



Oviductal characteristics, protein concentrations, and messenger ribonucleic acid expression in prepubertal ewe lambs, and mature ewes after natural or progestin-synchronized estrus  
by Amy Suzanne Jacobs

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Animal Science  
Montana State University  
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**Abstract:**

The objectives of this experiment were to determine if gross morphological characteristics, protein concentrations, messenger ribonucleic acid expression, and fluorescence intensity following immunostaining for sheep OSP of the ampulla and isthmus differed among pubertal ewe lambs or mature ewes after natural or progestin-synchronized estrus. Prepubertal ewe lambs ( $n = 5$ ), mature ewes that exhibited natural estrus ( $n = 4$ ), and mature ewes synchronized with progestin-impregnated sponges ( $n = 5$ ) were observed for estrus. Salpingectomies were performed aseptically via mid-ventral laparotomy under halothane anesthesia. Each oviduct was excised, trimmed of connective tissue, measured for length, and weighed. Ampullary and isthmus portions of each oviduct were flushed with 3 mL and 1.5 mL, respectively, of Delbecco's Phosphate Buffer Solution ( $\text{pH} = 7.2$ ). Small portions, approximately 4 mm in length, of the ampulla and isthmus from the ovulatory oviduct were also removed, placed in OCT, and snap frozen, immunofluorescent staining was performed on these sections. The ampullary and isthmus mucosal layers of the non-ovulatory oviduct were scraped with a sterile slide, placed in a cryotube with 1 mL of TRIzol<sup>®</sup> reagent, and flash frozen with liquid N<sub>2</sub>. Ribonucleic acid was isolated from these samples and real time reverse transcriptase-polymerase chain reaction was used to determine the absence or presence of the messenger ribonucleic acid coding for sheep oviduct specific glycoprotein. Protein content ( $\mu\text{g}$ ), or concentrations from the flushings, expressed as either  $\mu\text{g/g}$  or  $\mu\text{g/cm}$  for the ampulla and isthmus did not differ ( $P > 0.10$ ) among the groups. However, weight, length, and weight to length ratio for ampulla ( $P < 0.05$ ) and weight and weight to length ratio, but not length, for isthmus were greater ( $P < 0.05$ ) in mature ewes than in prepubertal ewe lambs. Messenger ribonucleic acid coding for the oviduct specific glycoprotein was present in the ampulla of ewes from each treatment group, but not in the isthmus. The immunostaining showed that the sheep oviduct specific protein was present in ampullary portions of the oviduct at higher intensity ( $P < 0.05$ ) than in isthmus portions. All treatment groups exhibited fluorescence. Progestin synchronization of mature ewes does not appear to affect gross oviductal characteristics, messenger ribonucleic acid expression or protein secretions. Other than weight, length, and weight to length ratio, no difference was observed between prepubertal and mature ewes.

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APPROVAL

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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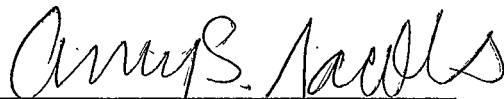
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### ABSTRACT

The objectives of this experiment were to determine if gross morphological characteristics, protein concentrations, messenger ribonucleic acid expression, and fluorescence intensity following immunostaining for sheep OSP of the ampulla and isthmus differed among pubertal ewe lambs or mature ewes after natural or progestin-synchronized estrus. Prepubertal ewe lambs ( $n = 5$ ), mature ewes that exhibited natural estrus ( $n = 4$ ), and mature ewes synchronized with progestin-impregnated sponges ( $n = 5$ ) were observed for estrus. Salpingectomies were performed aseptically via mid-ventral laparotomy under halothane anesthesia. Each oviduct was excised, trimmed of connective tissue, measured for length, and weighed. Ampullary and isthmic portions of each oviduct were flushed with 3 mL and 1.5 mL, respectively, of Delbecco's Phosphate Buffer Solution ( $\text{pH} = 7.2$ ). Small portions, approximately 4 mm in length, of the ampulla and isthmus from the ovulatory oviduct were also removed, placed in OCT, and snap frozen. Immunofluorescent staining was performed on these sections. The ampullary and isthmic mucosal layers of the non-ovulatory oviduct were scraped with a sterile slide, placed in a cryotube with 1 ml of TRIzol<sup>®</sup> reagent, and flash frozen with liquid  $\text{N}_2$ . Ribonucleic acid was isolated from these samples and real time reverse transcriptase-polymerase chain reaction was used to determine the absence or presence of the messenger ribonucleic acid coding for sheep oviduct specific glycoprotein. Protein content ( $\mu\text{g}$ ), or concentrations from the flushings, expressed as either  $\mu\text{g/g}$  or  $\mu\text{g/cm}$  for the ampulla and isthmus did not differ ( $P > 0.10$ ) among the groups. However, weight, length, and weight to length ratio for ampulla ( $P < 0.05$ ) and weight and weight to length ratio, but not length, for isthmus were greater ( $P < 0.05$ ) in mature ewes than in prepubertal ewe lambs. Messenger ribonucleic acid coding for the oviduct specific glycoprotein was present in the ampulla of ewes from each treatment group, but not in the isthmus. The immunostaining showed that the sheep oviduct specific protein was present in ampullary portions of the oviduct at higher intensity ( $P < 0.05$ ) than in isthmic portions. All treatment groups exhibited fluorescence. Progestin synchronization of mature ewes does not appear to affect gross oviductal characteristics, messenger ribonucleic acid expression or protein secretions. Other than weight, length, and weight to length ratio, no difference was observed between prepubertal and mature ewes.

## INTRODUCTION

The ability of female ruminants to reproduce efficiently is of great importance to producers. The sooner an animal begins its reproductive life, the sooner the producer begins to realize a profit from that animal. One of the major problems concerning reproductive efficiency in young female ruminants is reduced fertility associated with puberty. Studies have been conducted that indicate the problem is associated with early embryonic growth and development. There is limited information available explaining why the early embryonic growth and development differs between young and mature female ruminants. Similarly, failure of mature female ruminants to become pregnant following progestin treatments that synchronize estrus can also result in lower reproductive efficiency and higher reproductive costs.

The oviduct plays an important role in the establishment of pregnancy. It is the site of gamete transport, sperm capacitation, fertilization, and early embryonic development (Harper, 1994). Before these events occur the oviduct must undergo specific physiological and biochemical changes. The oviduct secretes many molecules which play a role in providing a proper environment to sustain oviductal-gamete and oviductal-embryo interactions. Oviduct specific glycoproteins (OSP) with high molecular weights have been identified and characterized in many mammalian species including swine, bovine, and ovine (Oliphant and Ross, 1982; Buhi et al., 2000). These proteins are secreted at a time when sperm capacitation, fertilization, and early embryonic growth occurs; they bind to zona pellucidae and sperm; are found in the perivitelline space; are associated with plasma membranes of

blastomeres; enhance *in vitro* embryonic development; and may provide protection from proteolytic enzymes during oviductal transport (Buhi et al., 2000). Therefore, oviductal proteins appear to play essential roles in processes regulating early embryonic development and survival. It is possible that the reduced fertility observed in prepubertal and progestin synchronized animals is due to changes in the types and amounts of these oviductal proteins. In this project the differences in gross morphology and protein secretions among prepubertal, and mature ewes following a progestin synchronized or natural estrus were investigated.

The following is a review of literature that describes of various aspects of puberty; the processes and factors involved with fertility at puberty, and progestin-synchronized estrus; and the role of the oviduct in these processes of the female ovine. Other species are included where data are not available for the ovine species.

## REVIEW OF LITERATURE

Importance of Puberty

The age at which an animal reaches puberty is important because it is when the animal begins its reproductive life. This time is even more important in seasonal breeding animals, like sheep, when failure to reach puberty can delay breeding for a year (Ferrell, 1991). This beginning of reproductive life is of great importance to livestock producers. The sooner these animals begin to reproduce, the faster they begin to produce revenue for the owner. Increasing the reproductive rate in animals with low numbers of offspring per year can potentially greatly increase production rate (Bradford et al., 1991). Failure of young female ruminants to become pregnant early in their lifetime can reduce the reproductive efficiency of production systems (Short et al., 1994).

Producers want ewes to reach puberty as soon as possible, so they can begin breeding and producing offspring and money. The total weight of lamb produced per breeding ewe is the most important economic product of a commercial sheep enterprise (Ercanbrack and Knight, 1998). Even if ewe lambs are not bred during their first year, they still have a higher production potential if they reach puberty during their first year (Hulet et al., 1969). Increases in flock production could be accomplished by only selecting early maturing ewes for replacements and breeding these ewes to have lambs by one year of age (Southam et al., 1971). It has also been shown that ewes bred first as lambs produce more lambs or more kilograms of lamb

during their lifetime then ewes bred at eighteen months of age (Bowstead, 1930). Simply stated, the efficiency of lamb production can be increased by breeding ewe lambs (Southam et al., 1971).

### Puberty

Puberty is an important transitional time in an animal's life. It is the time when the animal undergoes various physical and endocrinological changes to move from a sexually immature state to a state where reproduction is possible. Scientists have used many different definitions to describe puberty. It has been defined as the first estrus accompanied by ovulation (Drymundsson, 1983; Lewis, 1990), and the time "when mature gametes are first produced and reproductive activity is initiated" (Foster and Nagatani, 1999). Puberty has also been described as "a sequential process of events, each dependent upon previous circumstances, leading to sexual maturity"(Lewis, 1990).

There are differing opinions on what exactly prompts the animal to begin the pubertal transition. The physiological requirement for puberty in mammals is the development of specific hypothalamic neurons, which release gonadotropin-releasing hormone (GnRH) in appropriate quantities and frequencies (Senger, 1997). Thus, the major factor that limits the onset of puberty in the female is the failure of the hypothalamus, due to ovarian inhibition, to produce adequate amounts of GnRH to cause gonadotropin release.

It is known that various internal and external signals act on the brain to increase secretion of gonadotropins, which then act on the gonads to begin increasing production of sex steroids. The hypothalamus plays an important role in the onset of puberty in mammals. The female hypothalamus contains a tonic GnRH center and a preovulatory GnRH center, or surge center. Both centers must be functioning before the female can reach puberty. The surge center releases bursts of GnRH. These bursts of GnRH induce the release of luteinizing hormone (LH) from the anterior pituitary. In the prepubertal animal the LH pulses are not strong enough to cause any effect in the reproductive system due to the inhibitory feedback of estrogen. During the pubertal transition there is a decrease in the negative feedback of estradiol on GnRH secretion. It is possible that this decline in estradiol negative feedback could be due to a decrease in the number of estradiol receptors in the hypothalamus, although, the underlying reason for the decline in negative feedback is not completely understood (Kinder et al., 1995). This decrease in estradiol feedback allows for increases in the pulse frequency secretions of GnRH from the "tonic" release center in the median basal hypothalamus which in turn acts upon the luteotropic cells of the anterior pituitary to secrete high-frequency LH pulses resulting in a sustained increase in estrogen, which induces the gonadotropin surge, ovulation, and later corpus luteum formation (Lewis, 1990). The increased frequency of LH pulses enhances the development of ovarian follicles and indicates that the animal is now beginning the pubertal transition (Kinder et al., 1995).

It has been documented that all the necessary positive and negative feedback mechanisms needed for proper reproductive function are in place in the prepubertal ewe by at least the end of the first month of age, and that these are not limiting factors in the attainment of puberty (Foster and Karsch, 1975; Foster, 1984). The following section describes the role of various factors known to impact this process.

### Factors Affecting Puberty

#### Photoperiod

Many factors can influence the time at which a female reaches puberty, including, breed, season, photoperiod, body weight, nutrition, heterosis, and physical environment (Hafez, 1953; Hafez and Hafez, 2000). Photoperiod is a factor that greatly influences reproductive activity in ewes. Short photoperiods encourage reproductive function, while long photoperiods inhibit reproductive function (Legan and Karsch, 1980). Lambs born in the spring reach puberty in the fall, assuming they receive adequate nutrition. Lambs born in the fall do not reach puberty until ten to twelve months following birth.

Photoperiod has an endocrinological effect on the reproductive system and the subsequent attainment of puberty. Light stimulates the retina of the eye, and nerves transfer this impulse to an area of the hypothalamus called the suprachiasmatic nucleus. This impulse then travels to another area called the superior cervical ganglion of the sympathetic nervous system where inhibitory neurons make contact with cells in the pineal gland called pinealocytes. These cells secrete melatonin.

During the daytime, light activates this pathway, causing the inhibitory neurons to fire, and consequently inhibiting melatonin secretion. During the dark, the inhibitory neurons are not excited and melatonin is synthesized and secreted. Melatonin is important because it stimulates GnRH secretion and promotes cyclicity (Senger, 1997).

It has been hypothesized that changes in day length do not actually induce breeding activity, but just fine tune the endogenous rhythm already present in the animal. This concept was demonstrated by keeping animals in rooms with constant light or blinding them, so that changes in day length could not be detected. All the animals continued to show cyclicity, although this cyclicity became erratic in subsequent years (Lindsay, 1991).

### Social Cues

There are other external factors, in addition to photoperiod, which affect age of puberty in sheep. One of these factors is the social environment in which the ewes are reared. Research has been conducted which shows that the attainment of puberty in ewes has been enhanced by the presence of rams. Pheromones from the ram stimulate sensory neurons in the olfactory system and enhance the onset of puberty. The introduction of rams to ewes that have previously been isolated induces a rapid increase in LH pulse frequency. This is then followed by an LH surge, thus advancing puberty (Ungerfeld and Rubianes, 1999). The effectiveness of the presence of males in enhancing puberty in females is dependant on the animal's reproductive response to changing day lengths, or photoreceptiveness. The more an

animal is photoreceptive, the smaller the effect the presence of a male will have on advancing puberty (Lindsay, 1991).

### Nutritional State

Nutritional state also plays a major role in the attainment of puberty (Foster and Nagatani, 1999). In times when insufficient quantities of food are available, puberty can be delayed for months or even years. Kennedy and Mitra (1963) hypothesized that puberty is closely related to a change in somatic metabolism, and that the timing of puberty is based on energetics. It is known that inadequate nutrition retards growth and delays sexual maturation, and that high nutrition and rapid growth advance maturation. It has been postulated that timing of puberty is associated more closely with size rather than age. In sheep, it has also been noted that changes in pulse frequency of LH secretion is associated with body size more than age (Foster and Nagatani, 1999). When feed allowances were increased to attain a body weight of 45 kg by the middle of the breeding season, the incidence of puberty was increased from 12 to 88% (Southam et al., 1971). Increasing nutrient supply before breeding increases ovulation rate, conception rate, or numbers of offspring born (Ferrell, 1991).

Lambs provided with inadequate nutrition have a reduced GnRH release frequency as compared to lambs fed *ad libitum*. The GnRH pulse frequencies can then be changed quickly by increasing the level of nutrition. Although experiments have shown the effects of nutrition on puberty, the metabolic signals which link growth and reproduction are yet to be determined. Steiner et al. (1983) hypothesized

that glucose, insulin, or amino acids could provide information about increasing body size during development to increase GnRH secretion. Foster and Nagatani (1999) investigated the 16-kDa protein leptin and its role in affecting GnRH secretion. They found that leptin secretions as well as LH pulse frequency are suppressed during a forty-eight hour fast in rats. The fasting-induced decline in LH pulse frequency can be prevented by peripheral administration of leptin. They concluded that leptin might time puberty through triggering insulin-like growth factor I (IGF-I) secretion and increasing glucose availability. Insulin-like growth factor I is a factor that mediates the physiological effects of growth hormone and may be a signal for the timing of puberty (Foster and Nagatani, 1999). The ultimate reasons for nutritional, and eventually body size, effects on puberty are not completely known or understood, although these effects have been documented.

### Genetics

The attainment of puberty in ewe lambs is also influenced by many genetic and environmental factors and the interactions between these factors (Papachristoforou et al., 2000). Although season does affect puberty, the magnitude of this effect is under genetic control. This means that due to genetic differences various breeds may respond differently to photoperiod changes (Land, 1978). There are documented breed differences present for age at puberty (Dyrmundsson, 1981), as well as intrabreed genetic variation for age at puberty, although less is known about the latter type of variation (Bradford et al., 1991). Belibasaki and Kouimtzis (2000) found that rams from prolific Greek tropic breeds (e.g., Friesland and Chios) reached

puberty sooner than rams from less prolific Greek tropic breeds (e.g., Karagouniki and Serres). Prolific breeds reached puberty when their weight was approximately 50 and 55% of their adult weight, while less prolific breeds reached puberty when they had attained approximately 65 and 67% of their mature body weight.

Heterosis also influences age at puberty in sheep. Hohenboken and Cochran (1976) found that crossbred lambs have better reproductive performance than do purebred lambs. It is thought that heterosis contributes to earlier sexual development (Dickerson and Laster, 1975) and systematic crossbreeding programs have been employed to utilize this heterosis. Southam et al. (1971) found that Finnish Landrace x Rambouillet ewes attained puberty almost three weeks earlier than Rambouillet, Targhee, Columbia, and Dorset x Targhee ewe lambs. The Finnish Landrace x Rambouillet ewes also had the highest pregnancy rates as lambs in comparison to various other breeds. The levels of heterosis for reproduction components are relatively low and are estimated to be in the range of three to six percent (Bradford et al., 1991). Eventually crossbreeding and the use of prolific breeds will be thoroughly exploited, then the best option for improving reproduction will be to select for genetically superior animals (Ercanbrack and Knight, 1998).

#### Fertility at Puberty

Studies have shown that fertility associated with breeding at puberty is significantly lower than that of breeding at a later estrus in cattle (Byerley et al., 1987) and sheep (Hare and Bryant, 1985). There are many different hypotheses for

these observations. Various studies have focused on differences between oocytes from ewe lambs and adult ewes. It has been shown that the developmental competence of *in vitro*-matured oocytes from prepubertal animals is lower than that of adult oocytes. Lamb oocytes were observed to contain an increase in parthenogenetic activation and polyspermic penetration as compared with adult oocytes (Ledda et al., 2001).

O'Brien et al. (2000) compared oocytes from prepubertal and adult pigs in terms of their capacity to undergo *in vitro* maturation and fertilization, their glycolytic and oxidative metabolism following *in vitro* and *in vivo* maturation, and their ultrastructure. The study found that a high proportion of oocytes from piglets degenerated during *in vitro* maturation as compared to the rates from gilts and adult pigs, and that the proportion of oocytes from adult pigs undergoing maturation was significantly higher than those obtained from piglets and gilts. They concluded that oocytes from prepubertal pigs have different *in vitro* development capabilities, but appear to be similar to the metabolic and morphological characteristics of oocytes from adult pigs.

O'Brien et al. (1997) studied the developmental capacity and survival of oocytes from unstimulated prepubertal and normally cycling sheep matured and fertilized *in vitro*. They found that the portion of cleaved zygotes reaching blastocyst stage was significantly lower for oocytes from prepubertal sheep than those from adult sheep (15.4% and 34.1%, respectively). However, of the embryos that did reach blastocyst stage, both had equal capacity to develop completely. This demonstrates

that there is something fundamentally different in the development of oocytes up to the blastocyst stage between prepubertal and adult sheep. Furthermore, it has been demonstrated that treatment of prepubertal oocyte donors with steroid and gonadotropin hormones may improve oocyte cytoplasmic maturation because of an increase in the proportion of oocytes reaching blastocyst stage in culture (O'Brien et al. 1997). This difference may be related to the gonadotropin and steroid milieu associated with the developing ovary because treating prepubertal oocyte donors with steroids and gonadotropins increased the proportion of oocytes reaching the blastocyst stage.

Ledda et al. (2001) reported that prepubertal sheep oocytes have significant biological differences as compared with adult oocytes. Specifically, they analyzed the functional status of the cumulus-oocyte complex, protein synthesis during *in vitro* maturation, and maturation-promoting factor (MPF) fluctuations throughout meiotic progression between oocytes obtained from prepubertal and adult sheep. MPF is the universal cell cycle regulator of mitosis and meiosis. The study showed that prepubertal sheep oocytes had reduced synthetic activity, and lower MPF activity during M2 stage as compared to adult oocytes. This reduced synthetic activity may be due to decrease in coupling between cumulus cells and oocytes, which would also lead to reduced intercellular communication. This could affect biosynthetic activity. Any one of these deficiencies could lead to the reduction in developmental capacity observed in sheep prepubertal oocytes.

Another hypothesis is that an immature oviduct, and thus an improper environment for sperm transport, capacitation, and embryonic development, is responsible for the low developmental competence of embryos from prepubertal animals. A study was conducted to compare the oviduct secretions of calves to adult cows. The study discovered that there were no consistent age-dependant or estrous cycle-dependant differences among molecules (bovine estrus-associated glycoprotein, epidermal growth factor (EGF), transforming growth factor, EGF receptor, inhibin  $\beta_A$ , and inhibin  $\beta_B$  subunits) between prepubertal and adult cows (Modina et al., 1997). This data does not support the initial hypothesis that an immature oviduct is responsible for the low developmental competence of prepubertal animals.

Ewe lambs frequently experience their first estrus later in the season than do adults, thus they must be frequently mated at the pubertal estrus or shortly after. Hare and Bryant (1985) investigated the ovulation rate and embryo survival of ewe lambs mated at either the first, second, or third estrus. The fertility of ewe lambs increased by approximately 20% when mating occurred at the second rather than at the first estrus of the breeding season, although, little improvement occurred from mating at the third estrus.

McMillan and McDonald (1985) investigated the survival of ewe-lamb ova and adult-ewe ova in ewe-lamb recipients after natural estrus. They found that the ability of 8 to 16 cell Romney ewe-lamb embryos to develop to term was less than that of embryos from adult ewes (25% vs 52%, respectively), and that the *in vitro* developmental capacity of cleaved prepubertal lamb embryos was lower than those

from adult ewe embryos. This higher incidence of ruptured and indented zona pellucidea in ewe lamb embryos suggests further abnormality in prepubertal ewes. They eventually concluded that naturally ovulated ewe lamb embryos were less viable than those of adult ewes. However, they could not identify specific underlying cause(s) for this observation.

### Estrous Synchronization

One way to increase fertility rates in ewe lambs is to induce estrus. Stellflug et al. (2001) found that induction of estrus increased pregnancy rates in ewe lambs, and recommended that estrous induction be used in production systems where increased reproductive performance in ewe lambs is desired. There are many different ways to synchronize estrus in domestic female ruminants.

### Progestagen Treatments

One commonly used method is treatment with progestagen. However, the use of progestin-containing intravaginal sponges to synchronize estrus has resulted in lower conception rates as well as lower lambing rates in ewes bred at the synchronized estrus (Curl et al., 1966; Dewesse et al., 1970). Many producers use long-term progestagen treatments to synchronize estrus. A large number of animals exhibit estrus following these treatments, but the fertility rate is lower than following a natural estrus. It is thought that this decreased fertility rate is due to changes in the hormonal environment that results in asynchrony between estrus and ovulation as

well as alternations in sperm transport (Pearce and Robinson, 1985; Scaramuzzi et al., 1988).

Follicular development occurs in a wave-like pattern in ewes and is regulated by progesterone and gonadotropins. Supraluteal concentrations of progesterone decreases the growth of the dominant follicle and promote follicular turnover, while subluteal progesterone concentrations lengthen the life of the largest follicle and delay the appearance of the next wave. It is possible that during long term progestagen treatment a supraluteal effect is observed at the beginning of the treatment while a subluteal effect is present at the end. This would cause an aged dominant follicle to be present at the end of the treatment, which would ovulate and could be responsible for the reduced fertility. This has been documented in cattle, but the relationship between follicular lifespan and fertility has not been documented well in ewes (Vinoles et al. 2001).

MAP Pessaries. Vaginal pessaries impregnated with medroxyprogesterone acetate (MAP; 60mg) are commonly used to synchronize estrus. The devices are used for approximately twelve days, then removed. Vinoles et al. (2001) investigated the effects of length of progestagen treatment (12 d vs 6 d) on follicular dynamics, estrous synchronization, and pregnancy rate using MAP, with or without an equine chorionic gonadotropin (eCG) after MAP withdrawal. A lower pregnancy rate was observed after long-term progestagen treatment which was probably due to a slower follicle turnover that promoted the ovulation of old dominant follicles. Conversely, short term progestagen treatment resulted in higher pregnancy rates most likely due to

ovulation of newly recruited growing follicles. It was also observed that treatment with eCG had no advantage when used with long-term MAP treatment, and actually had a negative effect when used in combination with short-term MAP progestagen treatment.

Simonetti et al. (2000) conducted a study that investigated the effects of treatment with intravaginal sponges with varying amounts of MAP (60, 50, or 40 mg) on estrus, interval to estrus, and pregnancy rate. They postulated that since the amount of MAP absorbed is lower than the typical dose of 60 mg, smaller amounts could be used to gain the same results. In a previous study, Greyling et al. (1997) found that fertility following treatment with halved pessaries was higher than that with whole pessaries, although, in this study pregnancies following insemination, estrus incidence, and interval to estrus were similar among all three doses. They suggested that ewes could be treated with a 40 mg dose of MAP in estrus synchronization and artificial insemination programs.

Ungerfeld and Rubianes (1999) investigated the ram effect in combination with MAP treatments to shorten and synchronize the interval to estrus. The ram effect has been used to advance puberty, shorten lactational anestrus, and induce out-of-season estrus (Oldham and Gray, 1984). They found that the interval to estrus was shortened when the ram effect was used in conjunction with traditional MAP treatments. This was because it stimulated follicular growth and estrogen secretion by increasing the LH pulse frequency.

Even though MAP pessary treatments have been tested and proven to work, they are still associated with reduced fertility. It is possible that these treatments somehow alter the secretions of the oviduct, thus affecting fertility.

Other Progestagen Treatments. Sanchez et al. (1993) hypothesized that conception rates in cows would be increased if a corpus luteum (CL) was present during progestin treatment to synchronize estrus. They used norgestomet, a synthetic progestin, to synchronize estrus. This treatment has resulted in reduced pregnancy rates as in other progestin treatments. These decreased pregnancy rates after norgestomet treatment may be due to improper temporal relationship among estrus, the preovulatory LH surge, and ovulation, as well as, the delayed selection and/or maturation of the ovulatory follicle, or aberrant uterine function. They found that a larger percentage of females became pregnant after artificial insemination (AI) in a group that had norgestomet treatment with a CL present than in the group which had norgestomet treatment without a CL present. The difference may be due to greater concentrations of estrogen ( $E_2$ ) over a prolonged period of time in the group without a CL present. Pregnancy appears to be compromised in cows when  $E_2$  concentrations are increased for periods of time longer than those occurring during the normal estrus cycle. They concluded that this altered endocrine milieu might compromise reproduction by having a detrimental influence on the oocyte, gamete transport, or early embryonic development (Sanchez et al., 1993).

Another device used to synchronize estrus is the Controlled Internal Drug Releasing (CIDR) device. This is an intravaginal pessary which contains

progesterone. The device is usually used for 12 to 14 d, and after removal most animals show estrus within seventy-two hours. Van Cleeff et al. (1998) investigated the use of CIDR devices to synchronize estrus. They found that the application of two CIDR devices simultaneously for eight days beginning in the luteal phase resulted in a CIDR removal to surge interval of similar length when compared to a natural estrus cycle, and effectively synchronized and concentrated the timing of gonadotropin surges. This synchronization is probably from alteration of the ovarian follicle wave patterns (MacMillan and Peterson, 1993). Even though CIDRs can effectively synchronize estrus, they still result in reduced fertility. Van Cleeff et al. (1989) found that pregnancy rates were reduced in dairy heifers when a CIDR was inserted one to two days following insemination. This reduction in fertility may result from abnormal sperm transport or abnormal early embryonic development (MacMillan and Peterson, 1993). Although progestin treatments are effective in synchronizing estrus the resulting fertility is usually decreased. Since the main goal of producers is not just to synchronize estrus, but also to increase pregnancy rates, the use of these progestagen devices is not beneficial.

### Oviduct

#### Morphology

The oviduct is a muscular tube, consisting of three different sections, which conveys the ovulated oocyte to the uterine cavity, and is suspended by the mesosalpinx. Each of these three sections plays a valuable role in gamete transport

and fertilization. The infundibulum is the distal end of the oviduct that surrounds the ovary around the time of ovulation, and captures the newly ovulated oocyte. The infundibulum is covered with many finger-like projections called fimbriae. These projections increase the surface area of the infundibulum allowing it to cover the surface of the ovary. This aids in the capture of the newly ovulated oocyte. The infundibulum introduces the ova to macromolecular secretions and transports it towards the ampulla (Buhi et al., 2000). The infundibulum is continuous with the ampulla. The ampulla has a large diameter, and occupies one-half or more of the length of the oviduct. The ampulla is a site of major biosynthetic activity, macromolecular synthesis, and secretion and fluid production. The internal portions of the ampulla contain many mucosal folds with ciliated epithelium. This ciliated epithelium aids in the transport of oocytes and spermatozoa promoting fertilization (Senger, 1997). Immediately proximal to the ampulla is a site known as the ampullary-isthmic junction. This is the portion of the oviduct where fertilization is thought to occur. The next segment of the oviduct is called the isthmus. It has a smaller diameter than the ampulla, but with a thicker muscular wall, and fewer mucosal folds. The isthmus regulates sperm transport to the ampulla, passage of embryos to the uterus, and may also function as a sperm reservoir (Buhi et al., 2000). The isthmus connects directly to the uterus at a site known as the uterotubal junction.

The oviduct wall consists of three distinct tissue layers. The muscularis is the outermost layer, consisting of inner circular and outer longitudinal smooth muscle, and is responsible for transporting oocytes and spermatozoa to the site of fertilization.

The next layer is referred to as the submucosa. It contains the nerve supply, lymphatics, and vasculature of the oviduct. The innermost layer of tissue is called the mucosal layer (Senger, 1997). This layer contains simple columnar cells, many of which also have cilia. The ciliated cells are especially numerous in the infundibulum and ampulla. The cilia always beat in the direction of the uterus, facilitating the transport of the newly ovulated oocyte. There are also nonciliated, or secretory cells, present in this layer. These cells produce the secretions which sustain the spermatozoa until oocyte arrives, and provides the optimal oocyte environment. Ciliated and secretory cells are both profuse in the infundibulum and ampulla, although the ampulla appears to contain a greater number of secretory cells. The activity of secretory cells and the presence and length of cilia are all under hormonal control. During the follicular phase of the estrous cycle the number of ciliated cells is the largest, while during the luteal phase the ciliated cells decrease in number and the secretory cells become inactive (Kessel and Kardon, 1979). In addition to providing the proper mechanisms for gamete transport and fertilization to occur, the oviduct also contains many hormones (e.g. Estrogen, LH, Progesterone, Prostaglandin) and secretions (e.g. albumin, transferrin, immunoglobulins, cytokines, enzymes, oviduct specific glycoprotein, protease inhibitors, growth factors) which provide the proper endocrinological environment for these processes to occur (Senger, 1997).

## Hormonal Environment

Proteins and Peptides. There are many different hormones present in the oviducts at various times during the estrous cycle. One of these hormones is called luteinizing hormone (LH). It causes ovulation and transformation of the ovarian follicle to a corpus luteum (CL) and also plays a role in regulating gonadal physiology. Before ovulation a surge of LH occurs, inducing ovulation and luteinization of granulosa and theca cells. Luteinizing hormone also plays an important role in controlling oviductal motility and in the opening of the ampullary isthmic junction for spermatozoa. Gawronska et al. (1999) found that *in vitro* treatment with LH during the peri-ovulatory stage of the estrous cycle decreased the amplitude, frequency, and area under the curve (AUC) of the contractions of the isthmus, as well as the frequency and AUC of ampullary contractions. This relaxative action may also facilitate passage of embryos through the isthmus towards the uterus. Therefore, LH may be a major factor in the synchronization of events leading to fertilization of ova in the ampulla (Gawronska et al., 2000).

Steroids. Another hormone with effects on oviductal environment is progesterone ( $P_4$ ). It influences several physiological events in the oviduct including ciliogenesis, discharge of mucin into the lumen, and the rate of secretion and composition of oviductal fluid. An experiment by Stone and Miller (1978) found that in giving ewes varying combinations of estrogen and progesterone treatments the progesterone had almost no effect on the oviduct. Estrogen treatment resulted in

large increases in the rate of protein synthesis and cell content of RNA. Therefore they concluded that progesterone does not stimulate protein or RNA synthesis in the isthmus and has little effect on transcription or translation of principle cell types in the isthmus. When progesterone was administered to ovariectomized rats in addition to estrogen, uterine growth was inhibited. Typically, rats receiving only estrogen exhibited rapid uterine growth. This led to the conclusion that progesterone exhibits antiestrogenic activity (Clark et al., 1977). Progesterone also diminishes estrogen ( $E_2$ ) and  $P_4$  receptor levels in the uterine endometrium as well as the isthmic oviduct, but does not decrease protein:DNA and RNA:DNA ratios or protein synthesis (Stone et al., 1979). An experiment by Murray (1992) found that treatment with progesterone also decreases the synthesis and secretion of oviduct specific glycoproteins in ovariectomized sheep. Ovariectomized ewes were treated with estrogen for six days and progesterone for either two, four, or six days in the continued presence of estrogen. Oviductal flushings from these animals were analyzed on 10% SDS gels and the presence of oviductal specific proteins were not detected. In a similar experiment, it was discovered that treatment with progesterone also decreases the synthesis and secretion of oviduct specific glycoproteins in the rhesus monkey (Verhage et al., 1997).

Estrogen is another hormone with extensive effects in the female. Estrogen causes growth of isthmus oviduct and an increase in  $E_2$  receptor concentrations (Stone and Miller, 1978). Estrogens bind to intracellular receptor proteins, which regulate the expression of responsive genes within cells of target tissue. Brief physiological

estradiol treatment evoked obvious changes in gross uterine characteristics. Estradiol increased endometrial weight by 46%, and also up-regulated steady state concentrations of estrogen receptor (ER) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Mammals use this mRNA stabilization to up-regulate ER gene expression in the endometrium, which prepares it for production of steroid dependant secretions, which are critical to the survival of embryos in the early days of pregnancy (Ing and Ott, 1999).

When fertilization occurs the hormonal environment of the reproductive tract changes. On approximately day three of pregnancy, following fertilization, the fertilized egg leaves the oviduct and enters the uterus, coinciding with a rise in  $P_4$  and a decline in  $E_2$ . Estrogen causes synthesis and release of the oviduct specific glycoprotein (OSP) from the ampulla, but not the isthmus. After day three the embryo enters the uterus and the oviduct has completed its role in the productive process (Murray, 1993).

Prostaglandins. Prostaglandins are modified fatty acids, and some of the most physiologically active compounds in the body (Anderson et al., 2001). They stimulate smooth muscle contractions in the uterus and are involved in inflammatory responses. The most important prostaglandin's involved with reproduction are prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) and prostaglandin  $E_2$ . Prostaglandin  $E_2$  has been found to stimulate progesterone synthesis in the corpora lutea.  $PGF_{2\alpha}$  causes luteolysis, or destruction of the corpus luteum, but only if the animal is not pregnant (Senger, 1997). The function of  $PGF_{2\alpha}$  is primarily mediated by its plasma membrane

receptor. A knockout of this receptor in mice has shown that normal luteal regression does not occur without this receptor. Luteolysis occurs following activation of this receptor by  $\text{PGF}_{2\alpha}$  in a corpus luteum (Anderson et al., 2001).

Growth Factors. There is increasing evidence that oviductal fluid also contains numerous growth factors. Epidermal growth factor, transforming growth factor, and insulin-like growth factor were all found in oviductal fluid by radioimmunoassay from mated gilts. These factors may act in an autocrine or paracrine mode and regulate oviductal function or early embryonic growth and development, but it is hard to hypothesize which and when these factors may be important (Buhi et al., 2000).

#### Oviductal Secretions

The oviduct plays a vital role in the establishment of pregnancy. It is the site of gamete transport, sperm capacitation, fertilization, and early embryonic development. The oviduct secretes fluids which help maintain a proper environment for these processes to occur (DeSouza and Murray, 1995). There are two separate hypotheses concerning protein secretions and accumulations in the oviduct. The first is that the oviduct contains a passive source of protein, derived from serum and plasma as a transudate. The second hypothesis is that the oviduct is an active tissue that synthesizes and secretes macromolecules throughout the estrous cycle. Recent studies in swine, ovine, and bovine species have indicated that the oviduct is in fact a biologically active tissue, synthesizing and releasing proteins (Buhi et al., 2000). Oviductal fluid is the medium in which the egg is fertilized and where the first few

cell divisions take place (Sutton et al., 1984). Oviductal secretions contain many different proteins, amino acids (Walker et al., 1996), and other molecules; epidermal growth factor, transforming growth factor- $\alpha$ , and polypeptides associated with the interaction of sperm with oviductal cells (Ellington et al., 1993). Water is the largest component of oviductal fluid, while the most abundant proteins are albumin and immunoglobulin G. Oviductal fluid has a potassium concentration greater than that of plasma and a calcium ion concentration below that of plasma (Leese, 1988). Oviductal secretions also contain glucose, lactate, and pyruvate which are excellent exogenous substrates for spermatozoa (Leese, 1988). Walker et al. (1996) showed that supplementation of oviduct fluid with amino acids improved embryo development in the sheep and cow. The pH of oviductal fluid varies during the estrous cycle. During the follicular phase of the rhesus monkey it ranges from 7.1 to 7.3, and during ovulation and the luteal phase it ranges from 7.5 to 8.0. Oviductal fluid also keeps the surface epithelium of the oviduct moist, irrespective of whether there are gametes or embryos present within the lumen (Leese, 1988).

Oviductal secretions vary corresponding to the changing hormone concentrations during the estrous cycle and, if fertilized, the first few days of pregnancy. When samples of oviductal fluid were taken from ewes with indwelling catheters, the secretion volume was increased two to three fold during ovulation in response to  $E_2$ . Estrogen causes an increase in capillary blood flow to the oviduct in sheep. This increase may somehow stimulate the observed oviductal fluid production rate (Sutton et al., 1984).

The oviduct also produces a capacitating factor present in its secretions that allows spermatozoa to acquire fertility. This was determined by incubating sperm in estrual oviduct fluid from cannulated heifers and measuring the percentage of sperm that underwent capacitation. This was further verified by using the exposed sperm in an *in vitro* fertilization assay. Seventy percent of oocytes were penetrated using sperm incubated in oviduct fluid, while only one percent was penetrated in the control group. It was also determined that this factor has its highest capacitation activity during the estrual period (Parrish, 1989). Low levels of fertilization have been observed in domestic farm animals when using *in vitro* fertilization techniques, leading to the conclusion that there may be proteins in oviductal fluid which help facilitate fertilization in cattle and sheep (Sutton et al., 1984). This conclusion is supported by the observation that *in vitro* fertilization rates and *in vitro* embryonic development can be enhanced by adding oviductal epithelial cells or oviductal secretions (Murray, 1992). Due to their possible roles in fertilization and early embryonic development, it is these protein macromolecules that are of the most interest to researchers.

Oviduct Specific Glycoproteins. Some of the most important components of oviductal fluid are oviductal glycoproteins. These oviduct specific glycoproteins (OSPs) are synthesized and released from the oviduct during the estrogen dominated phase of the estrous cycle. The OSPs have been characterized in many mammalian species including pigs, sheep, and cows (Buhi et al., 2000). The primary amino acid sequence of the glycoprotein is highly conserved among mammalian species and the

protein core shares identity with chitinases (DeSouza and Murray, 1995). This conservation and appearance in a variety of mammalian species leads to the conclusion that OSPs may play an important role in the establishment of pregnancy.

Oviductal secretory mucins bind to sperm and increase their penetration through the zona pellucida, serve as a selective barrier to sperm transport, facilitate the final stage of sperm capacitation, and modulate the ascent of sperm to the fertilization site (DeSouza and Murray, 1995). Oviduct specific glycoproteins share these common features: induction by  $E_2$ , repression by  $P_4$ , localization to nonciliated epithelial cells, large molecular weight, and glycosylation. Oviductal secretions enhance *in vitro* fertilization rates and embryonic development (Murray, 1993). It is due to these qualities that we are investigating the presence of OSPs in the oviducts of prepubertal and mature ewes following natural or progestin-synchronized estrus.

Sheep OSP is a periodic acid-Schiff (PAS) positive protein with a molecular weight of 90-92,000 kDa. It is localized in secretory granules of nonciliated cells of the ampulla, and is not present in the isthmus. It is also unique to the oviduct and  $E$  induced (Murray, 1992). The oligosaccharide chains of the sheep OSP possibly contributes to its ability to associate with gametes and embryos. Oviduct specific glycoproteins from sheep, cows, and hamsters all contain a number of complex and hybrid o-linked glycan chains and play a role in sperm, egg, and embryo surface interactions. They are important because of the supportive role they play in fertilization and embryonic cleavage. They associate with sperm, oocytes, and the fertilized ovum (DeSouza and Murray, 1995). A study conducted by King and

Killian (1994) reported that bovine OSP binds to the sperm membrane and may play a role in enhancing sperm capacitation.

The sheep OSP is present in *in vivo* oviductal secretions at estrus until approximately day four of pregnancy, corresponding with the time that the embryo moves into the uterus (Murray, 1993; Sutton et al., 1984). In an experiment conducted by Murray (1993) the ipsilateral and contralateral oviducts to the CL synthesized and released similar amounts of the radiolabeled protein *in vitro*. *In vitro* fertilization rates are enhanced and significant *in vitro* embryo growth occurs in the presence of these oviductal secretions.

Oviductal proteins play an essential role in processes regulating early embryonic development and survival. The patterns of secretions from the oviduct can be altered by outside means. Oviductal proteins and mRNA's are regulated by steroids (Binelli et al., 1999). A change in the hormonal environment of the oviduct could alter the pattern of protein synthesis and contribute to the lower fertility observed in prepubertal and synchronized animals.

The regulation of synthetic activity seems to be greater in specific areas of the oviduct, namely the ampulla and infundibulum. The ampulla has been determined as the major site of biosynthetic activity and OSP expression. These areas are also the sites of fertilization and early embryonic development which suggests that the proteins secreted in these segments may play an important role in these events (Buhi et al., 2000).

## STATEMENT OF THE PROBLEM

There is a reduced fertility associated with breeding sheep at puberty, then breeding at a later date. Failure of these young female ruminants to become pregnant early in their lifetime can reduce production efficiency. Similarly, failure of mature females ruminants to become pregnant after progestin treatments that synchronize estrus can result in lower reproductive efficiency and higher reproductive costs.

The oviduct plays an important role in the early events that are required for the establishment of pregnancy. It is the site of gamete transport, sperm capacitation, fertilization, and early embryonic development. The oviduct also provides the optimum environment for these processes to occur.

The possibility that the oviduct and its secretions may contribute to lower than normal fertility rates associated with breeding at puberty or at estrus after progestin synchronization in sheep has not been addressed. The purpose of this project was to determine if oviductal protein secretions during pubertal transition of ewe lambs and ewes synchronized with progestins provide the necessary molecular support needed for early embryonic growth and development. Therefore, objectives of this experiment were: 1) to evaluate gross morphological characteristics among prepubertal ewe lambs and mature ewe exhibiting natural or progestin-synchronized estrus, 2) to evaluate protein content and concentrations from oviductal flushings in prepubertal ewe lambs and mature ewe exhibiting natural or progestin-synchronized estrus, 3) to evaluate mRNA expression of sheep oviduct specific glycoprotein in the ampulla (AMP) and isthmus (IST) of the oviduct among pubertal ewe lambs or

mature ewes after natural or progestin-synchronized estrus, and 4) to evaluate fluorescence intensity of sheep oviduct specific glycoprotein in tissue samples among prepubertal ewe lambs and mature ewe exhibiting natural or progestin-synchronized estrus.

## MATERIALS AND METHODS

Animal Care

This experiment, project number MONB000183, was conducted at the Fort Ellis Sheep Research Station, Bozeman, MT from early November to mid January (2000-2001). Rambouillet ewe lambs born mid to late April 2000 and mature Rambouillet ewes (three to six years of age) were both used in the experiment. All animals were handled and cared for according to a protocol approved by the Montana State University Institutional Animal Care and Use Committee, permit number 834. Ewes and ewe lambs were maintained in a single pasture, fed mixed-grass hay *ad libitum*, and given free access to water and a vitamin-mineral supplement.

Treatments

Mature ewes were observed for behavioral estrus twice daily with the aid of teaser rams. Ewes that had shown at least one estrous cycle of normal length (sixteen to eighteen days) were randomly assigned to receive either a progestin-impregnated vaginal pessary (n = 7), containing 60 mg of 6 $\alpha$ -methyl-17 $\alpha$ -hydroxyprogesterone acetate (Tuco Products Limited, Orangeville, Ontario, Canada) for twelve days, or no pessary (n = 6). Pessaries were removed on day twelve, and ewes were observed for estrus twice daily with the aid of teaser rams for the next five days.

Mature ewes that had not received a pessary and exhibited natural estrus (MNE; n = 4) and mature ewes that exhibited synchronized estrus following sponge

removal (MSE; n = 5) were removed from feed and water immediately after exhibiting estrus. Prepubertal ewe lambs (PP; n = 5) were also used in this experiment.

### Surgery and Data Collection

Salpingectomies were performed aseptically via mid-ventral laparotomy under halothane anesthesia twenty-four hours following estrus for MNE and MSE ewes, and eighteen hours following feed and water removal for prepubertal ewe lambs.

The reproductive tract of each ewe was exposed and the ovaries examined for the presence of corpora lutea (CL), corpora hemorrhagica (CH), corpora albicantia (CA), and large antral follicles (LAF; dia. > 10 mm). Ovaries of each MNE and MSE ewe contained at least one CA, a regressing CL, and either a CH or a LAF in the process of ovulating. Prepubertal ewe lambs had antral follicles but no visual evidence of regressed luteal structures or corpora hemorrhagica in the ovaries.

Immediately before removal of an oviduct, ligatures were placed at the utero-tubal, isthmic-ampullary, and ampullary-infundibular junctions of each oviduct to prevent migration of substances between segments of the oviduct.

Each oviduct was excised, trimmed of connective tissue, measured for length, and weighed. Ampullary (AMP) and isthmic (IST) portions of each ovulatory oviduct were flushed with 3 mL and 1.5 mL, respectively, of Delbecco's Phosphate Buffer Solution (pH = 7.2). Flushings from the AMP and IST were passed through a 0.45  $\mu\text{m}$  filter into 12 x 75 mm cryogenic tubes. Filtration of the oviductal flushings was

necessary for removal of cellular debris associated with the flushing process. Immediately following filtration each flushing was flash-frozen in liquid N<sub>2</sub>, and stored at -80°C until assayed for protein content and concentration. Small portions, approximately four millimeters in length, from the middle portion of the ampulla and isthmus from the ovulatory oviduct were also removed, placed in Tissue-Tek O.C.T.<sup>®</sup> (Sakura Finetek, Torrance, CA), snap frozen (see Appendix A), and kept for histological and immunocytochemical analyses. The AMP and IST mucosal layers of the non-ovulatory oviduct were also scraped with a sterile slide, placed in a cryotube with one milliliter of TRIzol<sup>®</sup> reagent (Life Technologies<sup>™</sup>, Gaithersburg, MD), and flash frozen with liquid N<sub>2</sub> for further molecular biological analysis.

### Laboratory experiments

#### Protein Content and Concentration

Oviductal flushings were thawed at 4°C overnight and vortexed. The quantity of fluid in each cryotube was measured volumetrically, using a Pipetman<sup>®</sup> (P 1000 and P 200). Five hundred micro liters of each sample were placed in separate tubes and kept for the assay. Protein concentrations in 100 µL of each AMP and IST flushing were assayed in duplicate for protein using BCA<sup>®</sup> assay kits (Pierce, Rockford, IL). Bovine serum albumin was used as the standard for the assay, and standard concentrations ranged from 0 to 200 µg/mL. Two hundred micro liters of each standard were used for the assay. One hundred micro liters of the samples added to 100 µl of DPBS were used for the assays. Working reagent was mixed at the time

of the assays, and 2 ml of this reagent was added to each tube. The tubes were then incubated at 62°C in a water bath for thirty minutes as described in the Pierce BCA<sup>®</sup> Protein Assay manual. Samples were removed from the water bath and placed in a cool water bath for two minutes. Samples were then placed in labeled cuvettes, and the absorbance was determined at 562 nm using a Shimadzu UV-VIS 1201 spectrophotometer. The absorbance of the standards was regressed on the protein concentration of the standards to establish a standard curve. The protein concentration of each oviductal flushing was determined from the average absorbance from the two samples. Concentrations were adjusted to one mL and the protein content of each AMP and IST sample was obtained by multiplying the flushing volume times the concentration per mL. Protein content of each AMP and IST was divided by their respective weight, length, and weight to length ratio to obtain protein concentrations per g, per cm, and per g•cm<sup>-1</sup>.

#### Ribonucleic Acid Isolation and Expression

Ribonucleic acid was isolated from the flash frozen AMP and IST mucosal layer samples using TRIzol<sup>®</sup> reagent (Life Technologies<sup>™</sup>, Gaithersburg, MD) and a protocol provided by Life Technologies<sup>™</sup>. To prevent degradation of the RNA, all reagents and pipette tips used in this protocol were RNase-free. The oviductal mucosal layer tissue samples flash frozen in 1ml TRIzol<sup>®</sup> were removed from -80°C and placed on ice for approximately two hours until samples had thawed. The samples were then homogenized using a sterile glass rod for approximately two minutes. Following homogenization the tissues had broken down and the TRIzol<sup>®</sup>

had become cloudy. Homogenized samples were then incubated at room temperature for five minutes. Following incubation, 0.2 ml of chloroform was added to the samples. The samples were then shaken by hand for fifteen seconds, and incubated again at room temperature for two to three minutes. Samples were centrifuged at 10,000 x *g* for fifteen minutes at 4°C. The samples had then separated into three distinct phases: a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The ribonucleic acid (RNA) was present in the colorless upper phase. This upper aqueous phase was then transferred to a fresh 1.5 ml PCR tube, and 0.5 ml of isopropyl alcohol was added to these samples. The samples were incubated at room temperature for ten minutes. Following incubation, the samples were centrifuged at 10,000 x *g* for ten minutes at 4°C. The RNA can sometimes be visible as a pellet on side of tube. The supernatant was removed with a pipette and the RNA pellet was washed with 1 ml of 75% ethanol and vortexed. The samples were centrifuged at 5,000 x *g* for five minutes at 4°C. The ethanol was poured off and the RNA pellet was briefly air-dried. The RNA pellet was then redissolved in RNase-free water and stored at -80°C until analyzed using reverse transcriptase-polymerase chain reaction.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) is a method used to quantify RNA expression. Real time RT-PCR is used to detect the presence of a specific RNA, reverse transcribe that RNA sequence into a complementary deoxyribonucleic acid (cDNA) sequence, and then amplify that cDNA by using primers specific for that sequence. Real time PCR is useful because it allows for the

direct detection of the PCR product. This is accomplished by measuring the increase of fluorescence at various time points from the binding of SYBR-Green to the double stranded DNA. Real time RT-PCR was used in this experiment to determine the absence or presence of the messenger ribonucleic acid (mRNA) coding for sheep OSP in each animal of the three treatment groups. The PCR primers used in this experiment were designed using the computer program Primer Express (PE Biosystems, Foster City, CA), and the sequences are given in Appendix B. Real time RT-PCR was performed on the RNA using a protocol developed from Dr. Donald Burgess (unpublished data); (Appendix C). One RNA sample was used without RT to provide a negative control for the PCR reactions. An internal control (mouse 18s ribosomal subunit) was also used to ensure the reaction was occurring properly. The PCR reactions were performed in a 5700 Real Time PCR machine (PE Biosystems). A no template control was used with the PCR reaction to ensure that there were no interactions between the reagents.

### Immunofluorescence

Cryostat sections (5um) were prepared from each four-millimeter sample taken from the AMP and IST portions of the oviduct. The sections were placed on positively charged slides, two sections per slide, and were either dried overnight at room temperature in a desiccator, or fixed in 10% formalin in Coplin jars followed by staining with hematoxylin and eosin y.

All immunofluorescent staining was performed in a humidity chamber. Following air-drying in a closed container the slides were fixed in acetone in Coplin

jars at 4°C for 10 minutes. The samples were then removed from the acetone, and air-dried for an additional 20 to 30 minutes at room temperature to remove any remaining acetone. Before the actual staining, lines were drawn on the slide above and below the slice using an immunopen, to prepare chambers for buffers and staining solutions. Sections were then rinsed, using a sterile glass pipette, in Delbecco's Phosphate Buffer Solution (DPBS) three times; two min for each rinse. Sections were not allowed to dry throughout the remainder of the procedure. Each section was blocked with a normal serum block containing DPBS with 2 % normal donkey serum and 0.2 % bovine serum albumin (BSA) for 30 min. Sections were rinsed with a buffer consisting of DPBS containing 0.2 % BSA. The primary antibody (rabbit anti-sheep OSP) in DPBS with 2 % donkey serum and 0.2 % BSA was then added to each section. Initially, a dilution panel was run to determine the optimal primary antibody concentration for the experiment. A 1:300 dilution for the primary antibody was determined to yield the best immunofluorescent result for this experiment. A negative control with a 1:300 dilution of rabbit IgG was also used instead of the primary antibody in this experiment, and the sections were incubated for 30 min at room temperature. Sections were then rinsed in DPBS with 0.2 % BSA. The secondary antibody-Fluorescein Isothiocyanate (FITC) conjugated (AffiniPure Donkey Anti-Rabbit IgG (heavy and light chains)) in DPBS with 2 % normal donkey serum and 0.2 % BSA was then added to the slides at a 1:100 dilution, and incubated at room temperature for 30 min. This reagent cross-reacted minimally with bovine and ovine serum proteins. Slides were placed in the humidified chamber which was

covered with aluminum foil to protect the slides from fluorescent light and sunlight. After the 30 min incubation in the FITC conjugated secondary antibody, sections were rinsed in neat DPBS five times; two min for each wash. A coverslip was mounted to each slide using Gel Mount™ (Biomedica Corporation, Foster City, CA) while sections were still wet with DPBS. They were laid flat to dry at room temperature, and remained in the dark until viewed with the microscope. Five separate controls were used in this experiment. They were slides treated with: only primary antibody; only secondary antibody; only rabbit IgG; only buffers; and rabbit IgG with secondary antibody. These slides were used for comparison to determine the amount of background fluorescence and auto-fluorescence present in the tissues.

#### Hematoxylin and Eosin Y Staining

Sections of frozen ampullary and isthmic tissue were mounted on slides and fixed with 10% buffered formalin at room temperature until stained with hematoxylin and counter-stained with eosin y. Slides were removed from the formalin and rinsed with three changes of distilled water, two min for each change, to remove any residual formalin. They were then placed in hematoxylin for one to one and a half min. Following immersion in hematoxylin, slides were rinsed very gently with running tap water for one min. Slides were then placed in bluing solution for one min, followed by another one-min tap water rinse. Thereafter, slides were placed in a solution of 70% alcohol for one min; immersed in eosin y for thirty sec to one min, and washed twice in 95%, then 100% alcohol (one minute per wash). The procedure was finished in a fume hood with two washes in Xylene substitute (one minute per

wash). Each slide was then cover slipped from the xylene solution using Permount (Fisher Scientific, Fair Lawn, NJ).

Some slides were also stained following acetone fixation, with never being exposed to formalin. The samples were stained using the same procedure outlined above, except they were fixed in acetone for ten minutes at 4°C instead of being placed in formalin. These sections were used to determine if fixation in formalin was too harsh for frozen oviductal sections.

#### Data Analysis

Data for AMP and IST weight, length, weight to length ratio, protein content, and protein concentrations were analyzed by ANOVA using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). The model included treatment only. Fisher's protected LSD tests were used to evaluate treatment means. Differences were considered significant at  $P < 0.05$ .

The presence or absence of the mRNA encoding for sheep OSP was determined by computer and graphical analysis. The presence or absence of OSP with the ampullary and isthmic mucosa was visually assessed by immunofluorescence microscopy. Sections were observed using a Zeiss Standard 20 epifluorescent microscope. To accomplish the evaluation, each section was divided into four microscopic fields by dividing each section by two diameters at right angles to each other. Sections were given a number, ranging from 0-3, corresponding to the intensity of the observed fluorescence. Intensity of fluorescence within each field

was then subjectively scored. A score of zero was assigned to fields that were completely black, and a score of three was assigned to fields that had intense localized fluorescence. Four scores were obtained for each of two sections from the ampulla and isthmus of each animal. These scores were then averaged and the averages were analyzed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). Model included treatment and oviduct segment. Differences were considered significant at  $P < 0.05$ .

## RESULTS

Gross Morphological Characteristics

Ampullary weight, length, and weight to length ratios were smaller ( $P < 0.05$ ) for PP ewes than those for either MNE or MSE ewes (Table 1). Whereas, ampullary weight, length, and weight to length ratios did not differ ( $P > 0.10$ ) between MNE and MSE ewes (Table 1).

Table 1. Least squares means for gross morphological characteristics of ampullae of prepubertal ewe lambs (PP), and mature ewes 24 h after natural estrus (MNE) or progestin-synchronized estrus (MSE)

Treatment	n	Weight (g)	Length (cm)	W/L ratio (g/cm)
PP	5	0.256 <sup>a</sup>	7.95 <sup>a</sup>	0.032 <sup>a</sup>
MNE	4	0.689 <sup>b</sup>	11.9 <sup>b</sup>	0.057 <sup>b</sup>
MSE	5	0.690 <sup>b</sup>	11.5 <sup>b</sup>	0.062 <sup>b</sup>
SEM		0.14	2.1	0.012

<sup>a,b</sup>Means within columns that lack a common superscript letter differ ( $P < 0.05$ ).

Isthmic weight and weight to length ratios were smaller ( $P < 0.05$ ) for PP ewes than those for either MNE or MSE ewes, however, IST length did not differ ( $P > 0.10$ ) among treatments (Table 2). Isthmic weight and weight to length ratios did not differ ( $P > 0.10$ ) between MNE and MSE ewes.

Table 2. Least squares means for gross morphological characteristics of isthmi of prepubertal ewe lambs (PP), and mature ewes 24 h after natural estrus (MNE) or progestin-synchronized estrus (MSE)

Treatment	n	Weight (g)	Length (cm)	W/L ratio (g/cm)
PP	5	0.104 <sup>a</sup>	5.12 <sup>b</sup>	0.021 <sup>a</sup>
MNE	4	0.244 <sup>b</sup>	5.95 <sup>b</sup>	0.041 <sup>b</sup>
MSE	5	0.275 <sup>b</sup>	7.00 <sup>b</sup>	0.04 <sup>b</sup>
SEM		0.05	1.25	0.006

<sup>a,b</sup>Means within columns that lack a common superscript letter differ ( $P < 0.05$ ).

#### Protein Content and Concentrations

Ampullary protein content, and concentrations expressed as either  $\mu\text{g}$  per g,  $\mu\text{g}$  per cm, or  $\mu\text{g}$  per  $\text{g}\cdot\text{cm}^{-1}$  did not differ ( $P > 0.10$ ) among PP ewe lambs, MNE and MSE ewes (Table 3).

Table 3. Least squares means for ampullary protein content and concentration per g, and per cm, and per weight to length ratio for prepubertal ewe lambs (PP), and mature ewes 24 h after natural estrus (MNE) or progestin-synchronized estrus (MSE)<sup>a</sup>

Treatment	n	Content		Concentrations	
		( $\mu\text{g}$ )	( $\mu\text{g}/\text{g}$ )	( $\mu\text{g}/\text{cm}$ )	( $\mu\text{g}/\text{g}\cdot\text{cm}^{-1}$ )
PP	5	131.2	501	16.0	4,089
MNE	4	213.0	357	19.0	3,956
MSE	5	231.8	373	22.4	4,027
SEM		77.9	212	11.0	1,926

<sup>a</sup>Means within columns did not differ ( $P > 0.10$ ).

Isthmic protein content, and concentrations expressed as either  $\mu\text{g}$  per g,  $\mu\text{g}$  per cm, or  $\mu\text{g}$  per  $\text{g}\cdot\text{cm}^{-1}$  did not differ ( $P > 0.10$ ) among PP ewe lambs, MNE and MSE ewes (Table 4).

Table 4. Least squares means for isthmic protein content and concentration per g, and per cm, and per weight to length ratio for prepubertal ewe lambs (PP), and mature ewes 24 h after natural estrus (MNE) or progestin-synchronized estrus (MSE)<sup>a</sup>

Treatment	n	Content		Concentrations	
		( $\mu\text{g}$ )	( $\mu\text{g}/\text{g}$ )	( $\mu\text{g}/\text{cm}$ )	( $\mu\text{g}/\text{g}\cdot\text{cm}^{-1}$ )
PP	5	59.0	621	11.3	3,288
MNE	4	107.7	502	19.7	2,731
MSE	5	118.7	475	18.3	3,103
	SEM	40.7	260	7.9	1,408

<sup>a</sup>Means within columns did not differ ( $P > 0.10$ ).

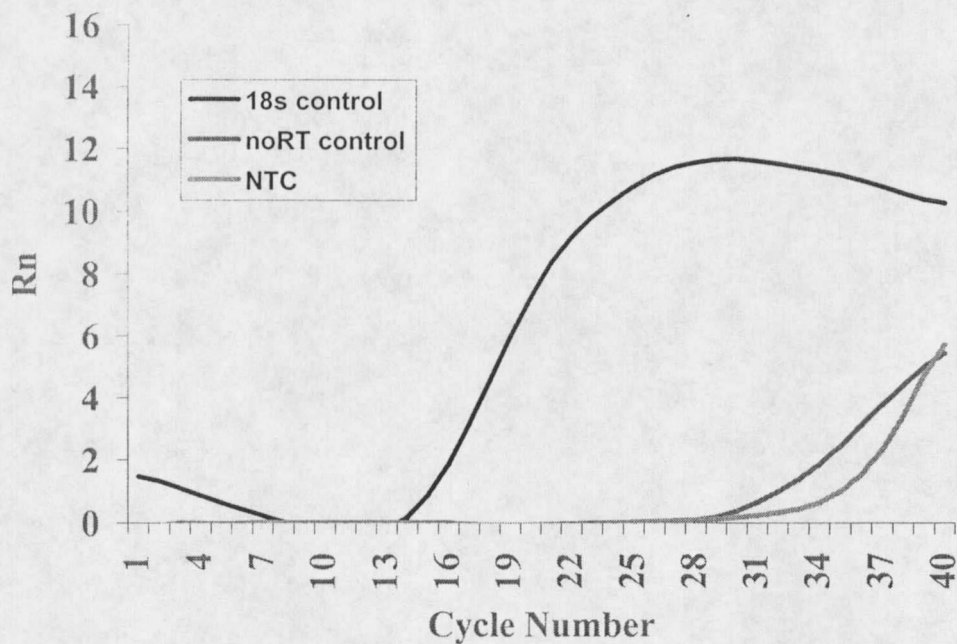
#### Messenger Ribonucleic Acid Expression

The RT-PCR results showed that mRNA coding for the sheep OSP was present in the ampulla of ewes from each treatment. No isthmic portions of the oviduct appeared to contain any mRNA coding for the sheep OSP which the primers could bind to and amplify.

Three separate controls were used to validate the use of RT-PCR to determine mRNA expression in oviductal segments. They were two were negative controls and one positive control. RNA from a mouse 18s ribosome subunit was used as a known positive control to make sure the PCR reaction proceeded properly. A no reverse transcriptase (RT) control was used to determine if there was any DNA contamination

or non-specific binding between primers and mRNA in the samples. A no template control (NTC) was also used to determine if the reagents had any nonspecific binding and interactions between each other. Figure 1 shows amplification plots for each control. The 18s control was the only one in which the cDNA was amplified and the reaction proceeded properly, which is consistent with it being a positive control. The other two controls did not have DNA amplification, which was to be expected.

Figure 1. Amplification plot of controls.

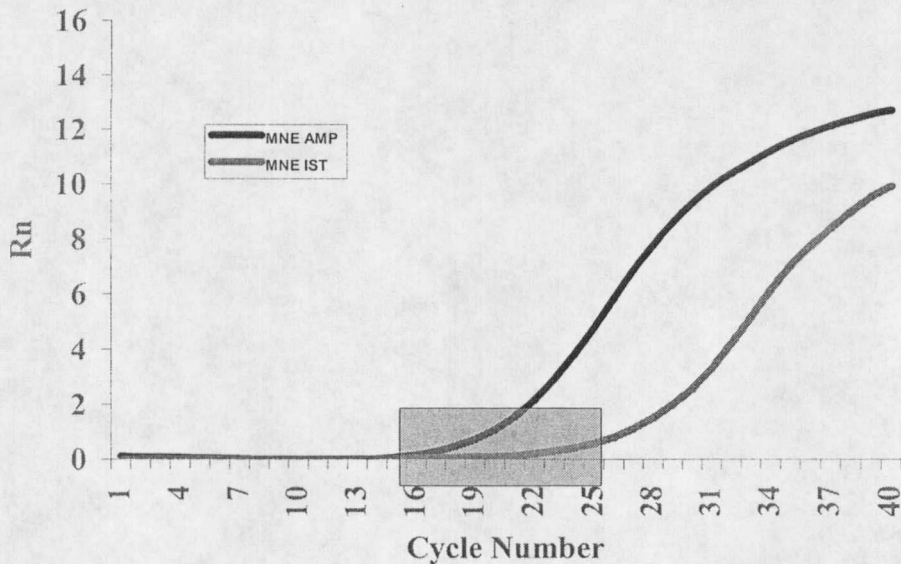


The most important part of an amplification curve is the initial exponential increase occurring around cycles 13 to 16 (Overbergh et al, 1999). This corresponds with primer binding and the amplification of complementary deoxyribonucleic acid

(cDNA). The SYBR-Green reagent used in this method only binds to double stranded (DS) DNA. The cumulative increase in fluorescence (Rn), or increase in amount of DS DNA, is measured and plotted against the cycle number to give the amplification graphs.

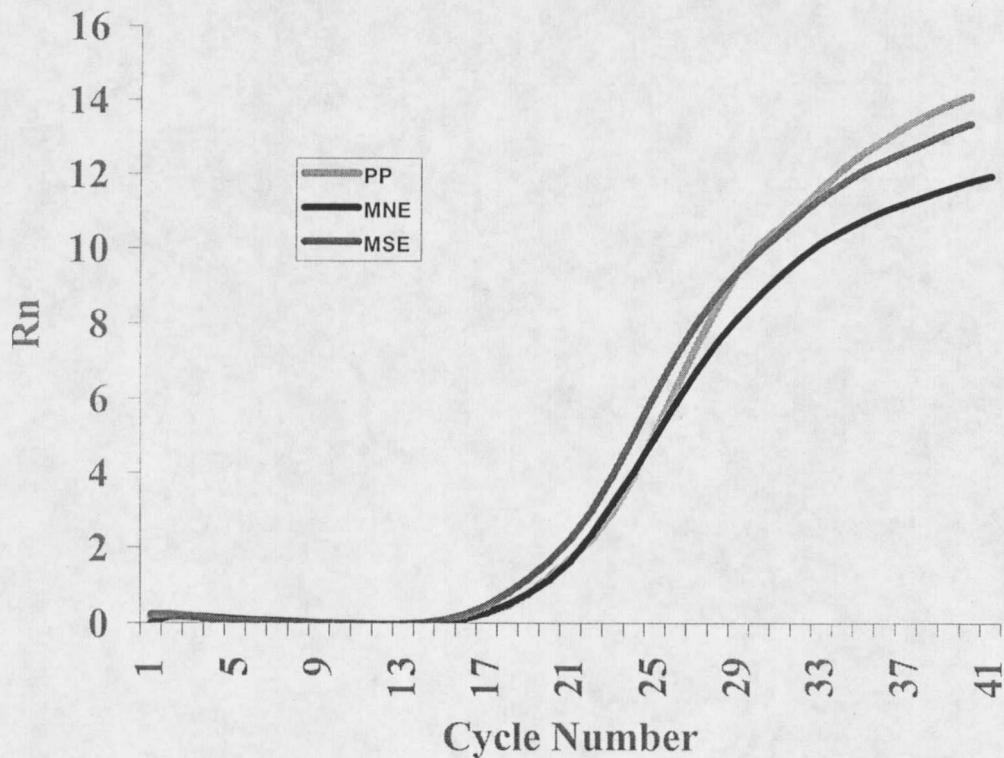
No mRNA coding for sheep OSP was detected in the IST of ewes of any treatment group (Figure 2). The highlighted portion of the graph shows the difference between the amplification of the cDNA in the AMP and IST. The AMP actually has cDNA present, which was bound to and amplified by the primers. This type of increase is comparable to the 18s positive control seen in Figure 1. Whereas, the IST curve does not have a significant increase in the highlighted portion of the graph.

Figure 2. Amplification curve for AMP and IST of MNE.



Messenger RNA coding for sheep OSP appeared to be present in the AMP of ewes of all three treatments (Figure 3). The plots for ewes in all three treatments showed a similar exponential increase in the Rn value in the important 13 to 16 cycle time-frame.

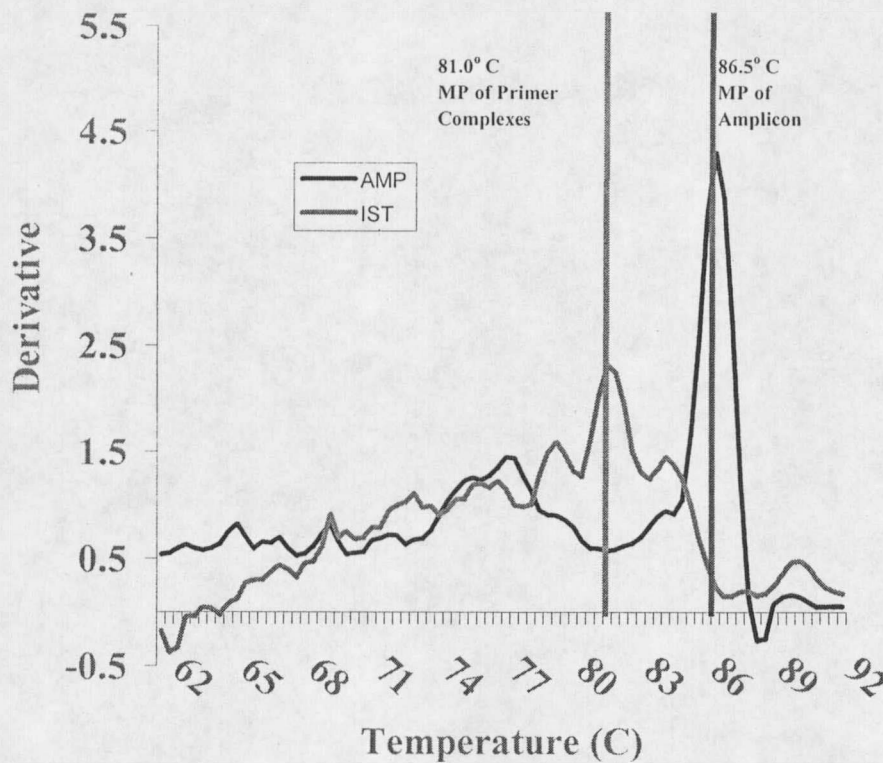
Figure 3. Amplification curve for AMP of PP, MNE, and MSE treatment groups.



To insure that DS DNA was being formed indicative of a message, the reactions dissociation curves were evaluated. A dissociation curve for a properly occurring reaction will have a clearly defined melting point, while an unsuccessful reaction will not have a defined melting point (Figure 4). The dissociation curve for

the RT-PCR product in the AMP showed a defined melting point corresponding to stable double stranded DNA. Whereas, the dissociation curve of the RT-PCR products for the IST shows numerous small peaks with no large defined one. This type of pattern corresponds to unstable double stranded DNA, most likely due to primer-dimer interactions.

Figure 4. Dissociation curve for AMP and IST



#### Hematoxylin and Eosin Y staining

Examination of the hematoxylin and eosin y stained slides at 100x and bright field illumination revealed the presence of ciliated and mucosal cells, blood

vessels, and the various tissue layers (Figures 5 and 6). This indicated that the cellular structures of the sections were intact.

Figure 5. H&E staining of AMP for MNE ewe.

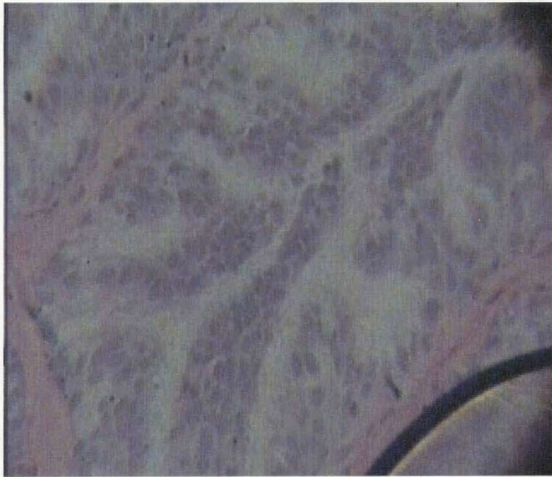
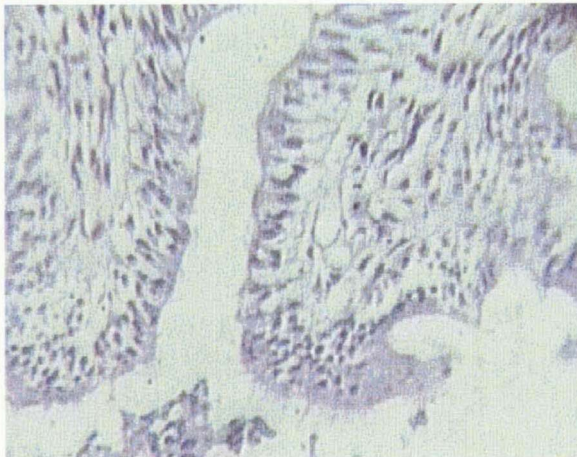


Figure 6. H&E staining of IST for MNE ewe



### Immunofluorescence

Following immunofluorescence staining, the slides were examined using a Zeiss epifluorescent system with 40x and 100x Ph2 achromatic phase contrast objectives under a blue filter number: CZ710 (Cromatechnology Corp.) using a deuterium light. Every slide had some degree of background and/or autofluorescence. The slides were observed and scored according to their fluorescence.

We found a distinct luminal-epithelial interface of fluorescent material in both the AMP and IST sections of each animal that exhibited intense fluorescent staining (Figures 7 and 8).

Figure 7. Fluorescent staining of AMP from an MNE ewe.

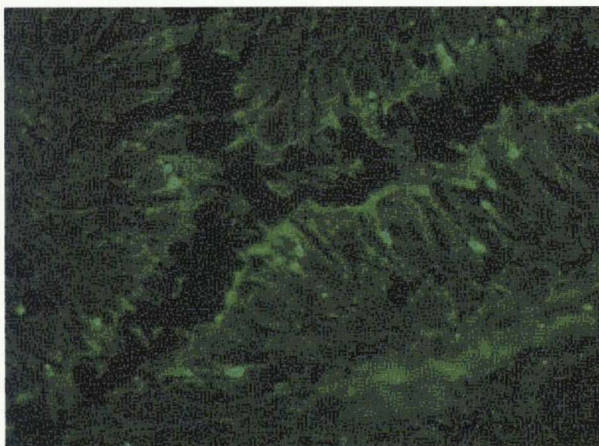


Figure 8. Fluorescent staining of IST from an MNE ewe.



Although not well characterized in the images (Figures 9 and 10), the PP sections did exhibit localized fluorescent staining when initially examined.

Figure 9. Fluorescent staining of AMP from PP ewe.

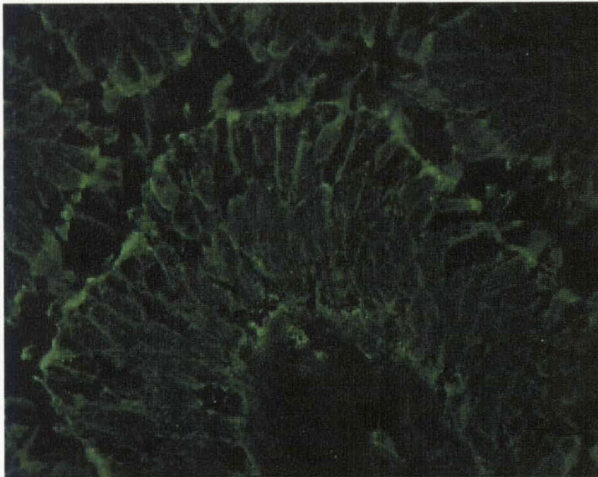
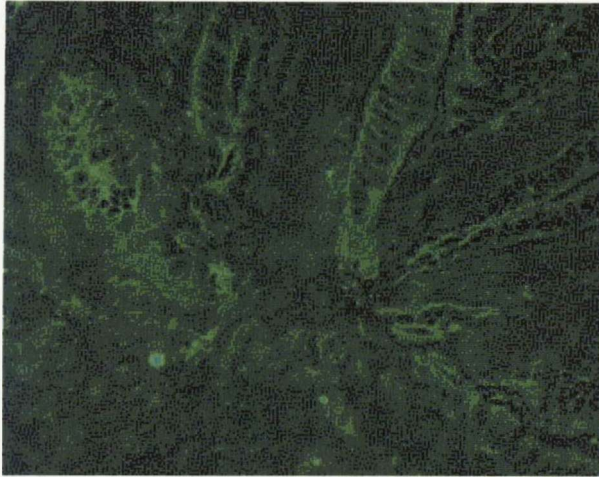


Figure 10. Fluorescent staining of IST from PP ewe.



The relative fluorescence (RF) intensity varied slightly among treatments, with MNE and MSE having the highest intensity, and the AMP being significantly more intense than the IST (see Table 5). Control sections exhibited some fluorescence, most scoring a one, which can be attributed to background and autofluorescence (Appendix D). No localized staining was observed in these slides (Appendix E).

Table 5. Least squares means for relative fluorescence for ampulla (AMP) and isthmus (IST) of prepubertal ewe lambs, and mature ewes 24 h after natural or progestin-synchronized estrus

Treatment	n	Relative Fluorescence		
		AMP	IST	
PP	5	2.35	1.75	
MNE	4	2.65	1.93	
MSE	5	2.50	2.07	
Mean		2.50 <sup>a</sup>	1.92 <sup>b</sup>	SEM=0.27

<sup>a,b</sup>Means within columns that lack a common superscript letter differ ( $P < 0.05$ ).

## DISCUSSION

In this present experiment, we evaluated gross morphological characteristics, protein contents and concentrations, mRNA expression, and fluorescent intensity following immunostaining in the ampulla and isthmus of the oviduct among pubertal ewe lambs and mature ewes twenty-four hours after the onset of either a natural or progestin-synchronized estrus. Specifically, we tested the hypotheses that weight, length, weight to length ratio, and protein content and concentrations, expressed as  $\mu\text{g}$  per g,  $\mu\text{g}$  per cm, or  $\mu\text{g}$  per  $\text{g}\cdot\text{cm}^{-1}$ , presence or absence of mRNA coding for sheep OSP, and fluorescence intensity following immunostaining of the AMP and IST do not differ among pubertal ewe lambs or mature ewes after natural or progestin-synchronized estrus. The rationale for this evaluation was to determine if there were any specific morphological or secretory characteristics of PP ewe lambs and MSE ewes that differed from those of MNE ewes. Such differences could explain the cause of lower fertility rates associated with breeding during the pubertal transition or after a progestin-synchronized estrus in sheep. Results of these investigations may also lead to further determination of the mechanism by which the oviduct influences fertilization and early embryonic development.

We found that AMP weight, length, and weight to length ratios of PP ewes were significantly smaller than those of either MNE or MSE ewes. Furthermore, except for the length, gross morphological characteristics of the IST were smaller in PP ewe lambs than in MNE or MSE ewes. These results are similar to those reported by Lewis and Berardinelli (2001) in a comprehensive study that evaluated the same gross

morphological characteristics among PP ewe lambs, first estrus ewe lambs, and mature ewes.

Oviductal secretions contain proteins, some of which represent a serum transudate, while others are synthesized and secreted by the epithelium. Oviduct-specific glycoproteins of high molecular weight have been identified and characterized in all mammalian species studied to date including; swine, bovine, and ovine (Oliphant et al., 1982; Buhi et al., 2000). These proteins are secreted at a time when sperm capacitation, fertilization, and early embryonic growth occurs; they bind to zona pellucidae and sperm; are found in the perivitelline space; are associated with plasma membranes of blastomeres; enhance in vitro embryonic development; and may provide protection from proteolytic enzymes during oviductal transport (Buhi et al., 2000). Therefore, oviductal proteins appear to play essential roles in processes regulating early embryonic development and survival.

In our study, we found that protein content, and concentrations expressed as either  $\mu\text{g per g}$ ,  $\mu\text{g per cm}$ , or  $\mu\text{g per g}\cdot\text{cm}^{-1}$  of either AMP or IST did not differ among PP ewe lambs, MNE and MSE ewes. Of particular interest is the fact that protein content and concentrations in AMP and IST of PP ewe lambs were as great as that found in both MNE and MSE ewes twenty-four hours after these ewes had exhibited estrus. The presence of such a large quantity of protein in these segments in PP ewe lambs, even though these segments were lighter and shorter, is difficult to explain. One interesting possibility for this finding may be that these proteins serve

to inhibit the processes that could result in fertilization at an immature stage of development.

Treating mature ewes with progestin-impregnated intravaginal sponges for a twelve-day period did not affect gross morphological characteristics, or protein content or concentrations in MSE ewes. To our knowledge, this is the first study that has examined these oviductal characteristics twenty-four hours after an estrus synchronized with a progestin in ewes. Thus, lower conception rates as well as lower lambing rates in ewes bred at a progestin-synchronized estrus (Curl et al., 1966; Dewesse et al., 1970) may not involve changes in gross morphology, or protein content or concentrations in the oviduct.

The RT-PCR results showed that mRNA coding for the sheep OSP was present in the ampulla of ewes from each treatment group. No isthmic portions of the oviduct appeared to contain any mRNA coding for sheep OSP. This means that the sheep OSP is not produced in the isthmus, but that does not mean that it cannot be present in the isthmus. These results are consistent with literature stating that OSP's are synthesized and secreted only in the ampullary portions of the oviduct (Murray, 1993). Although no previous experiments have been done on the differences in OSP localization in the oviduct between prepubertal and mature ewes following natural or progestin-synchronized estrus, it is surprising that the mRNA is present in all treatment groups.

The immunofluorescence work showed that sheep OSP was present in both AMP and IST portions of the oviduct in all animals (PP, MNE, and MSE). The AMP

exhibited higher fluorescence intensity than the IST ( $P < 0.05$ ), but there still was fluorescence in the IST in a measurable amount. The fluorescence was localized in the luminal-epithelial interphase of the oviduct in these animals. A possible explanation for this observation of OSP in the IST is that it traveled from the AMP to the IST along with other secretions during the estrous cycle.

In conclusion, the oviducts of PP ewe lambs are shorter and lighter than that of mature ewes although content and concentrations of proteins are as great as those of mature ewes. The physiological significance of this finding, during this period in development, is unknown. Furthermore, it does not appear that synchronization of estrus with progestins in ewes affects gross morphological or proteinaceous secretions of the oviduct. If OSP's are actually responsible for the differences in fertility between prepubertal and mature ewes, then the presence of mRNA coding for oviduct specific glycoprotein in both mature and prepubertal ewes is puzzling. Further work needs to be done to determine if the mRNA present is actually expressed and what portion of the cell the expression occurs in. This may give us further insight into the changes that occur during the pubertal transition, and a possible mechanism for the observed reduced fertility associated with pubertal and progestin-synchronized ewes.

The observation that sheep OSP is present in all three treatment groups (PP, MNE, MSE) is in harmony with the results that show the mRNA is present in all groups. The presence of OSP in the IST of all three groups, even though it is not as intense as the presence in the AMP, is perplexing. One possible explanation is it is produced in the AMP and migrates to the IST along with other oviductal secretions

during smooth muscle contractions at the beginning of the estrous cycle, or during fluid movement with ciliated cells. Further work needs to be done to confirm that the protein the primary antibody bound to is actually sheep OSP. Another interesting experiment would be to take samples from the beginning, middle, and end of the IST to determine if a gradient exists for the amount of OSP present.

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APPENDICES

APPENDIX A

SNAP FREEZING OF OVIDUCTAL SECTIONS

Samples of IST and AMP portions of the oviduct were taken following salpingectomy, measuring for length and weight, and flushing. These samples needed to be flash frozen in a specific manner to preserve the tissues for future slicing and immunofluorescence work.

- 1) Small samples, approximately 4mm in length, of IST and AMP portions of the oviduct were cut with razor blade
- 2) Samples were placed in a small plastic weigh boat and uniformly covered in OCT, taking care not to produce any bubbles or air pockets in OCT
- 3) Samples were then snap frozen using the following setup:
  - a. Styrofoam container filled with dry ice
  - b. Place a glass beaker in the dry ice and fill with hexane
- 4) Samples were then placed in hexane until OCT turned cloudy
- 5) Frozen samples were placed in Ziploc bags, labeled, and kept frozen at  $-80^{\circ}\text{C}$  until sliced and used in immunofluorescence work

APPENDIX B

PRIMERS FOR RT-PCR

Primers used for RT-PCR:

Forward: 5' CCAAAGGTGAGATTGCAACCC 3'

Reverse: 5' TCTGTCCAGTCTGAAGGCGC 3'

The primers were purchased from The Midland Certified Reagent Company (Midland, Texas) and were gel filtration (GF) grade. This means the product was desalted by gel filtration chromatography and is suitable for use as sequencing primers, or primers for DNA or RNA amplification.

APPENDIX C

REAL TIME RT-PCR METHODS

Real Time RT-PCR reaction

Real time reverse transcriptase- polymerase chain reaction (RT-PCR) is used to detect the presence of a specific RNA, reverse transcribe that into DNA, and amplify the DNA. Real time PCR is useful because it allows for the direct detection of the PCR product. This is accomplished by measuring the increase of fluorescence at various time points from the binding of SYBR-Green to the double stranded DNA. The basic protocol used for RT-PCR is as follows:

- 1) Isolated RNA was thawed on ice
- 2) The amount of RNA needed for reaction was determined by using a spectrometer and a 1:25 dilution
  - a. 4 $\mu$ l of RNA sample and 96 $\mu$ l of RNase free water placed in snap top tube (2 blanks of 100 $\mu$ l RNase free water were also prepared)
  - b. Following zeroing the spectrometer with blank, the absorbance of samples were measured at 260 nm
  - c. The amount of RNA ( $\mu$ l) needed is  $1/A_{260}$
- 3) Prepared a master mix for RT (multiply these amounts by the number of samples, be sure to include controls)
  - a. dNTPs            2 $\mu$ l
  - b. Oligo dt        0.5 $\mu$ l
  - c. 5x buffer        5 $\mu$ l
  - d. DNase            0.66 $\mu$ l

e. Mix well

- 4) Added sterile water to RNA (amount determined in step 2) to get final volume of 41.5 $\mu$ l
- 5) Added 8.16 $\mu$ l of master mix to each sample, vortex
- 6) Incubated at 37 C for 30 minutes
- 7) Heat inactivated at 75 C for 3 minutes
- 8) Cooled to room temperature on ice
- 9) Added 1 $\mu$ l M-MLV RT to each sample (don't add it to 1 sample for a no RT control), vortex
- 10) Incubated at 35 C for 1 hour
- 11) Heat inactivated at 75 C for 3 minutes
- 12) Master mix for PCR (added reagents in this order, multiplied amounts by number of samples):
  - a. 10x SYBR PCR buffer 2.5 $\mu$ l
  - b. 25mM MgCl<sub>2</sub> 3.0 $\mu$ l
  - c. dNTP blend 2.0 $\mu$ l
  - d. AmpliTag Gold (5 $\mu$ / $\mu$ l) 0.13 $\mu$ l
  - e. AmpErase UNG (1 $\mu$ / $\mu$ l) 0.25 $\mu$ l
  - f. Mix these, then separate into individual buffer solutions for each primer
- 13) Added primers (forward, reverse) 1 $\mu$ l each/sample at 5 $\mu$ M
- 14) Added 12.12 $\mu$ l sterile water/sample

- 15) Mix well
- 16) Added 3 $\mu$ l of cDNA sample to PCR tubes
- 17) Added 22 $\mu$ l of master mix to samples, be sure to leave a sample with no cDNA for a non template control
- 18) Put clear PCR caps on tubes and put in PCR machine
- 19) Set up PCR machine (label wells, set reaction volume to 25 $\mu$ l, hot start) and run reaction

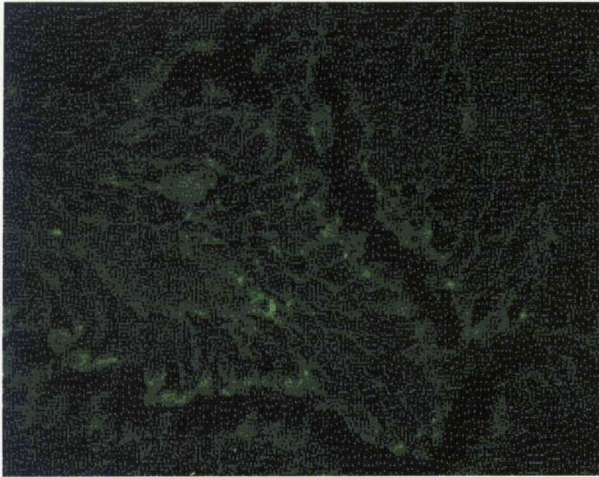
APPENDIX D

AVERAGE RF VALUES FOR BUFFERS

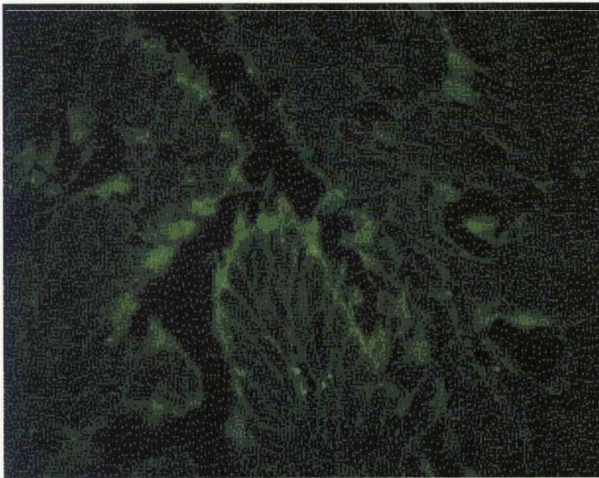
Oviduct Section	Animal	Treatment	buffers	primary	secondary	IgG	IgG+second
AMP	6677	MNE	1	1	1.125	1	1.375
IST	6677	MNE	1	1	1	1	1
AMP	6615	MSE	1	1	1	1	1.375
IST	6615	MSE	1	1	1	1	1
AMP	701	PP	1	1	1	1	1
IST	701	PP	1	1	1	1	1

APPENDIX E  
PICTURES OF CONTROL SLIDES

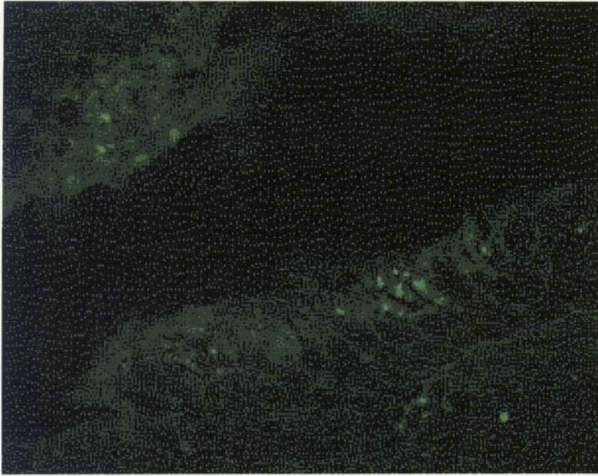
Negative control- picture of slide following incubation with only primary antibody



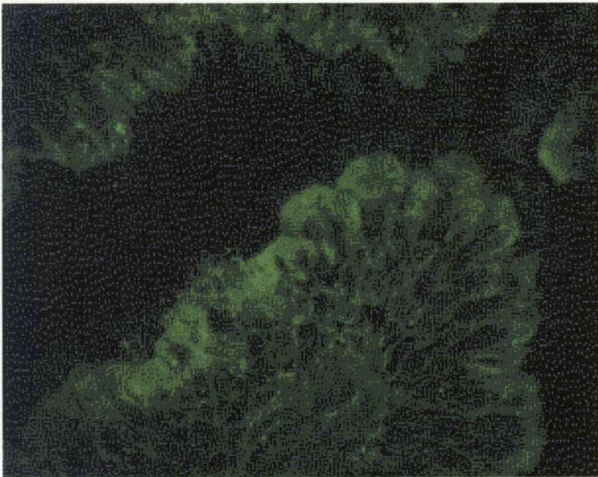
Negative control- picture of slide following addition of only buffer solutions



Negative control- picture of slide following incubation with only Rabbit IgG



Negative control- picture of slide following incubation with secondary antibody only



Negative control- picture of slide following incubation with Rabbit IgG and secondary antibody



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