of SAS. The model for serum P<sub>4</sub> concentration included fixed effects of treatment, day, and treatment x day. Average P<sub>4</sub> concentration was a repeated measure and was analyzed using the autoregressive (1) function. Significance was determined at  $P \leq 0.05$ . To separate values for treatment effects, day effects, and treatment x day interactions, CONTRAST statements and LSMEANS were utilized ( $P \leq 0.05$ ).

## **2.4. Results and Discussion**

### **2.4.1. Ewe Weight Change and Feed Intake**

By treatment design, there was no significant difference of two-day ewe weights and BCS due to treatment at the start and end of the trial ( $P \geq 0.23$ ; Table 2.2). Average daily hay

intake on a DM basis was  $1.71 \pm 0.009$  and  $1.57 \pm 0.014$  kg · head<sup>-1</sup> · d<sup>-1</sup> for CON and FLX treatments, respectively ( $P < 0.001$ ). By design, the CON treatment was offered more hay to simulate weight gains similar to the FLX treatment. The goal of the trial was to evaluate supplemental flax, not changes in CP and energy supply, on ewe fertility and reproductive efficiency. Average Flaxlic<sup>®</sup> Sheep Tub consumption for the FLX treatment was 150.82 g · head<sup>-1</sup> · d<sup>-1</sup> for the 35-day trial. Consumption was higher than the recommended tub intake of 56.70-113.40 g · head<sup>-1</sup> · d<sup>-1</sup>, likely due to the confined feeding situation. A pasture grazing situation, such as what the Flaxlic<sup>®</sup> Sheep Tub was intended to be used in, would require ewes to travel longer distances to reach the tubs. Tub intake would likely drop closer to the recommended rate. Total  $\omega$ -3 FA intake of CON ewes was 11.11 g · head<sup>-1</sup> · d<sup>-1</sup>. Though intake was higher than recommended, the total  $\omega$ -3 FA intake of FLX ewes was 11.27 g · head<sup>-1</sup> · d<sup>-1</sup>, which includes a 1.06 g · head<sup>-1</sup> · d<sup>-1</sup> contribution from the flax tubs. Omega-6/ $\omega$ -3 ratio for FLX ewes was 1:1.36, while  $\omega$ -6/ $\omega$ -3 FA ratio for CON ewes was 1:1.35. The increase in omega-3 FA coming from the flax tubs was not sufficient to alter the  $\omega$ -6/ $\omega$ -3 FA when compared to the CON ration.

**Table 2.2.** Impact of Flaxlic<sup>®</sup> Sheep Tub on Initial and Final Weights of Ewes during a Flushing Feeding Period

Item	Treatment			P-Value <sup>3</sup>
	CON	FLX	SEM	
Initial Weight, kg	71.0	70.8	0.19	0.47
Final Weight, kg	69.5	70.1	0.37	0.23
Initial BCS <sup>2</sup>	3.1	3.1	0.02	0.45
Final BCS <sup>2</sup>	3.0	3.0	0.05	0.89

<sup>1</sup>FLX = Flaxlic<sup>®</sup> Sheep Tub supplemented ewes; CON =control ewes; SEM = Standard Error of the Mean. <sup>2</sup>Body condition score; scale of 1-5; Kenyon et al., 2014.

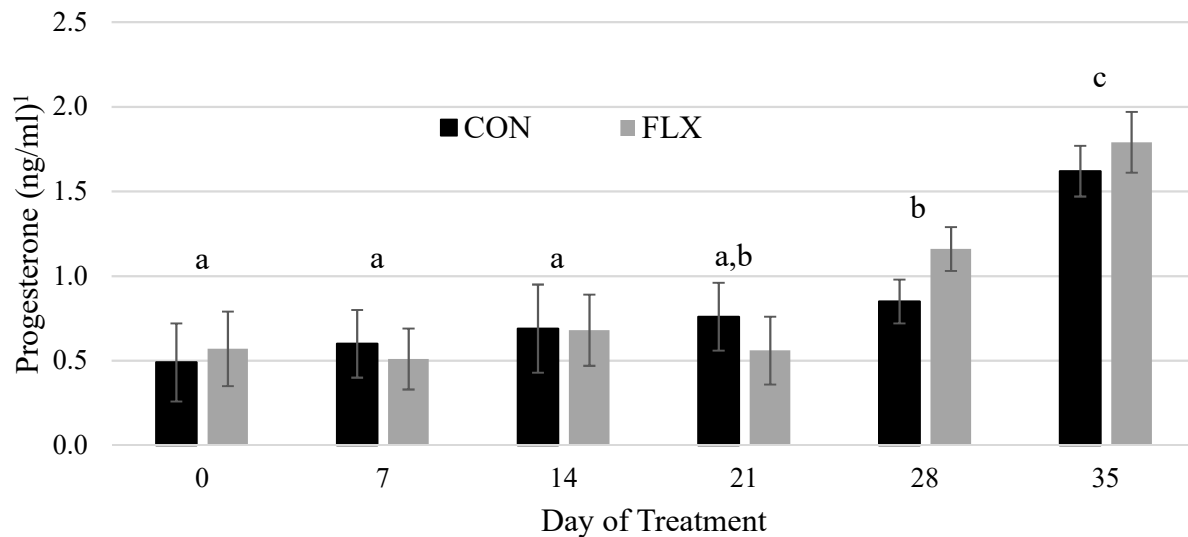
<sup>3</sup>P-value across treatments (n=12 for FLX and CON treatments).

#### 2.4.2. Circulating Progesterone

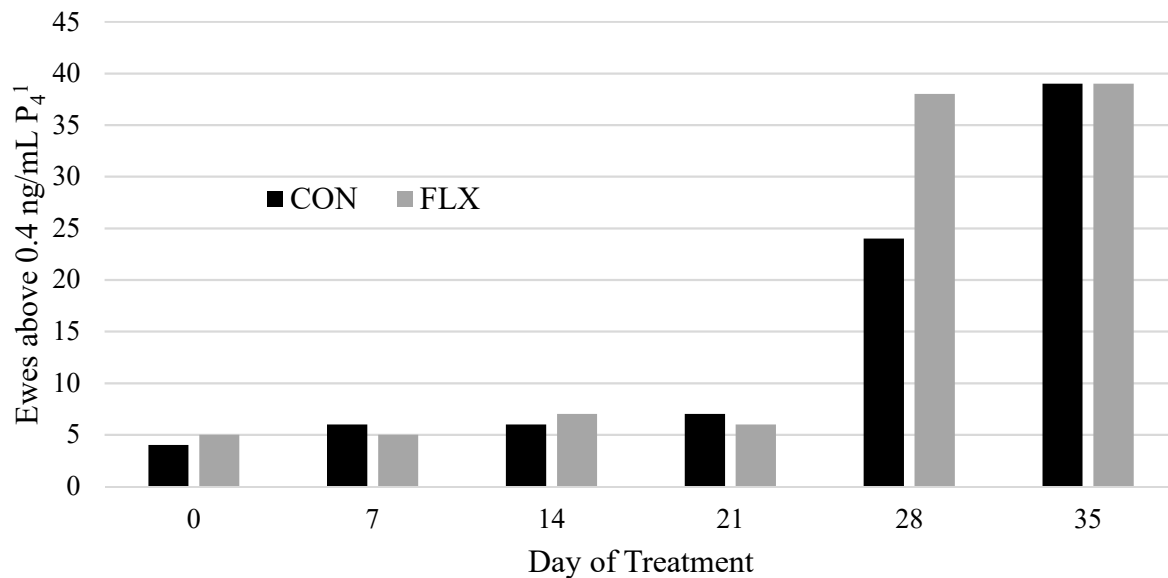
Ewe cyclicity was determined when a concentration of over 0.4 ng/ml of serum P<sub>4</sub> was present (Quirke et al., 1985; Wright et al., 2002; Santos et al., 2018). In the present study,



particular attention was given to the trend rather than the concentration. There was no treatment x day interaction ( $P = 0.82$ ) for serum  $P_4$  concentration. There also was no treatment effect ( $P = 0.83$ ). However, there was a day effect ( $P < 0.001$ ; Figure 2.1.). Progesterone increased as day increased. The increase is likely due to ram exposure due to increased cyclicality.



**Figure 2.1.** Impacts of Flaxlic® Sheep Tub on Serum Progesterone Concentration by Day of Tub Exposure During the Flushing Period. <sup>1</sup>Serum progesterone concentration of Flaxlic® Sheep Tub fed ewes (FLX) versus control ewes (CON). The threshold for cyclicality was stated as a value over 0.4 ng/ml (Quirke et al. 1985; Wright et al., 2002; Santos et al., 2018). <sup>a-</sup>Groups with different letters differ ( $P < 0.001$ ) between days.



**Figure 2.2.** Impacts of Flaxlic® Sheep Tub on Ewe Cyclicity by Day of Tub Exposure During the Flushing Period. <sup>1</sup>Number of ewes above the 0.4 ng/ml progesterone (P<sub>4</sub>) concentration on a given day; Flaxlic® Sheep Tub fed ewes (FLX) versus control ewes (CON). The threshold for cyclicity was stated as a value over 0.4 ng/ml (Quirke et al. 1985; Wright et al., 2002; Santos et al., 2018).

Progesterone concentration on d 0 through 21 were lower than d 28 and 35 ( $P < 0.001$ ). In addition, the rams were run along the fence line after d 17, perhaps leading to active cyclicity afterwards due to the male effect, as reported in sheep and goats (Walkden-Brown et al., 1993; Rosa and Bryant, 2002; Rivas-Muñoz et al., 2007; Delgadillo et al., 2009). These results are in agreement with Ambrose et al. (2006) and Hutchinson et al. (2012) who reported no difference in P<sub>4</sub> concentration between control and flaxseed fed dairy cows. Dairy cattle are very different from sheep in management and production level. Ambrose fed 427.5 g of  $\omega$ -3 FA in mechanically rolled flaxseed to mature dairy cows with an average weight of 650 kg. Ambrose et al. (2006) fed 4 times the amount of  $\omega$ -3 FAs per kg bodyweight compared to the present study, which fed a daily  $\omega$ -3 FA intake of 10.31 g · head<sup>-1</sup> · d<sup>-1</sup>. This may explain why a difference was not found in the present study. In agreement to this hypothesis, the results in the present ewe study are contrary to studies who reported significant increases in P<sub>4</sub> concentration in flax fed cows (Lessard et al., 2003; Petit and Twagiramungu, 2006). Both Petit and Twagiramungu

(2006) and Lessard et al. (2003) fed flax as whole flaxseed, finding a significant increase in P<sub>4</sub>. In contrast, both Ambrose et al. (2006) and Hutchinson et al. (2012) fed flax in a processed form (oil and mechanically rolled flax, respectively). The present study fed flax in a highly processed form, utilizing both flax oil and flaxseed meal in the formulation of the Flaxlic<sup>®</sup> Sheep Tub. The processed flax may have been more vulnerable to hydrogenation by rumen microbes, while the pericarp of whole flaxseed protected the oils until further digestion occurred in the small intestine (Lashkari et al., 2015). Santos et al. (2008) notes further research is required to fully understand how long chain fatty acids affect the ruminant animal, whether the effects reported are due to fatty acids in the product fed or due to the biohydrogenated forms of those fatty acids after rumen digestion. To add further evidence to this argument, Luna et al. (2008), who used whole flaxseed, found increased ALA in the blood of sheep. These studies may infer rumen protection of ALA is required to elicit an effect on the reproductive performance of the ruminant animal.

### **2.4.3. Lambing**

There was no interaction between treatment and age for 1<sup>st</sup> cycle or overall pregnancy rate, prolificacy rate, or lambing rate ( $P \geq 0.13$ ; Table 2.3). There were no differences between treatments for 1<sup>st</sup> cycle pregnancy rate, prolificacy rate, or lambing rate ( $P \geq 0.26$ ). There were also no differences between FLX and CON ewes for overall pregnancy rate, prolificacy rate, or lambing rate ( $P \geq 0.26$ ).

**Table 2.3.** Impact of Flaxlic® Sheep Tub Supplementation During the Flushing Period on Ewe Lambing Data in the First Cycle and for the Overall Lambing Period

Item <sup>2</sup>			Treatment <sup>1</sup>		P-value <sup>3</sup>
			CON	FLX	TRT
First Cycle	Pregnancy	Mean	69%	72%	0.76
		SEM	4.4%	4.2%	
	Prolificacy	Mean	148%	153%	0.26
		SEM	6.0%	6.6%	
Lambing	Mean	103%	112%	0.31	
	SEM	7.7%	8.1%		
Overall	Pregnancy	Mean	97%	96%	0.61
		SEM	1.7%	1.9%	
	Prolificacy	Mean	147%	149%	0.86
		SEM	5.0%	5.7%	
	Lambing	Mean	145%	145%	0.89
		SEM	6.0%	6.0%	

<sup>1</sup>FLX = Flaxlic® Sheep Tub supplemented ewes; CON=control ewes.

<sup>2</sup>Pregnancy = percentage pregnant per ewe exposed; Prolificacy = lambs per ewe lambled; Lambing rate = lambs per ewe exposed; SEM = standard error of the mean.

<sup>3</sup>P-value between treatments (TRT;  $n = 12$  for FLX and CON treatments).

More focus should be placed on 1<sup>st</sup> cycle findings rather than overall results of the lambing season. The ewes were taken off flax supplementation upon becoming comingled with rams for breeding. Therefore, the flax supplementation effect would only be exhibited for a limited amount of time. This would especially be true if the reproductive system was only affected while being supplemented. Subsequent ovulation during the breeding period would only occur within the first 16 to 21 days. Previous studies reported increased pregnancy rates (Ambrose et al., 2006; Silvestre et al., 2011), improved conception, and decreased embryo mortality (Ambrose et al., 2006; Petit and Twagiramungu, 2006). These improvements were not reflected in the present study's 1<sup>st</sup> cycle pregnancy, lambing, or prolificacy rates. As mentioned previously, Ambrose et al. (2006) fed flax at an ALA concentration that was four times as high as the present study by weight. The rolled flaxseed from Ambrose et al. (2006) would also be more vulnerable to biohydrogenation than whole flaxseed (Lashkari et al., 2015). The feeding level of flaxseed in the present study may not be at a level to sufficiently affect these pregnancy

parameters. Increased prolificacy via increased ovulations was found by Trujillo and Borughton (1995), which disagrees with the present study. The ability of Flaxlic<sup>®</sup> Sheep Tub supplementation to improve pregnancy, lambing, and prolificacy rates may become more pronounced when flax is fed in larger quantities, and age is blocked by pen. Age impacted both overall and first cycle lambing and prolificacy rates.

## 2.5. Conclusions

Addition of a Flaxlic<sup>®</sup> Sheep Tub did not significantly improve pregnancy parameters or influence progesterone concentration level. An important aspect of feeding these components is to not only increase the  $\omega$ -3 FAs but also to decrease the  $\omega$ -6 to  $\omega$ -3 FA ratio in the diet and therefore in the system of the targeted animal. However, if the  $\omega$ -3 FAs cannot make it through the rumen environment without being biohydrogenated, the effect may be lost altogether. Utilization of whole flaxseed or rumen protected ALA may be the answer. Studies using processed flaxseed did not find improved progesterone concentration. However, some did find improved conception. Perhaps this improved conception is due to the products of hydrogenation of  $\omega$ -3 FAs from the processed flaxseed. Studies utilizing whole flaxseed, protected by the pericarp, found increased progesterone concentrations in the blood of dairy cows and increased ALA in the milk of ewes. This may imply the use of whole flaxseed is more efficiently utilized by the ruminant and thus more beneficial to reproduction. More research is required to confirm the hypothesis that rumen protection is warranted to elicit desirable responses in reproductive performance in sheep and ruminants in general.

The desirable ratio of  $\omega$ -6/ $\omega$ -3 FAs is not yet known for reproductive improvement in female ruminants. More research with specific focus on controlling  $\omega$ -6/ $\omega$ -3 ratio is required to discover the most desirable ratio for the ruminant female. In the present study, the as-fed  $\omega$ -6/ $\omega$ -3

FA ratio was 1:1.36 for FLX ewes and 1:1.35 for CON ewes. These ratios were not different enough to elicit any responses, as shown by the present study.

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### 3. IMPACTS OF FLAX ON MALE REPRODUCTIVE TRAITS WHEN SUPPLEMENTED PRIOR TO BREEDING IN SHEEP

#### 3.1. Abstract

Fertility in rams is important in range sheep operations. Prior to breeding, fertility can be improved by improving nutritional management. Fatty acid supplementation has been shown to improve male reproductive characteristics, such as sperm motility, concentration, and morphology. Supplementation with flax prior to breeding is a potential strategy to increase  $\omega$ -3 FAs in the diet. The objective of this study was to evaluate the effectiveness of flax supplementation on serum testosterone concentration and semen quality. One-hundred twenty Rambouillet ram lambs ( $42 \pm 2.78$  kg) were randomly assigned to 24 pens (5 rams/pen;  $n = 12$ ) and fed for 112 days. Rams were assigned to either receive a Flaxlic® Sheep Tub (**FLX**) or a control (**CON**). Tubs were weighed on d 0, 14, 28, 48, 64, 92, 103, and 112. Tubs weighing less than 5 kg were replaced with new tubs. Weight data for all rams was taken on d 0, 28, 56, 85, and 112, with 2-d weights taken on d -1 and 0 and d 111 and 112. Serum for testosterone concentration analysis, semen for quality analysis, and scrotal circumference measurements were collected on d 83-84 and 111-112. Average daily gain (**ADG**) for FLX rams was not different from CON ( $0.73 \pm 0.10$  and  $0.78 \pm 0.11$ , respectively;  $P = 0.25$ ). No differences were observed for testosterone concentrations between CON and FLX treatments ( $208.04$  ng·dl<sup>-1</sup>;  $P = 0.70$ ). There were no significant differences in scrotal circumference, sperm motility, sperm morphology, or sperm concentration ( $P \geq 0.15$ ). These results are in contrast to others who fed flax to rams. However, lack of changes to semen parameters are in agreement with a study feeding a Flaxlic® Tub to bulls. Therefore, flaxseed supplementation in a tub form is not recommended to growing ram lambs for the improvement of semen quality.

### 3.2. Introduction

Reproduction is a vital component for any range sheep operation. The male side of reproduction is a component of overall productivity that can be overlooked. One way to improve a ram's performance during the breeding season is to improve the nutrients provided leading up to breeding. Not only will this help rams regain lost condition from the previous season, but it may also stimulate improved spermatogenesis and sperm cell function. Adding extra nutrients to the pre-breeding ration, such as essential fatty acids, has been shown to further improve a male's reproductive efficiency (Tou et al., 1999; Zanini et al., 2003; Baiomy and Mottelib, 2009; Yan et al., 2013; Esmaceli et al., 2014; Jafaroghli et al., 2014; Moallem et al., 2015; Shah et al., 2016)

Flaxseed provides two essential fatty acids (FA): Alpha-linolenic acid (ALA; C18:3  $\omega$ -3), an omega-3 ( $\omega$ -3) FA and linoleic acid (LA: C18:2  $\omega$ -6), an omega-6 ( $\omega$ -6) FA. Flax is approximately 45% oil. Of the total fats in flax oil, about 57% is ALA and 16% percent is LA (Morris, 2007). Flax is an excellent supplier of  $\omega$ -3 FA and contains a very low  $\omega$ -6 to  $\omega$ -3 FA ratio (1:3.56).

Flaxseed in particular has been shown to improve sperm motility and progressive motility in bulls (Moallem et al., 2015). Flaxseed has also been shown to increase levels of the reproductive hormones such as gonadotropin-releasing hormone, follicle stimulating hormone (Yan et al., 2013), luteinizing hormone (Yan et al., 2013), and testosterone (Baiomy and Mottelib., 2009; Yan et al., 2013; Esmaceli et al., 2014). Supplementing male sheep with flaxseed prior to breeding may be a way to improve semen quality and thereby improve fertility. Our hypothesis was the supplementation of flaxseed would increase testosterone in the blood and therefore improve spermatogenesis and reproductive performance while preventing sperm

abnormalities. The objective of the present study was to supplement flaxseed in an applied setting using Flaxlic® Sheep Tubs during a 112-d period leading up to the breeding season.

### 3.3. Materials and Methods

All procedures were approved by the Animal Care and Use Committee of North Dakota State University (NDSU; protocol #A18059). This study was conducted at the NDSU Hettinger Research Extension Center in Hettinger, ND.

#### 3.3.1. Experimental Design

Rambouillet ram lambs ( $n = 120$ ) were selected from the NDSU Hettinger Research Extension Center flock. At 60 days of age, lambs were weaned and vaccinated for *Clostridium perfringens* type C and D and tetanus. On d -1, ram lambs (approximately 4 months of age;  $42 \pm 2.78$  kg) were randomly assigned to 24 pens (5 rams/pen;  $25.2 \text{ m}^2/\text{ram}$ ), with pen serving as the experimental unit. Rams were fed a basal ration with a Flaxlic® Sheep Tub (FLX;  $n = 12$ ) or a basal ration alone (CON;  $n = 12$ ). The basal ration was a total mixed ration (TMR) made up of 60% soybean hulls, 10% corn, 15% soybean meal, and 15% Market Lamb Supplement (dry matter basis; Southwest Feed, Inc.). The basal ration was balanced to meet the CP and TDN requirements of a 40 kg lamb gaining 300 g/d (Table 3.1; NRC, 2007). The ration was mixed in a mixer-grinder (GEHL mix-all, Model 170; West Bend, WI) and provided to the rams ad libitum via bulk feeders (98 cm of bunk space per ram). Orts were taken on day 87 and 112 and tested for nutrient composition. Samples were sent to Midwest Laboratories (Omaha, NE) for nutrient analysis. Dry matter (calculated from the moisture measurement, method 930.15; Association of Analytical Communities [AOAC] Int., 1990), acid detergent fiber (ADF; ANKOM Tech. Method), crude protein (CP; method 990.03; AOAC Int., 2006), total digestible nutrients (TDN),

and minerals (method 985.01 modified; AOAC Int., 2006) were measured. Omega-6 and  $\omega$ -3 FA were analyzed as well (method 996.06; AOAC Int., 2012). The ingredients for the Flaxlic<sup>®</sup> Sheep Tub by inclusion level are beet molasses, ground flaxseed (21%), flaxseed oil (6.4%), soybeans (45%), and select vitamins and minerals (Table 3.1). Flaxlic<sup>®</sup> Sheep Tub weights were taken on d 0, 14, 28, 48, 64, 92, 103, and 112 to monitor ram tub intake. Flaxlic<sup>®</sup> Sheep Tubs that fell below 5 kg were replaced with new tubs. Rams were allowed 12-hour access to the tubs from 8 PM to 8 AM in the first two weeks. Intake during this time was below the recommendation level of 56.70-113.40 g · head<sup>-1</sup> · d<sup>-1</sup>. From d 14 until the trial finished on d 112, FLX rams were allowed 24-hour access to the tubs to increase intake.

**Table 3.1.** Nutrient Composition of the Basal Ration and Flaxlic<sup>®</sup> Sheep Tub

Nutrient, % DM <sup>1,3</sup>	TMR <sup>2</sup>	Flaxlic <sup>®</sup> Sheep Tub
DM (% as fed)	86.66	-
ADF	28.9	2.5
CP	22.5	12.0
TDN	72.3	-
S	0.30	-
P	0.42	1.0
K	1.54	2.5
Mg	0.36	-
Ca	1.34	1.0-1.5
Na	0.25	-
Fe	683 ppm	-
Mn	91.7 ppm	0.12 ppm
Cu	13.0 ppm	0 ppm
Zn	129 ppm	1200 ppm
$\omega$ -3 Fatty Acids	0.15 g·100g <sup>-1</sup>	0.07 g·100g <sup>-1</sup>
$\omega$ -6 Fatty Acids	0.71 g·100g <sup>-1</sup>	0.025 g·100g <sup>-1</sup>

<sup>1</sup>TMR = total mixed ration; Most measurements reported on a dry matter basis; fatty acid analysis reported on an as fed basis. <sup>2</sup>60% pelleted soybean hulls, 15% soybean meal, 15% Southwest Grain Market Lamb Supplement, 10% whole corn; dry matter basis.

<sup>3</sup>DM = dry matter; ADF = Acid Detergent Fiber; CP = crude protein.

The basal ration had a measured  $\omega$ -6/ $\omega$ -3 FA ratio of 4.7:1. The Flaxlic<sup>®</sup> Sheep Tub had an  $\omega$ -6/ $\omega$ -3 FA ratio of 1:2.8. In total, CON rams received an average of 13.5 g of  $\omega$ -6 FAs and 2.87 g of  $\omega$ -3 FAs per day based on the CON group's average intake. FLX rams received 12.86 g of  $\omega$ -6 FAs and 2.75 g of  $\omega$ -3 FAs per day based on the FLX group's average intake. The overall ratio is 4.73:1 and 4.69:1 for CON and FLX groups, respectively.

Two-day weights and body condition score (1-5 scale; Kenyon et al., 2014) were taken on d -1 and 0 and d 111 and 112, with ram body weight recorded every 28 d to monitor ram health. Ram scrotal circumferences were taken during a 2-day period alongside semen collection, on d 83-84, and again on d 111-112. With a standing ram, both testes were retained to the base of the scrotum, where circumference was measured of the scrotal tissue and the two testes combined (Martin et al., 1994). Four rams were removed due to non-treatment related death prior to d 84 (two FLX, two CON). One ram was removed due to non-treatment related death prior to day 112 (FLX).

Blood and semen were collected over a two-day period on d 83 and 84, then again on d 111 and 112. Blood was collected via jugular venipuncture using a 21-gauge 3.81 cm needle (Vacurette blood collection needle) into a Serum Separator Tube blood tube (SST, VWR Inc.) and placed on ice. Samples were centrifuged at 10 °C for 10 min at 3300 x g for serum collection. Testosterone samples were analyzed at North Dakota State University using the Immulite Immunoassay system (IMMUNULITE 1000 Total Testosterone; LKTW1; Siemens Diagnostic, Los Angeles, CA). The intraassay and interassay coefficients of variation (CV) were 8.6% and 13.1%, respectively. Average testosterone concentrations for the quality control samples were 115.3, 587.75, and 1134.5 ng/dl for low, medium, and high samples, respectively. The limit of detection was 50.0 ng/dl.

Semen was collected via electro-ejaculation over a two-day period on d 83 and 84, then again on d 111 and 112. Rams were stimulated until a successful ejaculation occurred into a plastic collection sheath, up to three times. The first successful ejaculate from each ram was evaluated. Ejaculates were placed into a cooler held at 35 °C. Contents of a sheath were transferred to a 2.5 mL conical tube for volume determination. Within 20 minutes of collection, a sample of semen was diluted with buffer (Easy Buffer B, IMV Technologies U.S.A., Maple Grove, MN) to a target cell count of 60 to 80 cells per field. Diluted semen was placed into 20 µm capillary chamber slide (Leja products B.V., Netherlands) and loaded into a computer assisted semen analysis machine (IVOS II, Hamilton Throne, Beverly, MA). Each of 10 fields were assessed. Notable measurements for abnormalities included bent tail percent of total sperm, proximal droplet percent of total sperm, and distal droplet percent of total sperm. Quantity and mobility measurements included total concentration (million cells/ml), sperm count (concentration of sperm per milliliter x total volume of the ejaculate), semen volume (ml), motile concentration (million cells/ml), motile count (concentration of motile sperm per milliliter x total volume of the ejaculate), motile sperm as a percent of total sperm, progressive concentration (million cells/ml), progressive sperm count (concentration of progressively motile sperm per milliliter x total volume of the ejaculate), progressive sperm as a percent of total sperm, and static sperm as a percent of total sperm.

### **3.3.2. Statistical Analyses**

Ram body measurements, serum testosterone concentrations, and semen analysis results were analyzed in a completely random design using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC), with pen serving as experimental unit and variance component structure. Pen nested in treatment was a random variable. Testosterone concentration and semen characteristics

were repeated measures. Class variables were day and treatment. Models included fixed effects of treatment, day, and treatment x day. If a treatment x day interaction was not found, the model was run again without the interaction. Significance was determined at  $P \leq 0.05$ . To separate day effects and treatment x day interactions, LSMEANS and CONTRAST statements were utilized ( $P \leq 0.05$ ).

### **3.4. Results and Discussion**

#### **3.4.1. Ram Weight Change and Feed Intake**

There were no treatment x day interactions ( $P \geq 0.41$ ) for ram weight or BCS. Initial and final ram weight or BCS did not differ between treatments (Table 3.2;  $P \geq 0.25$ ). As the rams were given free access to feed with or without the addition of the Flaxlic<sup>®</sup> Sheep Tub, this result was expected. However, there was a day effect for weight gain ( $P < 0.001$ ), which was also expected due to the growth of a ram lamb into a mature ram. There was a treatment x day interaction ( $P = 0.04$ ) between treatments for average daily gain (ADG). Between d 28 and 56, the CON treatment had a higher ADG than the FLX group ( $0.74 \pm 0.32$  and  $0.53 \pm 0.44$ , respectively;  $P = 0.04$ ). The CON treatment gained more over time than the FLX group. These results are in contrast with Pesta and Drouillard (2010) who reported increased ADG and improved feed efficiency between treatments in Flaxlic<sup>®</sup> Tub fed bulls ( $P < 0.05$ ).



**Table 3.2.** Impact of Flaxlic® Sheep Tubs on Weight and Body Measurements in Rambouillet Ram Lambs

Item <sup>2</sup>	Treatment <sup>1</sup>			P-Value <sup>3</sup>
	CON	FLX	SEM	
Initial Weight, kg	41.8	42.2	2.78	0.82
Final Weight, kg	81.6	79.5	2.80	0.25
Initial BCS	3.00	2.99	0.02	0.72
Final BCS	3.62	3.58	0.05	0.65
DM Intake, kg·hd <sup>-1</sup> ·d <sup>-1</sup>	1.91	1.81	0.05	0.23
ADG, kg/d	0.78	0.73	0.03	0.25
G:F	5.42	5.47	0.10	0.77
SC d 83-84, cm	31.8	32.1	0.29	0.56
SC d 111-112, cm	34.4	34.2	0.33	0.72

<sup>1</sup>FLX= Flaxlic® Sheep Tub supplemented ewes; CON=control ewes; SEM= Standard error of the mean.

<sup>2</sup>BCS= Body condition score; scale of 1-5; Kenyon et al. (2014); DM = Dry matter; ADG= Average daily gain; G:F= Gain to feed; SC= Scrotal circumference. <sup>3</sup>P-value across treatments (n=12).

There was no effect of treatment on daily dry matter intake between CON and FLX treatments ( $1.91 \pm 0.05$  and  $1.81 \pm 0.05$  kg · hd<sup>-1</sup> · d<sup>-1</sup>, respectively;  $P = 0.23$ ). FLX intake of the Flaxlic® Sheep Tub was  $42.52$  g · hd<sup>-1</sup> · d<sup>-1</sup>, 2.2% of their total feed intake per day. Gain to feed (G:F) was not different between CON and FLX treatments ( $5.42 \pm 0.10$  and  $4.47 \pm 0.10$ , respectively;  $P = 0.77$ ). Pesta and Drouillard (2010) conversely reported the control group of bulls had higher feed intakes than Flaxlic® Tub supplemented bulls. Pesta and Drouillard (2010) also reported improved G:F ratios not found in the present study.

The present study results were opposite to the similar study in bulls, possibly indicating a difference in physiological response to flax between bulls and rams. This may be due to a species-specific response that does not occur in sheep. Another explanation may be the rams were not eating enough of the Flaxlic® Sheep Tub compared to the intake of the bull. Rambouillet rams may require more flax per pound of bodyweight than bulls.

### **3.4.2. Reproductive Traits**

#### ***3.4.2.1. Scrotal Circumference***

There were no treatment x day interactions for scrotal circumference (SC;  $P = 0.34$ ). There was a day effect between d 83 and 84 and d 111 and 112 (31.92 and 34.32 cm, respectively;  $P < 0.001$ ), as expected of maturing ram lambs (Camela et al., 2018). There were no treatment effects for overall SC ( $P = 0.72$ ) or on d 83-84 (Table 3.2;  $P = 0.56$ ). The lack of change in SC is in agreement with Baiomy and Mottelib (2009), who also found no change in SC between flaxseed supplemented and control rams. Baiomy and Mottelib (2009) used flax oil, which was less protected from biohydrogenation than feed types such as whole flaxseed (Lashkari et al., 2015). The present study also used a less protected form of flax in a tub, made up of flax oil and flaxseed meal. Changes in scrotal circumference are affected by maturity and season. Rams were in the correct season to stimulate changes in SC. However, Rambouillets are known for being late maturing (Foote et al., 1970). Therefore, the lack of change in SC may simply be due to the rams' immaturity. Extending the trial for a longer period in the Rambouillet breed may reveal differences between treatments.

#### ***3.4.2.2. Testosterone***

Half of the samples' testosterone concentration levels were too low for accurate measurement. Therefore, only values greater than 50 ng/dl were utilized. There were no treatment x day interactions ( $P = 0.99$ ). Serum testosterone was not different between treatments (213.13 and 213.12 ng/dl, respectively;  $P = 0.99$ ). A day effect was observed between d 83 and 84 and d 111 and 112 (179.2 and 247.0 ng/dl, respectively;  $P = 0.02$ ). This increase in testosterone over time is in unison with the increase in scrotal circumference by day. Baiomy and Mottelib (2009) also measured testosterone in flax-fed vs control rams, reporting an increase in the flax-fed rams over

the control after two months of treatment. The rams used were Ossimi, a breed from Egypt, which may explain a difference in testosterone response to flaxseed components due to differing climate, breed, and management systems. Baiomy and Mottelib (2009) also had an experimental unit of ram, with four rams per treatment, totaling 12 animals. Due to the small sample size, there is a higher risk of a type I error. The present study did not find differences in testosterone between treatments. However, as previously stated, biohydrogenation may have left ALA and LA unavailable for absorption by the small intestine (Kronberg et al., 2012; Lashkari et al., 2015).

#### **3.4.2.3. Sperm Morphological Abnormalities**

There were no treatment x day interactions ( $P \geq 0.08$ ) or treatment effects ( $P \geq 0.62$ ) for sperm abnormalities (Table 3.3). Percentage of bent tails and total abnormalities were affected by day ( $P \leq 0.04$ ). Bent tail percentage decreased from 9.34 to 5.65 % from d 83-84 to d 111-112, respectively. Total abnormalities dropped from 41.24% on d 83-84 to 35.89% on d 111-112, respectively. Both bent tail percentage and total abnormalities decreased as rams aged. Therefore, this improvement is likely due to the rams' increasing maturity, as abnormalities are reported to decrease as males reach maturity (Bartlett, 1982). In rams, semen reaches optimal quality by 2 to 3 years of age (Badi et al., 2018). These results are in agreement with Pesta and Drouillard (2010), who reported no differences between control and Flaxlic<sup>®</sup> supplemented bulls for percent normal sperm. The results are in contrast with Baiomy and Mottelib (2009) who found decreased abnormal sperm in  $\omega$ -3 FA supplemented rams.

The rams in the present study were fed processed flax oil and flaxseed meal in the form of a tub, in addition to the high levels of omega-6 FA found in the basal ration (Table 3.1). The FAs becoming biohydrogenated in both the bulls' and the rams' Flaxlic<sup>®</sup> tubs could explain why

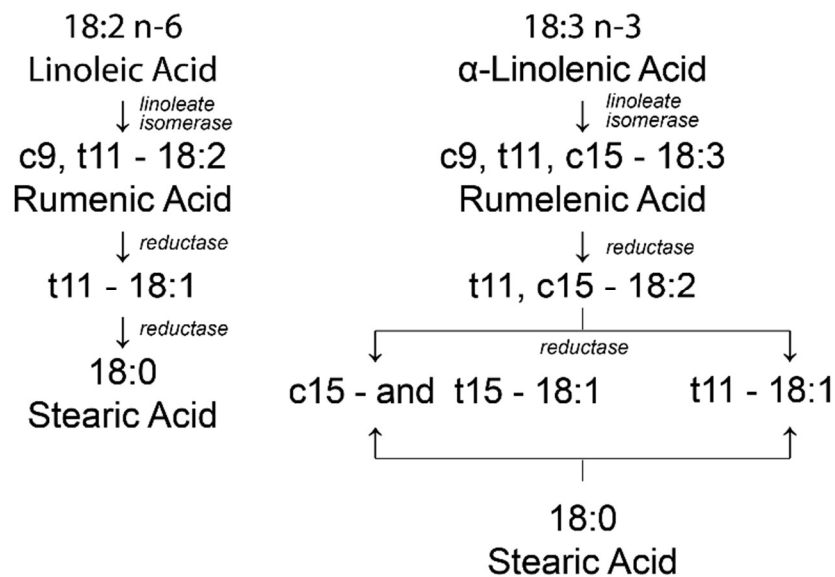
**Table 3.3.** Impacts of Flaxlic® Sheep Tub Supplementation on Semen Characteristics in Rambouillet Ram Lambs

Item <sup>3</sup>	Treatment <sup>1</sup>		SEM	P - Value <sup>2</sup>
	CON	FLX		TRT
Overall bent tail, %	7.55	7.52	0.78	0.98
D 84	9.32	9.37	1.38	
D112	5.71	5.58	0.58	0.98
Overall distal droplet, %	4.89	5.03	0.34	0.86
D 84	5.40	5.10	0.56	
D112	4.38	4.99	0.40	0.87
Overall proximal droplets, %	24.71	24.64	1.23	0.97
D 84	23.94	25.14	1.87	
D112	25.49	24.12	1.60	0.62
Overall volume, ml	0.66	0.67	0.06	0.91
D 84	0.57	0.47	0.05	
D112	0.75	0.88	0.10	0.87
Overall total sperm count <sup>4</sup>	377.44	400.50	19.50	0.49
D 84	325.23	371.48	29.20	
D112	431.55	430.05	24.46	0.48
Overall sperm concentration, million cells/ ml	994.75	1053.30	92.66	0.67
D 84	720.23	850.65	118.00	
D112	1279.25	1259.66	136.85	0.67
Overall motile concentration, million cells/ ml	548.36	628.58	68.43	0.49
D 84	474.67	612.96	100.00	
D112	620.68	643.34	93.30	0.50
Overall motile sperm count <sup>4</sup>	192.76	216.53	16.06	0.44
D 84	189.64	236.79	26.30	
D112	195.81	197.38	18.80	0.43
Overall motile sperm, %	45.61	47.47	2.48	0.70
D 84	47.53	49.29	3.75	
D112	43.73	45.68	3.27	0.70
Overall progressive concentration, million cells/ml	457.66	465.91	48.69	0.91
D 84	434.30	501.63	83.30	
D112	478.32	432.79	54.25	0.89
Overall progressive sperm count <sup>4</sup>	166.29	167.58	12.28	0.96
D 84	176.24	192.61	21.50	
D112	157.48	144.38	12.43	0.94
Overall progressive sperm, %	39.84	37.64	2.20	0.55
D 84	43.74	39.73	3.42	
D112	36.39	35.62	2.78	0.52
Overall static sperm, %	56.42	52.44	2.54	0.33
D 84	55.81	50.67	3.87	
D112	57.07	54.32	3.29	0.34

<sup>1</sup>CON = basal ration; FLX = basal ration with Flaxlic® Sheep Tub; SEM = Standard Error of the Mean.

<sup>2</sup>P-values considered significant at  $P < 0.05$ ; TRT = treatment effects; <sup>3</sup>Concentrations given in million per ml of semen; percentages given as % of total sperm <sup>4</sup>Count reported as concentration of identified sperm per milliliter multiplied by the total volume of the ejaculate entered initially into the IVOS.

a response was not found in either study. Kronberg et al. (2012) found higher levels of ALA in the blood and tissues of lambs fed flax protected from ALA hydrogenation. Despite this, Baiomy and Mottelib (2009) found decreased abnormal sperm in rams supplemented with unprotected flax oil. The fault in the present study may be with a combination of hydrogenation of ALA and the high  $\omega$ -6/ $\omega$ -3 ratio. The products of biohydrogenation may be beneficial to spermatogenesis, even with the products of  $\omega$ -6 FA biohydrogenation. Bacteria that specifically break down long chain FA have been specifically found and researched in sheep (Kemp et al., 1984; Belenguer et al., 2010). Linoleic acid is broken down into rumenic acid, further into vaccenic acid, and further still to stearic acid (18:0; Belenguer et al., 2010). Alpha-linolenic acid also can be broken down to stearic acid, however there are more steps, including rumelenic acid (Figure 3.1; Belenguer et al., 2010). Perhaps it is the components of these pathways that are absorbed by the small intestine and used in the body. The different concentrations of  $\omega$ -6 and  $\omega$ -3 FA between FLX and CON groups may not end up so different after biohydrogenation.



**Figure 3.1.** Biohydrogenation of  $\alpha$ -Linolenic Acid and Linoleic Acid by the Rumen Environment. Adapted from Hobson and Steward (1997).

#### **3.4.2.4. Volume and Concentration**

There were no treatment x day interactions ( $P \geq 0.15$ ) for semen volume or concentration. There were also no effects of treatment on semen volume, total count, or concentration ( $P \geq 0.48$ ). The lack of change in concentration between treatments is in contrast with studies in both rams and bulls (Baiomy and Mottelib, 2009 and Shah et al., 2016, respectively). Both studies had a total samples size of 12 and an  $n$  of 4 per treatment. Both Baiomy and Mottelib (2009) and Shah et al. (2016) used flax oil. For these studies, it is possible the products of biohydrogenation from the rumen contribute to the improved sperm morphology, rather than ALA itself. Also, the high  $\omega$ -6/ $\omega$ -3 ratio of the present study may also hinder the improvement of spermatogenesis.

There was a day effect ( $P \leq 0.03$ ) for ejaculate volume, sperm concentration, and total sperm count. Ejaculate volume increased from 0.52 ml on d 83-84 to 0.82 ml on d 111-112 ( $P < 0.001$ ). Total concentration increased ( $P < 0.001$ ) from 784.86 million cells per ml on d 83-84 to 1269.45 million cells per ml on d 111-112. Total sperm count also increased ( $P = 0.005$ ) from 348.15 sperm cells per ml on d 83-84 to 430.80 sperm cells per ml on d 111-112. An increase in total concentration by day is expected in maturing ram lambs (Badi et al., 2018).

#### **3.4.2.5. Motility**

There were no treatment x day effects ( $P \geq 0.18$ ) for any motility measurement. There was also no effect of treatment ( $P \geq 0.42$ ) for any motility measurement. There was a day effect ( $P = 0.05$ ) for progressive sperm count, decreasing from 184.85 sperm per ml on d 83-84 to 150.75 sperm per ml on d 111-112. It is unclear why progressive sperm count decreased, as this value should have increased as the rams aged. This finding disagrees with the statement that rams reach optimum seminal quality at 2 to 3 years of age (Badi et al., 2018). However, the other

measurements of progressively motile sperm, including percentage and concentration of progressively motile sperm, were not different between days ( $P \geq 0.13$ ).

The lack of difference between treatments is in agreement with Pesta and Drouillard, (2010) who found no differences between control and Flaxlic<sup>®</sup> supplemented bulls for sperm motility. The results are in contrast with multiple studies who found increased motility and mass movement in both rams and bulls (Baiomy and Mottelib, 2009; Jafaroghli et al., 2014; Shah et al., 2016). Jafaroghli et al. (2014) used fish oil in their study in rams, similar to flax oil used in Baiomy and Mottelib (2009). Both Baiomy and Mottelib (2009) and Jafaroghli et al. (2014) supplemented oils to increase  $\omega$ -3 FAs and found improved sperm motility. Again, perhaps it is the breakdown of ALA that contributes positively to spermatogenesis and motility, rather than ALA itself. As stated previously, the lack of improvement in the present study could also be affected by the high  $\omega$ -6/ $\omega$ -3 ratio caused by the basal ration.

### **3.5. Conclusions**

The addition of a Flaxlic<sup>®</sup> Sheep Tub did not improve reproductive parameters or influence testosterone concentration level. The objective of feeding the Flaxlic<sup>®</sup> Sheep Tub was to increase the availability of ALA to the testes to improve spermatogenesis. To make  $\omega$ -3 FAs as ALA available, they must make it through the rumen environment to be absorbed by the small intestine. In the present study, the Flaxlic<sup>®</sup> Sheep Tub's  $\omega$ -3 FAs were supplemented via flaxseed meal and flax oil. These substances are much more processed than the whole seed. The advantage of feeding whole flaxseed is the pericarp of the seed is harder for the rumen to break down than a rolled and roasted seed, which would break the pericarp. If the  $\omega$ -3 FAs cannot make it through the rumen environment without being hydrogenated, their positive effects may be lost altogether. Research on protecting ALA from the rumen has been positive. However,

studies reporting positive impacts to spermatogenesis use different types of oils to supplement  $\omega$ -3 FAs, which is unprotected from rumen biohydrogenation. These studies could infer the present study simply did not offer enough flaxseed in these forms to impact spermatogenesis.

Research with specific focus on controlling  $\omega$ -6/ $\omega$ -3 ratio is required to discover the most desirable ratio for the ruminant male. Due to the similar  $\omega$ -6/ $\omega$ -3 ratios between CON and FLX rations, any improvement shown was not due to the ratio. The overall ratios for the present study are 4.73:1 and 4.69:1 for CON and FLX groups, respectively. The rams were fed too much soybean and too little flaxseed in the ration to achieve a ratio goal of 1:1. However, this ratio was developed in monogastrics, and may not hold true in ruminants, especially when biohydrogenation is involved. Even so, the present study's basal ration should be re-evaluated to decrease  $\omega$ -6 FAs. Doing this may help preserve the beneficial effects of  $\omega$ -3 FA and further explain the effect of biohydrogenation on the Flaxlic<sup>®</sup> Sheep Tub's ingredients and confirm whether or not this tub's formulation is beneficial for male sheep. Although a couple positive effects were found in the present study, more parameters were affected by flax supplementation in other studies. The next step may be to compare the Flaxlic<sup>®</sup> Sheep Tub as is to one formulated using whole flaxseed rather than flaxseed meal, to confirm the effect of biohydrogenation and  $\omega$ -6/ $\omega$ -3 FA ratios on the reproductive characteristics of Rambouillet rams. Even without measuring ALA in the blood, intake of ALA alone with tub supplementation is not enough to increase  $\omega$ -3 FA in the diet alone.

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#### 4. IMPLICATIONS AND FUTURE RESEARCH

Supplementation using Flaxlic<sup>®</sup> tubs was not effective in altering reproductive parameters in either ewes or rams. In addition, the rations' feed composition may have affected intake. This is especially evident in the ram trial, which fed 75% of the basal ration as soybeans, either in meal (15%) or hull form (60%). Soybeans are high in omega-6 ( $\omega$ -6) fatty acids (FA), adding 0.71 g of  $\omega$ -6 FA per 100 g of feed. The ewe trial in chapter two did not include soybean content in the basal ration. The Flaxlic<sup>®</sup> Sheep tub itself had low levels, but only supplied 2.5 g of  $\omega$ -6 FAs per 100 g of the tub. Perhaps this implies omega-3 ( $\omega$ -3) FAs were not supplemented in high enough levels to change any measured parameters, or the  $\omega$ -6 and  $\omega$ -3 FA were biohydrogenated by the rumen before they could be absorbed by the small intestine (Lashkari et al., 2015). This would make sense, as Kronberg et al. (2012) reported protecting alpha-linolenic acid (ALA) from rumen biohydrogenation leads to increased ALA in the blood and tissue.

Lin et al. (2016) suggests the ideal  $\omega$ -6 to  $\omega$ -3 FA ratio for boar reproductive improvement is 1:1. Matching  $\omega$ -6 with  $\omega$ -3 is complicated, however, as a typical ration can be as high as 30:1. Significant focus on feedstuffs added to a ration is required. Flaxseed's  $\omega$ -6/ $\omega$ -3 ratio is 1:3.56, making it a good source of  $\omega$ -3 FA. The Flaxlic<sup>®</sup> Sheep Tub's ratio is 1:2.8, potentially making the product a good way to integrate flax into the ration. However, when fed with large quantities of soybean in various forms, such as the ration in chapter three, the positive contribution of  $\omega$ -3 FAs by flax are diminished. Unfortunately, feeding soy-based products is a common practice in the sheep industry. Zanini et al. (2003) reported cockerels fed flaxseed oil in the ration with an  $\omega$ -6/ $\omega$ -3 ratio of 1:1.14 had lower fertility values than ratios of 6.62:1 and greater. However, their fertility was improved by the addition of vitamin E to the diet. This study puts into question whether an insufficient ratio of a diet can be improved by adding other

components. There also is not any known study that developed a definitive ideal ratio for ruminants. There is still much that can be researched in this area.

More research is required to completely understand the impacts of  $\omega$ -6/ $\omega$ -3 FA ratios. As the 1:1 recommended ratio was derived from monogastrics, more research on rumen degradation and ratio required for ruminants is needed. More focus on the level and type of  $\omega$ -3 FAs fed should be used to determine whether or not significant reproductive changes will occur when lower  $\omega$ -6/ $\omega$ -3 FA ratios are fed. This could be assessed by supplementing rumen-protected, processed, and unprocessed feeds rich in  $\omega$ -3 FA, such as flax, chia seed or fish oil, while also maintaining relatively low  $\omega$ -6 FA levels. The most beneficial level of  $\omega$ -3 FAs for ruminants must be found while also keeping  $\omega$ -6 to  $\omega$ -3 FA ratios relatively low.

Both the ewe and ram trials used the Flaxlic<sup>®</sup> Sheep Tub. The ewes were fed a basal ration of hay with an average  $\omega$ -6/ $\omega$ -3 ratio of 1:1.36. The rams were fed a basal TMR ration with an average  $\omega$ -6/ $\omega$ -3 FA ratio of 4.71:1. FLX and CON treatments in both trials were not different between treatments for any reproductive parameters. Ensuring a proper  $\omega$ -6/ $\omega$ -3 FA ratio has to do with the complete ration rather than specific ingredients added to the ration. For the ewe ration, the ratio was lower than 1:1, but both treatments were this way due to the high omega-3s in the basal ration. For the ram ration, the soybeans in the basal ration caused the ratio of both treatments to increase dramatically. The use of a high energy ration was necessary for these rams, as growing lambs. For the ram side, it would be interesting to repeat the trial using mature rams and a hay-based ration, similar to the ewe trial ration.

#### **4.1. Literature Cited**

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**APPENDIX. EFFECT OF OMEGA-3 FATTY ACID SUPPLEMENTATION ON MALE  
AND FEMALE REPRODUCTIVE TRAITS**

**Table A.1.** Summary of Literature Pertaining to Omega-3 Fatty Acids and Reproductive Characteristics in Females<sup>1</sup>

Role	Effect	Author <sup>2</sup>
Ovulatory Follicle Size	Increase	Ambrose et al., 2006
Conception	Increase	Ambrose et al., 2006
Ovulation Rate	Increase	Trujillo and Borughton, 1995
Serum Progesterone	Increase (No change)	Lessard et al., 2003; Petit and Twagiramungu, 2006 (Hutchinson et al., 2012)
Prostaglandin E <sub>2</sub> and/or its Receptor	Increase/Decrease	Fly and Johnson, 1990; Shahnazi et al., 2018
Pregnancy Loss	Decrease	Silvestre et al., 2011; Ambrose et al., 2006
Serum Prostaglandin F <sub>2α</sub>	Decrease	Fly and Johnson, 1990; Shahnazi et al., 2018
Total Prostaglandins	Decrease	Fly and Johnson, 1990; Shahnazi et al., 2018
Biologically Active Estrogen	Decrease	Bu and Lephart, 2005; Muti et al., 2000; Brooks et al., 2004
Embryo Implantation and Growth	Decrease	Petit et al., 2008; Shahnazi et al., 2018
Offspring Growth and Reproductive traits	No change	Leal Soares, 2010

<sup>1</sup>A summarized literature review for female reproductive traits and hormones affected by flax in any form.

<sup>2</sup>Authors referencing the given effect from flax



**Table A.2.** Summary of Literature Pertaining to Omega-3 Fatty Acids and Reproductive Characteristics in Males<sup>1</sup>

Role	Effect	Author <sup>2</sup>
Libido	Increase	Shah et al., 2016; Estienne et al., 2008
Sperm Activity (% Motile, Mass Movement)	Increase	Jafaroghli et al., 2014; Shah et al., 2016; Baiomy and Mottelib, 2009
Ejaculate Volume	Increase	Jafaroghli et al., 2014; Shah et al., 2016; Baiomy and Mottelib, 2009
Testosterone	Increase	Baiomy and Mottelib, 2009
Percent Live and Normal	Increase	Jafaroghli et al., 2014; Shah et al., 2016; Baiomy and Mottelib, 2009
Sperm Concentration	Increase	Shah et al., 2016; Baiomy and Mottelib, 2009
Percent Abnormal Sperm	Decrease	Baiomy and Mottelib, 2009
Seminiferous Tubule Volume	Decrease	Sprando et al., 2000
Scrotal Circumference	No change	Baiomy and Mottelib, 2009
Overall Testis Structure	No change	Sprando et al., 2000
Spermatogenesis	No change	Sprando et al., 2000

<sup>1</sup>A summarized literature review for male reproductive traits and hormones affected by flax in any form.

<sup>2</sup>Authors referencing the given effect from flax