

AN ABSTRACT OF THE DISSERTATION OF

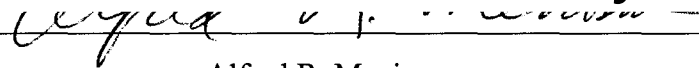
Susan A. Powell for the degree of Doctor of Philosophy in Animal Science
presented on August 16, 1999.

Title: The Relationship of Estradiol to Embryo-Maternal Interactions in the Llama.

Abstract

Redacted for Privacy

approved:



Alfred R. Menino

In the first experiment, estradiol production was measured in cultured embryos collected on Days 7, 9, 11, 13 and 15 post-mating. Estradiol was detected in Day 7 embryos and production increased approximately 50-fold ($P < 0.05$) in medium recovered from Day 13 compared to Day 11 embryos and was greatest ($P < 0.05$) from Day 15 embryos.

The second experiment evaluated the luteotrophic effect of estradiol in the llama. Daily injections of vehicle only, 5 or 10 mg estradiol benzoate (EB) in isopropylmyristate were administered on Days 7-15 (Day 0 = hCG ovulation induction). Mean progesterone levels were greater ($P < 0.05$) on Days 14, 15, 16, and 17 in llamas injected with 10 mg EB compared to llamas injected with vehicle or 5 mg EB.

The final experiment analyzed differences in estrogen receptor expression in the corpus luteum (CL), ovary, and uterus using Reverse Transcription-Polymerase Chain Reaction. A significant linear regression ($P < 0.05$) was detected for both $ER\alpha$ (increased from Days 7-11), and $ER\beta$ (decreased from Days 7-11) in llama CL. $ER\alpha$ expression in ovary was higher ($P < 0.05$) compared to Days 7 and 9 CL.

ER α expression in CL was lower on Day 9 ($P < 0.05$) than on Days 7 and 11 in pregnant animals. ER α expression decreased ($P < 0.01$) from Days 7-11 in uterus and expression was lower in pregnant versus non-pregnant uterus ($P < 0.10$). ER β expression was higher in pregnant versus non-pregnant uterus ($P < 0.10$). No differences ($P > 0.10$) in ER α or ER β expression were detected in endometrium due to reproductive status or days post-mating. A decrease ($P < 0.01$) in ER α expression was detected in pregnant uterus from Days 7-13. ER α expression was lower ($P = 0.12$) in juvenile uterus versus pubertal females. ER β expression in non-pregnant uterus was different ($P < 0.05$) by days post-mating and reproductive status.

These data suggest that estradiol produced by the embryo may be involved in embryo migration and maternal recognition of pregnancy in the llama, and these events may be mediated by differential expression of ER subtypes.

© Copyright by Susan A. Powell

August 16, 1999

All Rights Reserved

**The Relationship of Estradiol to
Embryo-Maternal Interactions
in the Llama**

by

Susan A. Powell

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented August 16, 1999

Commencement June 2000

Doctor of Philosophy dissertation of Susan A. Powell presented on August 16,
1999

APPROVED:

Redacted for Privacy

Major Professor, representing Animal Science

Redacted for Privacy

Head of Department of Animal Sciences

Redacted for Privacy

Dean of Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Redacted for Privacy

Susan A. Powell, Author

ACKNOWLEDGMENT

It does not seem possible that this journey nears its end and a new journey awaits. In the almost four years I have been at OSU, numerous people have touched my life and helped me achieve my goals. First, I would like to thank Brad Smith, Dave Froman, Jim Moore, and Victor Hsu for serving on my program committee. My major professor, Fred Menino, who gave me the opportunity to work on a project that was challenging and unique and kept me motivated through the rain and muck and “let’s do this experiment one more time” again. My experiences here and my success at this project would not have been possible without his support. And thanks “Coach” for getting me to dust off the cleats and mitt and allowing me to play softball again. Thanks also to the entire Menino family for their friendship and support and a big thank you to Holly Menino for her help in the lab and with the llamas and her friendship. Dave Froman deserves thanks for his wisdom, advice, challenges and candor. Thanks for always being there when I needed help. This project would not have been possible without Brad Smith and the OSU Vet School. Thanks for virtually unlimited access to animals and for their upkeep and care while I was using them for the various projects. Karen Timm deserves special thanks for her help, always being available even though she was in the middle of a sabbatical.

My life was made easier by the willingness of several people who volunteered to help with the llamas. Despite bad weather, many people braved the wind and rain to help with the llamas. First and foremost, Nancy Hollingshead who taught me everything I needed to know about the llamas, was always able to help me find new animals for projects, transported animals when I needed them and helped in more ways than can be named. Without Nancy, my research would not have happened. I am eternally grateful to Nancy for her commitment to this project and her friendship. Thanks to Wendy Black, Stephanie Latham, and Angie Larwood

for helping with the llamas and being a friend. Bill Black, fellow graduate student, was always there to help with animals no matter what day of the week or amount of mud. Thanks too for helping me win against the computer and keeping the lab interesting and fun, not to mention \$2 when I needed it.

I have truly enjoyed my studies here at OSU and friendships with many fellow graduate students past and present have enriched my life, Derek McLean, Tim and Kelly Hazzard, Kelly Haun, Heather Baumholtz, Ugur Salli, Lisa Milliman, Yuko Eguchi, Stephanie Greenway, and all graduate students who have “shared the pain” more commonly known as “writing a thesis”.

Specials thanks to my best friend Sara Supancic. It was a rocky start but I couldn't have asked for a better friend, one who will baby-sit too. I know we can stay close even with distance between us, because you are a special friend that is a rare gift. Thanks so much for your friendship and love, Biscuit too.

Last, but not least, I must thank my family for supporting me in my decision to return to school. My husband, Alan, I know it hasn't been easy but thanks for hanging in there and trust me it's all downhill from here, I hope. My parents for believing in me, letting go and helping in any way they could. Most of all thanks to my son Luke. Some day he will understand what this means and I hope that he too can reach for his dreams and achieve them. This is for you Luke, this thesis and my life are dedicated to you.

TABLE OF CONTENTS

	<u>Page</u>
CHAPTER 1. Introduction	1
CHAPTER 2: Review of Literature ..	6
Reproduction in the Female SAC	6
Reproduction in the Male SAC	18
Reproductive Oriented Behavior.....	19
Maternal Recognition of Pregnancy.....	22
Embryo Migration.....	25
Estrogen as a Mediator of Female Reproductive Function	26
References	30
CHAPTER 3: Estradiol Production by the Preimplantation Llama Embryo.	37
Abstract	37
Introduction	37
Materials and Methods.....	39
Results	41
Discussion	43
References	48

TABLE OF CONTENTS (Continued)

	<u>Page</u>
CHAPTER 4: Administration of Estradiol During the Mid to Late Luteal Phase Extends the Corpus Luteum Lifespan and Enhances Progesterone Production in the Llama.....	50
Abstract	50
Introduction	51
Materials and Methods	52
Results	54
Discussion	58
References	61
CHAPTER 5: Expression of Estrogen Receptors α and β in the Pregnant and Non- Pregnant Llama Uterus and Corpus Luteum.	63
Abstract	63
Introduction	64
Materials and Methods	67
Results	71
Discussion	86
References	93
CHAPTER 6: Summary.....	95
Bibliography	97

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
3.1. Estradiol production by preimplantation llama embryos collected on Days 7, 9, 11, 13, and 15.....	44
3.2. Estradiol production by preimplantation llama embryos collected on Days 7, 9, and 11	45
4.1. Serum progesterone levels in non-pregnant, ovulatory llamas receiving vehicle only, 5 mg or 10 mg EB in soybean oil injections from Days 7-15 post-hCG	55
4.2. Serum progesterone levels in non-pregnant, ovulatory llamas receiving soybean oil or saline injections from Days 7-15 post-mating/hCG	56
4.3. Serum progesterone levels in non-pregnant, ovulatory llamas receiving vehicle only, 5 mg or 10 mg EB in isopropylmyristate injections from Days 7-15 post-hCG.....	57
4.4. Serum estradiol levels on Days 5, 9, 15, and 20 post-hCG in non-pregnant, ovulatory llamas receiving 0, 5, or 10 mg EB in isopropylmyristate from Days 7-15 post-hCG.....	59
5.1. Llama ER α nucleotide sequence.....	72
5.2. Llama ER β nucleotide sequence.	73
5.3. Gene expression of ER α , ER β and β -actin in the pregnant and non-pregnant llama uterus.	74
5.4. Gene expression of ER α , ER β and β -actin in the pregnant and non-pregnant llama endometrium.	75
5.5. Gene expression of ER α , ER β and β -actin in the pregnant and non-pregnant llama CL and ovary.....	76
5.6. Expression of ER α and ER β in llama CL on Days 7, 9 and 11 post-mating.....	77

LIST OF FIGURES (Continued)

<u>Figure</u>	<u>Page</u>
5.7. Expression of ER α and ER β in pregnant and non-pregnant llama CL on Days 7, 9, 11 and 13 post-mating and ovary recovered from follicular phase females.	78
5.8. Expression of ER α in pregnant and non-pregnant llama CL on Days 7, 9, 11 and 13 post-mating.....	79
5.9. Expression of ER α in llama CL on Days 7, 9, 11 and 13 of pregnancy	81
5.10. Expression of ER α and ER β in llama uterus on Days 7, 9 and 11 post-mating.....	82
5.11. Expression of ER α and ER β in llama endometrium on Days 7, 9 and 11 post-mating.....	83
5.12. Expression of ER α and ER β in pregnant llama uterus, Days 7, 9, 11 and 13 post-mating.....	84
5.13. Expression of ER α and ER β in pregnant llama endometrium on Days 7, 9, 11, and 13 post-mating.....	85
5.14. Expression of ER α and ER β in uteri recovered from non-pregnant llamas on Days 7, 9 and 11 post-mating, non-ovulated and juvenile females.....	87
5.15. Expression of ER α and ER β in endometrium recovered from non-pregnant llamas on Days 7, 9 and 11 post-mating, and non-ovulated females	88
5.16. Expression of ER β in non-pregnant llama endometrium on Days 7, 9 and 11 post-mating.....	89

LIST OF TABLES

<u>Figure</u>	<u>Page</u>
3.1. Recovery of embryos from superovulated donors	42
5.1. PCR primer sequences for ER α , ER β and β -actin.....	70
5.2. Summary of ER α and ER β expression in the llama CL, uterus, and endometrium	90

THE RELATIONSHIP OF ESTRADIOL TO EMBRYO-MATERNAL INTERACTIONS IN THE LLAMA

CHAPTER 1

INTRODUCTION

Llamas have been used for centuries in South America as a food and fiber animal. The camelid family is twice as old as the cattle family and they have evolved specialized features allowing them to survive in areas of marginal food supply and severe environmental conditions. Ancestors of present-day camelids originated and evolved on the plains of North America 40 to 45 million years ago (Franklin, 1981). Camelid ancestors migrated to other continents 2 to 3 million years ago and evolved into the present day Family Camelidae consisting of the Old World camelids (OWC) and the South American camelids (SAC). OWC include the two-humped bactrian camel of Asia and the one-humped Arabian (dromedary) camel. SAC include the wild vicuña and guanaco as well as the domesticated llama and alpaca.

SAC are the most important large herbivores in South America because of their ecological dominance and importance to man (Franklin, 1981). Vicuñas are generally accepted as a separate taxonomic unit based on satellite DNA patterns, while llamas are considered to be a domestic form of the guanaco (Vidal-Rioja et al., 1994). Alpacas have two distinct breeds based on coat type, the huacayo and the suri, that have DNA patterns intermediate to the other three species. However, current literature has yet to define an exact taxonomic placement for the alpaca (Franklin, 1981; Vidal-Rioja et al., 1994; Novoa and Wheeler, 1988). There is evidence to suggest that alpacas are more closely related to vicuñas than to either the guanaco or the llama. Alpacas and vicuñas have similar dentition which is different from the dentition of both llamas and guanacos (Novoa and Wheeler, 1988).

SAC are generally found in the altiplano region of the Andes Mountains. Vicuñas are restricted to the Central Andean *puna* (a high altitude grassland), while the guanaco is widespread from Northern Peru to Southern Chile and centered in the Patagonia of Argentina. No wild herds of llamas or alpacas exist. Most llamas are found in Bolivia, alpacas and vicuñas in Peru, and guanacos in Argentina (Franklin, 1981).

SAC are a major and strategic resource to man. In their native land, their use as a pack animal and products such meat, hide, fiber, fuel (dung), medicines, and use in religious ceremonies have been an indispensable and effective form of land use in a formidable environment for thousands of years (Brownan, 1974). The pre-Incan culture *Qollas* of the southern highlands west of Lake Titicaca had a reputation throughout the Andes as llama breeders, controlling vast herds of llamas and alpacas (Murra, 1965). The Inca Empire's culture and economy revolved around the llama, a prime source of wealth, both for the individual and the state. Llamas provided transportation carrying goods and supplies. This allowed the construction of buildings, temples, irrigation products, and highways, in addition to military expansion, food commerce and mining (Murra, 1965). Alpacas have been domesticated for nearly 7,000 years and are among the oldest domesticated animals in the world (Wheeler, 1994). Llama and alpaca numbers drastically declined in the century following the Spanish invasion of the Central Andes. Uncontrolled slaughter, disruption of the Incan social order, and introduction of sheep were responsible for the decline (Franklin, 1981). Today it is estimated that there are over 3 million alpacas, 3.5 million llamas, 125,000 vicunas and 605,000 guanacos in South America (Gordon, 1997). Llamas and alpacas remain an important economic resource in South America.

Increased popularity of the llama as a pet and pack animal, in addition to their use as a source of fiber, has led to an increase in the numbers of SAC in North America during the past 17 years. Current estimates suggest that there are 150,000-200,000 llamas, 20,000 alpacas and a few hundred guanacos and vicuñas in North

America. Approximately 10% of the North American llama and alpaca population are found here in Oregon. The average cost for a llama is approximately \$2,000 while an average alpaca costs approximately \$12,000. High quality breeding females and males can cost substantially more.

Embryonic mortality is a significant factor contributing to reduced reproductive efficiency in many mammals. Substantial prenatal mortality is observed in all mammals, although there are significant differences between species in the extent and timing of embryonic death. As with other farm species, llamas and alpacas experience a high incidence of early embryonic mortality. Embryonic loss may be due to an abnormal embryo, a maternal environment unable to support normal development or a combination of these factors.

Defining the causes of early embryonic loss is particularly important in livestock due to potential economic losses resulting from decreased production. In mated ewes some 20-40% of ovulations are not represented by live births (Edey, 1979). Failure of fertilization accounts for only a small proportion of this loss. The greatest incidence of embryonic loss occurs after fertilization and before implantation or during the first 3 weeks of gestation. A similar pattern of loss has been observed in cattle (Ayalon, 1978). In pigs, embryo loss is estimated to be 40% (Hafez, 1993). Early studies in alpacas estimated that 50% of breeding age females failed to produce young each year despite fertilization rates of 80% or greater following natural mating (Fernandez-Baca et al., 1970). In a later study, Bravo et al. (1987) observed that reproductive wastage in female alpacas of different ages and reproductive status reached 83.2% (ova, embryo, fetal loss). However, no embryo-fetal losses were observed from 90 days post-mating to term. Factors responsible for this high incidence of wastage are not known. However, since this study was conducted on alpacas living in their natural environment, nutritional and other environmental constraints may have been a factor in the exceedingly high rate of loss.

While the overall fertility rate in the North American llama population is considerably higher than 50%, early embryonic mortality remains an important issue limiting the rate of reproduction in the llama. The llama embryo is known to migrate from the right to the left uterine horn, with approximately 95% of pregnancies occurring in the left uterine horn (Fernandez-Baca et al., 1970). Improper embryo migration, in addition to other factors, may be a reason for early pregnancy loss in the llama. An important issue in addressing the problem of early embryo loss has been the lack of basic information concerning early embryonic development, maternal recognition of pregnancy, and uterine implantation in camelids. Knowledge gained from studies of early embryonic development may yield practical applications which can be used to increase embryo survival.

Therefore, the goal of this research was to investigate events in early embryo development and maternal-fetal interactions involved in pregnancy recognition with the long-term goal of identifying potential factor(s) contributing to embryonic mortality and reduced fertility in the llama. The specific objectives of these studies were to determine 1) if the llama embryo produces estradiol, 2) if estradiol is luteotrophic in the llama and thus a possible candidate as the maternal recognition of pregnancy signal, and 3) differences in estrogen receptor subtype expression in the uterus and ovary as possible factors involved in embryo migration.

REFERENCES

- Ayalon N. A review of embryonic mortality in cattle. *J Reprod Fert* 1978; 54:483-493.
- Bravo PW, Sumar J, Rivera SG, Foote WC. Reproductive wastage in the female alpaca. In: *Improving Reproductive Performance of Small Ruminants*. Utah State University, Logan 1987:155-159.
- Brownan, DL. Pastoral nomadism in the Andes. *Current Anthropology* 1974; 15:188-196.
- Edey, TN. Embryo Mortality. In: Tomes GJ, Robertson DE, Lightfoot RJ, Haresign W (eds.), *Sheep Breeding*. London: Butterworths; 1979: 315-325.
- Fernandez-Baca S, Hansel W, Novoa C. Embryonic Mortality in the Alpaca. *Biol Reprod* 1970; 3:243-251.
- Franklin WL. In: *Mammalian Biology in South America*. Vol 6, Special Publication Series Pymatuning, Biology, ecology and relationship to man of the South American camelids. Laboratory of Ecology, University of Pittsburgh, Linesville, PA, 1981:457-489.
- Gordon I. *Controlled Reproduction in Horses, Deer, and Camelids*. University Press, Cambridge, UK, 1997:189-208.
- Hafez ESE (Ed). *Reproduction in Farm Animals*. 6th edition, Lea and Febiger, Philadelphia, PA; 1993.
- Murra JV. Herds and herders in the Incan state. In: Leeds A, Vayda AP (eds.), *Man, Culture, and Animals*. Washington D.C.: American Association for the Advancement of Science, 1965: Pub no. 78.
- Novoa C, Wheeler JC. Llamas and alpacas. In: Mason IL (ed.), *Evolution of Domestic Animals*. London: Longman Press; 1988: 116-144.
- Vidal-Rioja LA, Zambelli A, Semorle L. An assessment of the relationships among species of Camelidae by satellite DNA comparison. *Hereditas* 1994; 121:283-290.
- Wheeler J. Ancient alpacas and modern myths. *Alpacas*, 1994; Winter:19-21.

CHAPTER 2

REVIEW OF LITERATURE

Reproduction in the Female SAC

Anatomy. The reproductive tract of the female llama is similar to other domestic species (cow, horse, sheep). The ovaries are located within the pelvic cavity caudal and lateral to the uterotubal junction (Bravo, 1994). They are oval, resembling a peanut, and in the inactive state measure approximately 1.5 x 1.0 x 0.5 cm. However, the ovaries may double in size due to the presence of multiple follicles or the presence of a corpus luteum (CL; Johnson, 1989). Multiple developing follicles are typically present on the active ovary. The CL, if present, attains its full size at approximately 14 mm in diameter (Fernandez-Baca, 1993). The CL is deeply embedded in the ovarian parenchyma with a large portion protruding from the ovary (Johnson, 1989). The ovary is completely covered by a large, well-developed bursa. (Bravo, 1994).

The oviducts are long, tortuous and quite firm with the unique feature of distinct papillae at the uterotubal junction (Smith et al., 1994). Each papilla contains a sphincter (Fowler, 1989) that prevents retrograde flow of fluid from the uterus to the oviducts (but not orthograde flow) making salpingitis rare (Johnson, 1989).

In situ, the uterine body and two uterine horns resemble the letter “Y”, and the short uterine horns curl slightly ventral towards the ovarian end (Smith et al., 1994). Each uterine horn measures approximately 2 x 6 cm in the nongravid state (Johnson, 1989). An intercornual septum separates the caudal portion of the uterine horns and becomes especially prominent during pregnancy (Fowler, 1989). The septum is not complete to the cervix. A small uterine body is present that measures approximately 2.5 x 2.5 cm (Johnson, 1989). Both horns are equal in size in nulliparous females. However, because 95-98% of pregnancies implant in the left

horn, an asymmetry is seen upon subsequent examination of even a nongravid tract with a multiparous left horn measuring approximately 3 x 10 cm (Johnson, 1989).

The cervix has been described as having 2 to 3 cartilaginous rings which are not as distinct as the annular rings of the bovine (Johnson, 1989). Smith et al. (1994) describe the cervix as having a single spiral fold that makes 2 or 3 turns, giving the appearance of 2 or 3 cervical rings. The cervical lumen traverses in a clockwise direction (Johnson, 1989). The spiral fold of the cervix becomes soft and pliable during the time of receptivity allowing the male to deposit semen into the uterus (Smith et al., 1994). The short cartilaginous process of the penis serves to dilate the cervix during prolonged copulation as the penis spins forward in a clockwise direction (Johnson, 1989). The cervix increases in tone and closes tightly when a CL is present on the ovary (Smith et al., 1994). The external os protrudes slightly into the vagina (Johnson, 1989).

The remainder of the genital tract is unremarkable. The vagina is approximately 20-25 cm in length and 3 cm in diameter. A small vulva is present and the vulvar lips do not swell during sexual receptivity. The clitoris is well developed (Johnson, 1989).

Follicular Dynamics. Camelids do not exhibit estrous cycles as seen in other domestic species such as the cow, sheep, and horse. Laparoscopic and ultrasonic studies of ovaries indicate that follicles grow in waves that tend to overlap (Bravo and Sumar, 1989; Adams et al., 1990; Bravo et al., 1990b). The interwave interval, defined as the time (days) between the emergence of successive dominant follicles of 8 to 12 mm, has been determined to be 11 to 20 days in the llama (Adams et al., 1990; Bravo et al., 1990b), and 8 to 12 days in the alpaca (Bravo and Sumar, 1989). The ovaries alternate in the development of dominant follicles in 81% to 85% of the cases, with one ovary presenting a large follicle while the other is relatively quiescent (Smith et al., 1994). The follicular wave is divided into three stages; growing, mature, and regressing with each stage lasting approximately 4 days (Bravo et al., 1990b). A normal ovary contains two to three small follicles 3 mm in

diameter, and follicles may increase to 6 mm without showing dominance (Johnson, 1989). Beyond 6 mm, one follicle normally becomes dominant with the dominant follicle suppressing the development of other follicles in the same cohort (Bravo et al., 1990b). The follicular waves overlap and a subsequent follicle will become dominant within two to three days of the onset of atresia of the previous dominant follicle (Bravo and Sumar, 1989). A lag time between initiation of atresia of the dominant follicle and the start of the rapid growth phase of the next dominant follicle can be as short as one day (Bravo et al., 1990b). The succession of dominant follicles is repeated in this wave fashion until copulation induces ovulation and a CL forms (Bravo and Fowler, 1990).

Estradiol-17 β ($E_2\beta$) in plasma and estrone sulfate in urine are positively related to follicular waves. Concentrations of 10 to 15 pg/ml of $E_2\beta$ and 20 to 30 ng/mg creatinine of urinary estrone sulfate correlate with follicles 8 to 12 mm in size (Bravo et al., 1990b). In addition, while plasma $E_2\beta$ is positively associated with follicular activity, urinary estrogen conjugate concentrations are a better reflection of ovarian follicular activity (Bravo et al., 1990b). Progesterone (P_4) is usually only present at basal concentrations (less than or equal to 0.5 ng/ml) in the absence of a CL (Adam et al., 1989).

Follicular activity continues in waves for llamas during pregnancy. Ovarian follicular activity, represented by the presence of follicles at least 6 mm in diameter irrespective of the location of the CL, was shown to be present until 6 months of gestation. Thereafter, only small follicles (3 mm or less) were found (Smith et al., 1994). Adams et al. (1990) examined the effects of lactational and reproductive status on ovarian activity. Lactational status or type of mating (vasectomized vs. intact male) did not affect growth rate of the preovulatory follicle. The presence of a CL and/or lactation depresses follicular activity. The interwave interval was found to be 2.5 days shorter for lactating compared to non-lactating llamas. Pregnancy also shortens the interwave interval. The interwave interval for

ovulatory (vasectomy-mated) females is shorter than anovulatory (non-mated) females. Pregnant females had the shortest interwave interval (15 days) of all groups (Adams et al., 1990). Additionally, the presence of a CL was associated with fewer detected follicles. Although not explained, this study demonstrated longer interwave intervals for anovulatory llamas (20 versus 11 days) when compared to other reports. Regardless, this study demonstrated the depression of follicular activity with lactation or the presence of a CL.

Sexual Receptivity. Due to the nature of the follicular waves with a dominant follicle developing as the previous dominant follicle regresses, the female shows extended periods of sexual receptivity. In the absence of any ovulatory stimuli, females will show continuous receptivity for as long as 30 to 40 days, with occasional periods of non-acceptance (Fernandez-Baca et al., 1970b). Studies have demonstrated no relationship between sexual behavior and ovarian follicular size (Bravo, 1994). Females show differing degrees of receptivity that is independent of follicular size (Sumar et al., 1993). Females are essentially continuously receptive during follicular development. Refusal of the male by the female at this time is more a behavioral than hormonal effect (Pollard et al., 1994). Most females will refuse the male approximately 48 hours post-mating and will continue to refuse the male as long as a CL is present. If a non-fertile mating has occurred, females will return to receptivity approximately 12-14 days post mating.

Pregnancy diagnosis is often made by observing the female's receptivity to a male approximately 15 to 18 days post breeding. Ideally, all nonpregnant females should be receptive to mating, whereas during pregnancy serum P_4 is elevated to a level to inhibit receptivity (Fernandez-Baca et al, 1970c). Accuracy of this observation method requires knowledge of the exact breeding date. This test mating should be repeated for several days, ideally with at least two males, to eliminate the possibility of either a behavioral refusal or a brief non-receptive period. In addition, the female, if pregnant, should be retested at 30, 60, and 90 days since an increased incidence of pregnancy losses has been reported during the

first three months of gestation in alpacas (Fernandez-Baca et al., 1970a). It should also be kept in mind that this is a presumptive indication of pregnancy. Inaccurate breeding dates, a retained CL, or behavioral factors may give a false indication of pregnancy (Smith et al., 1994).

SAC are not true seasonal breeders. They exhibit ovarian activity throughout the year and may breed, conceive and give birth at any time of the year (Smith et al., 1994). However, in Peru breeding and birthing are seasonal and occur in summer. This phenomenon has been related to improved nutrition, due to forage availability, in the spring. Seasonal breeding of alpacas has been practiced by the Peruvians so that parturition occurs during the rainy season when pasture growth is available for optimal raising of the young (San Martin et al., 1968).

Ovulation. All camelids are induced ovulators with ovulation normally initiated by breeding to an intact or vasectomized male (Novoa, 1970; England et al., 1969; Fernandez-Baca et al., 1970c). Ovulation occurs within 24 to 26 hours post-copulation in alpacas (Novoa, 1970; San Martin et al., 1968) and 1.8 to 2 days post copulation in llamas (Adams et al., 1991; Bravo and Sumar, 1989; Bravo et al., 1990a). In rare cases ovulation may occur spontaneously, but it usually requires the neural stimulation of the penis penetrating the cervix, plus the leg clasp of the male (Fernandez-Baca et al., 1970c). Spontaneous ovulation rates of 3-15 % have been reported with and without contact with a male (Adam et al., 1989; Bravo et al., 1990a; Fernandez-Baca, 1993). However, these ovulations may not truly be spontaneous since females were not completely deprived of visual, olfactory, and auditory stimuli (usually during exposure to a vasectomized male for heat checking) which may have had some effect (Fernandez-Baca, 1993). Reed (1996), observed ovulations in females during the course of studies when animals were not exposed to males or ovulation-inducing treatments. Mid-luteal ovulations, both spontaneous and copulation induced, have been reported (Fowler, 1989; England et al., 1969).

Induction of ovulation may be accomplished with human chorionic gonadotropin (hCG) with ovulation occurring approximately 24 hours after injection (San Martin et al., 1968). Doses as low as 25 IU of hCG given intramuscularly have been reported to be effective in inducing ovulation (England, 1969). Ovulation can also be induced by gonadotropin-releasing hormone (GnRH). In llamas, a single intravenous dose of 1 µg GnRH/kg BW was effective in inducing ovulation (Reed, 1996) or a single intramuscular injection of 0.1 mg of GnRH (Wiepz and Chapman, 1985).

Endocrine Responses to Mating. Afferent impulses, originating from neural stimulation of the cervix during mating, ultimately reach the hypothalamus, causing release of GnRH and subsequent release of pituitary luteinizing hormone (LH) (Bravo, 1994). A significant increase in serum LH concentration was observed within 15 minutes after the onset of copulation, with the peak of the preovulatory surge of LH occurring at 2 hours post-coitus. Values returned to basal levels by 7 hours post-coitus (Bravo et al., 1990a). $E_2\beta$ concentrations were unchanged through 18 hours after copulation but decreased significantly by 48 hours after copulation. The first significant P_4 increase occurred at 3 days after copulation with values increasing through day 10.

The amount of LH released is important in provoking ovulation. The amount of LH released in response to mating, the administration of hCG, or GnRH is dependent on the size of the dominant follicle on the ovary (Bravo et al., 1991a). With follicles 7 mm or greater, the preovulatory rise of LH is evident and ovulation usually occurs within 24 to 30 hours in alpacas (1.8 to 2 days in llamas). In contrast, only small amounts of LH are released when only smaller follicles (< 7 mm) are present and ovulation does not occur. However, only 89% of females ovulate under ideal conditions (Smith et al., 1994). Females not ovulating do not have an increase in LH (Bravo et al., 1992). Mating of females with a regressing follicle provokes luteinization of the follicle without ovulation followed by a

shortened luteal phase. Luteinized follicles have a mean life span of 5.1 days (Bravo et al., 1991a). Multiple matings do not provoke the release of more LH. In the presence of a growing or mature follicle ≥ 7 mm, the preovulatory surge of LH is induced by the initial mating. Subsequent breeding 6 or 24 hours later show the same pattern of LH release as non-ovulatory and non-mated females (Bravo et al., 1992a). Subsequent breeding may, however, increase conception rates. Approximately 50% of alpacas ovulate greater than 30 hours after the copulatory stimulus, so multiple matings within 24 hours may provide adequate spermatozoa to fertilize ova in those females that ovulate late. However, studies have not shown statistically significant improvements in fertilization with multiple matings (Fernandez-Baca et al., 1970c).

The Corpus Luteum. The CL forms as a result of ovulation (Fernandez-Baca et al, 1970b). The CL can be detected ultrasonically by day 3 post-ovulation (Adams et al., 1991). Serum P_4 concentrations ≥ 1 ng/ml are detectable 3 to 4 days after ovulation and if conception does not take place, the CL starts to regress by approximately day 10 or 11 post-ovulation (Adam et al., 1989). The CL reaches its maximum size at days 6 to 9 in nonpregnant llamas and day 21 to 22 in the pregnant llama (Adams et al., 1991). The P_4 content of the CL reaches its maximum at day 4 and stays high until day 12, and is regressed completely by day 13 to 16 (Adams et al., 1991). Females may return to receptive behavior as early as day 12 post-ovulation (Fernandez-Baca et al, 1970b). Prostaglandins (PG) provoke the demise of the CL as evidenced by the presence of successive surges of 15-keto-13, 14-dihydro-PGF- 2α 10 to 12 days after a sterile mating (Sumar et al., 1988).

In nonpregnant llamas, plasma P_4 concentrations increase above basal levels 4 days post ovulation (≥ 1 ng/ml), peak at day 6 (4 to 5 ng/ml) and begin to decrease sharply at day 8 (Adams et al., 1991). In pregnant females, a transient drop in plasma P_4 below 2 ng/ml was observed between days 8 to 10 in llamas (Adams et al., 1991) and days 11 to 13 in alpacas (Fernandez-Baca et al, 1970b). In pregnant

llamas, by day 10 and later, plasma P_4 concentrations remain at ≥ 2 ng/ml, with another slight but significant decrease below mean P_4 concentration between days 18 - 24 (Adams et al., 1991). This has led to the hypothesis that the conceptus may be a factor in the rescue of the CL at a predetermined time of regression, which is reflected by an increase in size and function after an initial decrease (Smith et al., 1994). Ultrasound examination of the llama CL has shown corresponding changes in CL diameter and serum P_4 concentrations (Adams et al., 1991). Serum P_4 concentrations ≥ 1 ng/ml at day 21 after breeding is an indirect indication of pregnancy but may also be the result of a retained CL (Leon et al., 1990).

The secretion of P_4 by the CL is necessary to sustain pregnancy throughout gestation (Leon et al., 1990). Ablation of the CL in the pregnant llama or alpaca terminates pregnancy in 24 hours (Sumar and Bravo, 1991). If the CL is removed at 11 months of gestation, the pregnancy will continue to term, however the fetus will have a low birth weight (San Martin et al., 1968).

Puberty. The term puberty, first estrus accompanied by ovulation, does not apply to camelids due to the nature of their ovulatory cycles and, as such, puberty is difficult to define in these species. Puberty has been reported to occur as early as 5 months and as late as 2 to 3 years (Fowler, 1989). Bravo and Sumar (1989) reported that alpaca females are capable of mature ovarian activity by 11-12 months of age. In addition, 12-14 month old females do not differ in sexual behavior from adult females (Sumar et al., 1993). However, many young females with follicles ≥ 7 mm were anovulatory, suggesting that at this age the hypothalamic-pituitary axis requires additional stimuli in order to provide an ovulatory surge of LH (Sumar et al., 1993). The onset of puberty is greatly affected by nutritional status, as evidenced in the report by Smith et al. (1994) where 60% of adult body weight was required.

Pregnancy. The gestation period in llamas and alpacas is 335 to 365 days (Smith et al., 1994). A highly unusual observation in camelid pregnancy is an implantation rate of 95 to 98% in the left uterine horn (Fernandez-Baca et al.,

1970a). Furthermore, the CL is located on the right ovary in approximately 50% of these pregnancies indicating that an embryo conceived on the right must migrate to the left horn prior to implantation (Fernandez-Baca et al., 1979; Bravo and Varela, 1993; Fernandez-Baca et al., 1970a). The mechanism of how this occurs has not been elucidated. The histology and morphology of the left and right horns appear to be identical. The precise time of fetal membrane attachment has not been determined. Johnson (1989) reported that implantation begins at about day 30 and is complete by day 90, similar to the horse. Alpaca embryos have been found unattached to the uterus up to 30 days post-coitus (Fowler, 1989).

A possible explanation for the occurrence of almost exclusive left horn pregnancies was proposed by Fernandez-Baca et al. (1979) who postulated that the left uterine horn exerted both systemic and local luteolytic effects, while the right uterine horn exerted only local luteolytic effects. This was demonstrated by removing one or both uterine horns in alpacas that had a CL present on each ovary. Maintenance of both CLs was observed upon removal of both uterine horns. Removal of only the left horn prolonged the lifespan of the CL on the left, but the CL on the right showed early regression. Removal of only the right horn caused early regression of both the right and left CL. In support of this theory of differential luteolytic effects of the uterine horns, Del Campo et al. (1996) found a striking difference in the uterine vasculature of the SAC compared to other farm species. This group examined the vascular anatomy of llama and alpaca uteri and observed that 95% (30/32) of the females had a large branch of the right uterine artery that entered the left horn and a large venous branch of the left uterine vein that crossed over to the right uterine vein. Thus, PG released from the left uterine horn can drain into this additional uterine vein which crosses over to the right uterine vein and lies in close apposition to the right ovarian artery. This vascular arrangement would allow PG to reach the right ovary and regress any CL that may be present. In the remaining 5% (2/32) the vasculature was reversed, with an

additional right uterine vein branch crossing over and draining into the left uterine vein.

P₄ is elevated throughout pregnancy (≥ 1 ng/ml) and starts to decline 3 days before parturition (Leon et al., 1990). Estrogens are elevated during the last month of gestation when urinary estrone sulfate and pregnanediol glucuronide (PdG) are elevated. PdG starts to decline 5 days before parturition and estrone sulfate remains elevated until the initiation of expulsion of the term fetus (Bravo et al., 1991b).

Despite the approximate 10% to 15% occurrence of multiple ovulations (Fernandez-Baca, 1993; Bravo et al., 1990a; Adam et al., 1989), live-birth twins are an exceedingly rare occurrence in camelids with a 0% occurrence of alpaca twins reported (Fernandez-Baca et al., 1970a). Fowler (1990) reported 16 pairs of live-birth llama twins in the U.S. between September 1981 and November 1988. Several of these twins subsequently died. Twin conceptuses observed at 21 to 28 days of gestation are eventually aborted, resorbed, mummified, or stillborn. Occasionally, one twin is resorbed and the other carried to term (Smith et al., 1994). Free martinism has been reported in a female llama born to a male twin (Leipold et al., 1994).

Fowler and Olander (1990) described the placenta of the llama as epitheliochorial, similar to the mare or sow, with patchy areas of dense folded papillation. By day 60, the placenta has established a vascular network and full attachment to the uterine mucosa is completed by 60 to 90 days (Fowler, 1989). Between days 70 to 90, the surface of the chorion becomes dotted with numerous half-circular, domed projections that fit into corresponding depressions in the uterine mucosa. The attachment is tenuous and the chorion may be peeled from the mucosa with no resistance (Fowler, 1989). As pregnancy advances, the domes elongate to become rugose papillae. The elongation and development of leaf-like ridges increases the surface area and depth of interdigitation within the endometrium, serving to strengthen the attachment. The structure differs from that of the equine chorion, which is composed of extensively branched villi (Fowler and

Olander, 1990). It has been hypothesized that the nature of the placenta accounts for the lack of significant transplacental transfer of maternal immunoglobulins to the llama fetus (Smith et al., 1994). Most of the passive immunity passes to the newborn llama in colostral milk (Johnson, 1989).

A unique feature is the presence of an extra fetal membrane. This membrane is not maternally derived, but arises from fetal epidermis developing during the first trimester (Fowler and Olander, 1990). This membrane is an opaque whitish membrane approximately 1-2 mm thick (Fowler, 1989). It encases the entire fetal body and is lubricated by amniotic fluid facilitating the delivery of the conceptus (Merkt et al., 1988). The extraembryonic membrane is attached to the neonate at the mucocutaneous junctions such as the lips, nostrils, eyelids, ears, anus, vulva, and prepuce (Fowler, 1989). The extraembryonic membrane does not cover the nostrils or the mouth and, as such, poses little danger of postpartum suffocation if it is not immediately removed from the neonate (Fowler and Olander, 1990). Even with little movement or abrasion, the extraembryonic membrane soon dries out and withers away (Fowler, 1989). The extraembryonic membrane on premature fetuses is thicker and more durable than that of a full term fetus (Fowler and Olander, 1990). The function of this membrane is not known, but it is thought to be a high altitude, water conserving adaptation to the environment and may serve to lubricate the fetus for delivery (Smith et al., 1994). This suggestion is based on the observation of a minimal amount of uterine fluid in late term llama pregnancies.

Parturition. Parturition in llamas and alpacas usually occurs during the morning hours, probably as an adaptation to high altitude and cold environment (Bravo, 1994). Llamas in North America have been known to give birth at night and they seem to be able to regulate parturition as a voluntary activity (Fowler, 1989). Too much attention and excitement may delay delivery. Parturition lasts approximately 2 to 3 hours and occurs in three stages: stage 1, the prodromic stage (lasting 1.5 - 2 hours) begins with cervical relaxation and uterine contractions, stage 2 is the expulsion of the fetus (8-24 minutes), and stage 3 is placental expulsion

(usually occurring within 1-2 hours; Fowler, 1989). Normal delivery is in cranial presentation, dorso-sacral position, with the female usually standing (Bravo, 1994). The cria is covered with the epidermal membrane that quickly dries and disintegrates (Fowler and Olander, 1990). The dam does not lick the cria nor does she eat the placenta (Bravo, 1994).

Shortly after expulsion of the placenta, uterine involution begins and continues rapidly (Paul-Murphy, 1989). There is a dramatic reduction in uterine size, and expulsion of lochia occurs within the first 12 to 24 hours and may persist for 5 to 7 days (Smith et al., 1994). CL regression is complete by 3 days post-partum (Bravo et al, 1995). Post-partum females may be sexually receptive shortly after parturition. Ovarian follicular activity resumes 5 days post-partum with the ability to ovulate occurring between days 10-14 post-partum (Bravo et al, 1995). However, the uterus does not complete full involution until approximately 21 days post-partum (Bravo et al, 1995). Rebreeding the female is recommended between 20-30 days post-partum as this will allow the dam to potentially produce one offspring per year (Bravo et al., 1994).

Reproductive Aging. Llama pregnancies may occur into old age (> 15 years of age), however ovarian function may wane or cease entirely (Fowler, 1994). A few female llamas have had successful pregnancies in their 20's (Fowler, 1994). Ideally, a healthy female llama could produce a cria every year from the time she is 2 until she is 20. Several factors may contribute to the cessation of an aging female's reproductive function. These factors include previous uterine infection with subsequent scarring, diminished activity of the endocrine system, exhaustion of the finite number of potential follicles at birth, inadequate P₄ production from the CL, or other non-reproductive aging disorders (such as arthritis) that make mating difficult.

Reproduction in the Male SAC

Anatomy. The male anatomy is somewhat unique. The male SAC penis is approximately 35 to 40 cm in length and similar to the penis of the ram (Hoffman and Fowler, 1995). The penis is fibroelastic and has a pre-scrotal sigmoid flexure. The glans penis ends with a specialized cartilaginous process that is oriented clockwise and the adjacent urethral opening is at the base of the cartilaginous process rather than the tip (Bravo and Johnson, 1994). This cartilaginous process functions to penetrate the cervix and deposit semen into the uterine horns during copulation (Johnson, 1989; Smith et al., 1994). A prepuce with a posterior orientation covers the penis, thus males urinate in a caudal direction (Bravo and Johnson, 1994). Powerful protractor prepuce muscles pull the penis forward during erection. The prepuce is attached to the penis in the newborn. The detachment of the penis is initiated at 12 to 15 months of age, and starts from the tip and continues to the base. This process is related to testosterone concentrations and is usually complete by 22 to 26 months of age (Bravo et al., 1992b).

The testes of a mature llama are approximately 3 cm wide by 6 cm long by 3 cm deep and weigh approximately 24 g (Smith et al., 1994). The testes are non-pendulous and carried close to the body. The scrotum is small and has a location and orientation similar to the boar and dog (Bravo and Johnson, 1994). The scrotum's muscular structure allows it to contract in cold weather and relax slightly in warm weather (Bravo and Johnson, 1994). The accessory sex glands include a small prostate gland dorsolateral to the pelvic urethra and paired bulbourethral glands lateral to the base of the penis (Smith et al., 1994). Male camelids do not have seminal vesicles (Bravo and Johnson, 1994). Total seminal volume is small, approximately 3 ml. Lichtenwalner et al., (1996b) examined semen characteristics of 10 llamas by collection using an artificial vagina. A mean concentration of 1 million sperm/ml with 23.7% motility was observed. The low values for motility were speculated to be due to the viscous nature of llama semen.

Puberty. Puberty in the male is delayed as compared to the female. Histologic studies of alpaca testes indicate the presence of Leydig cells and spermatozoa between 15-18 months of age (Montalvo et al., 1979). Although spermatozoa have been detected in animals as young as 10 to 12 months of age, maturity is not reached until detachment of the preputial adhesions (Sumar, 1983). Yearling males display sexual interest in females, however intromission can not occur with the presence of adhesions. Approximately 70% of males are free of the adhesions at 2 years of age, and 100% at 3 years of age (Sumar, 1996). In other farm species, adhesions usually detach before viable spermatozoa are present in the ejaculate (Hafez, 1993).

Growth of the testes is slow, reaching a plateau at 30 months of age (Bravo and Johnson, 1994). Testosterone (T) concentrations are low during the first 19 months of age (35-90 pg/ml), increase exponentially at 21 months of age (300 pg/ml), and reach a plateau at 30 months of age (650 pg/ml; Bravo et al., 1992b). Reed (1996) measured the response of LH and T to GnRH challenge in male llamas 8 to 20 months of age. Reed observed no response of LH or T six hours after 1 µg/kg GnRH challenge in males 8 to 20 months of age. T levels only increased in llamas at 20 months of age 6 hours after GnRH challenge. This is in contrast to lambs that exhibited an increase in LH upon GnRH challenge at 6 weeks with a response that decreased in magnitude and was delayed at later ages. T response to GnRH in lambs increased with age (Wilson and Lapwood, 1979). Most alpacas and llamas are first utilized for breeding purposes at 2 to 3 years of age.

Reproductive Oriented Behavior

Communication. Camelids exhibit distinct behavior and communication prevalent in natural and domesticated environments. Interactions are both visible and audible including ear and tail signals as well as vocalizations. Many of these behaviors are associated with reproduction (Fowler, 1989). In the wild, camelids

congregate in family groups led by a territorial male. The territorial male is aggressive towards other males and his communicative behaviors are strongly linked to reproductive activities. All other mature males are excluded from the family group. In domesticated herds, the same arrangement is observed but to a lesser degree (Franklin, 1981; Hoffman and Fowler, 1995).

Body postures are highly identifiable and frequently used to communicate. Males commonly demonstrate a "broadside display" to warn off other mature males. The posture is a sideways rigid stance, ears pinned back, mouth open slightly, and nose pointed up. Non-receptive females will also display this behavior to warn off the male (Hoffman and Fowler, 1995).

Communication and behavior is most notable through vocalizations, many associated with reproduction. Humming is a bonding sound and can indicate loneliness or a sign of worry but is never an aggressive behavior. Mothers and cria often hum to each other (Hoffman and Fowler, 1995). Spitting and orgling are two universal communicative traits of camelids. Spitting occurs at times of displeasure, and is also performed by non-receptive females to discourage an interested male. Orgling is a distinctive guttural vocalization made by the male camelid throughout copulation. The distinctive Flehmen response is another reproductive oriented behavior common to both camelids and ruminants. The response occurs after the male sniffs a female's dung pile or vulva. He tilts his head backward while curling the upper lip to determine the reproductive state of the female (Franklin, 1981). There is evidence to suggest that the males detect receptive females by detecting estrogen conjugates in the female's urine (Bourke et al., 1992).

Mating. Domesticated female camelids can breed year round. However, SAC breed seasonally during the rainy season in their native environment (Smith et al., 1994; San Martin et al., 1968). When a male is introduced to a herd of females, mating behavior will occur immediately with a receptive female. The female will lie down in the "cush" position (sternal recumbency) and she will calmly accept the male. During copulation, the male makes the orgling vocalization. Often other

receptive females will lie down beside the mating pair (Smith et al., 1994; Fernandez-Baca, 1993). Copulation lasts for 3 to 65 minutes with an average duration of 20 minutes, which is entirely determined by the male.

Ejaculation begins early during copulation and lasts for the duration of mating (Fernandez-Baca et al., 1970c). Llamas have been described as continuous ejaculators, discharging semen continuously throughout the period of copulation (Johnson 1989). However, Lichtenwalner et al. (1996a) used transrectal digital palpation of urethral pulses to define the ejaculatory pattern of llamas during copulation. During the first 4 minutes of copulation, intermittent urethral pulses occurred at a slow rate. After 4 minutes large numbers of urethral pulses occurred, and each cluster was accompanied by a whole-body strain. Each cluster was composed of 4 to 5 urethral pulses and 18 to 19 of these clusters occurred during the period of copulation. Each cluster lasted approximately 20 seconds in duration with approximately 1 cluster per minute. Between clusters, a few urethral pulses occurred sporadically and at a slow rate. The authors proposed that each cluster of urethral pulses accompanied by a whole body strain is a single distinct ejaculation and that the male llama ejaculates 18 to 19 times during a single 22-minute copulation.

In a separate study, Lichtenwalner et al. (1996b) observed emission and ejaculation throughout copulation. Sperm concentrations increased significantly after 10 to 15 minutes of copulation. If ejaculation was continuous, as previously proposed, one would expect sperm concentrations to decrease due to increasing dilution by accessory sex gland secretions following an initial emission of spermatozoa into the pelvic urethra followed by slow expulsion. Increasing sperm concentrations would be consistent with the theory that the male llama undergoes multiple ejaculations and emissions, rather than a single ejaculation and a prolonged, continuous emission.

Maternal Recognition of Pregnancy

Maternal recognition of pregnancy may be the most important factor influencing survival of the embryo and a successful pregnancy. Maternal recognition of pregnancy is the series of events in which the maternal reproductive tract responds to a signal from the conceptus preventing the events which lead to destruction of the CL/or luteolysis. The signal from the conceptus must be produced at a precise time and in adequate amounts to maintain CL function and adequate P_4 production. P_4 stimulates and maintains endometrial functions that are necessary for early embryonic development, implantation, placentation, and successful fetal and placental development. A luteotrophic substance is one that acts directly on the CL to maintain its functional and structural integrity. Luteinizing hormone, sometimes referred to as the master hormone regulating luteal function, is released by gonadotropes of the anterior pituitary, and stimulates the follicle to differentiate into a CL after ovulation. LH is required to maintain the normal secretory function of the CL during the luteal phase of the ovarian cycle. However, an additional luteotrophin is needed in some species to switch the CL from a CL of the ovarian cycle to a CL of pregnancy.

Primates have a uterine-independent ovarian cycle. Luteolytic events responsible for regression of the CL at the end of a menstrual cycle result from intraovarian effects of PG, oxytocin, or other luteolytic agents (Johnson and Everitt, 1995). Chorionic gonadotropin (CG) produced by the primate placenta is the luteotrophic signal in primates and appears to act directly on the CL via LH receptors. Luteolysis is thereby inhibited by the luteotrophic signal provided by CG.

In contrast, sub-primates have a uterine-dependent ovarian cycle. Conceptuses of these species produce proteins, steroids and/or PG which inhibit uterine production of $PGF_{2\alpha}$, the potent luteolytic substance. In ruminants, a conceptus-secreted protein has been identified as an anti-luteolytic agent. Ovine trophoblast

protein-1 (oTP-1), a Type-1 interferon (IFN), is secreted by the mononuclear cells of the trophoblast between days 10 and 20 of pregnancy in the sheep (Imakawa et al, 1987). This trophoblastic protein has a molecular weight of 19,000, high amino acid sequence homology with IFN- ω (α_{II}), and potent antiviral, antiproliferative and immunosuppressive activities (Pontzer et al., 1988). A homologous protein, bovine trophoblast protein-1 (bTP-1), is produced by the bovine conceptus at the time of maternal recognition of pregnancy with maximal secretion between days 16-19 of pregnancy (Helmer et al., 1987). Homology between oTP-1 and bTP-1 has been determined to be 90% and 80% at the nucleotide level and the amino acid sequence level, respectively (Roberts et al., 1992). The goat gene shares 96% nucleotide sequence similarity to oTP-1 (Leaman and Roberts, 1992). Because of the unique developmental expression of oTP-1 and bTP-1 and similarity to other Type 1 interferons (α , β , and ω), oTP-1 and bTP-1 were reclassified as IFN τ (τ for trophoblast) by the committee on IFN nomenclature of the International Interferon Society (Roberts et al., 1992).

Successful establishment of pregnancy in ruminants requires that the pulsatile release of uterine PGF_{2 α} be eliminated or diminished to prevent the cascade of luteolytic events leading to the demise of the CL and loss of P₄ production. IFN τ produced by the ruminant conceptus during the period of pregnancy recognition inhibits development of the endometrial luteolytic mechanism and pulsatile release of PGF_{2 α} (Bazer, 1992). Endometrial production of luteolytic PGF_{2 α} pulses in ruminants is dependent on the effects of ovarian steroids, which regulate oxytocin receptor (OTR) gene expression (Wathes and Lamming, 1995). Estrogen increases estrogen receptor (ER), progesterone receptor (PR) and OTR gene expression in the endometrium (Spencer et al., 1995). Oxytocin, from the CL or posterior pituitary, binds to endometrial OTR receptors causing PGF_{2 α} secretion. This cascade leads to the pulsatile secretion of PGF_{2 α} , and hence luteolysis. IFN- τ exerts a paracrine, antiestrogenic effect on the endometrium to suppress ER and OTR expression

(Spencer et al., 1995). Spencer and Bazer (1996) demonstrated that oIFN- τ suppresses transcription of the ER and OTR genes in the ovine endometrium. They concluded that the antiluteolytic action of IFN- τ prevents the expression of the ER gene and prevents estrogen-induced increases in OTR gene expression in the endometrium. Consequently, the pulsatile release of PGF $_{2\alpha}$ is dampened and regression of the CL is prevented. Genes for trophoblast interferons have been found in sheep, goats, cattle, musk oxen, and giraffes, but not in swine, hippopotamus, horses, zebras or llamas (Leaman and Roberts, 1992).

The ovarian cycle in swine is also uterine-dependent. In the cycling pig, PGF $_{2\alpha}$ is secreted in an endocrine direction, towards the uterine vasculature and transported to the CL to exert a luteolytic effect (Bazer, 1989). However, in pregnant pigs the direction of secretion of PGF $_{2\alpha}$ is exocrine, into the uterine lumen, where it is sequestered to exert its biological effects in utero or be metabolized, thus preventing luteolysis (Bazer, 1992). It is thought that estradiol secreted by the porcine conceptus induces endometrial receptors for prolactin, which may allow prolactin to induce calcium cycling across the epithelium and redirect secretion of PGF $_{2\alpha}$ into the uterine lumen (Bazer et al., 1989). In the pig, maternal recognition of pregnancy signals from pig blastocysts begin at approximately Day 11 and coincide with the initiation of estrogen synthesis by the blastocyst and blastocyst elongation. Furthermore, administration of exogenous estrogens between Days 11 and 15 of the estrous cycle prolongs luteal function in pigs to an average of about 120 days (Geisert et al., 1982). In this manner, estradiol may also have a direct luteotrophic effect in addition to its role in redirecting PGF $_{2\alpha}$ release into the uterine lumen.

Maternal recognition of pregnancy in the horse has not been fully elucidated. The equine conceptus migrates between uterine horns 12-14 times per day between Days 12 and 18 of pregnancy to inhibit endometrial PGF $_{2\alpha}$ production and protect the CL (Ginther, 1984). Migration of the equine conceptus is thought to be

necessary to prevent luteolysis, as the equine conceptus does not elongate like swine and ruminant embryos (McDowell et al., 1988). Coincubation of conceptus and endometrium reduces $\text{PGF}_{2\alpha}$ production by endometrium in the mare (Berglund et al., 1982). The substance produced by the conceptus to prevent endometrial $\text{PGF}_{2\alpha}$ production is not known. In addition, equine conceptuses produce increasing amounts of $\text{E}_2\beta$ between Days 8 and 20 of gestation. However, attempts to prolong the lifespan in mares by injecting estrogens have provided conflicting results (Sharp et al., 1989).

Embryo Migration

Intrauterine migration and approximate equidistant spacing of embryos are an inherent characteristic of litter-bearing species. In the sow, intrauterine migration and spacing occurs between Days 7 and 12 of gestation (Dhindsa et al., 1967). Increased synthesis of estradiol by the porcine embryo occurs concomitantly with migration of the embryos and increased myometrial activity in vitro (Pope et al., 1982a). In addition, flushings of Day 12 pregnant gilts overcame the inhibitory effects of indomethacin, suggesting an indirect action of estradiol on myometrial activity. In another study, small spherical beads of Silastic glue, containing physiological amounts of $\text{E}_2\beta$, have been observed to stimulate migration as compared with cholesterol-impregnated beads (Pope et al., 1982b). Estrogen produced by the conceptus may be able to override the quiescent influence of P_4 , thereby inducing local myometrial contractility and allow embryo migration and spacing in this polyovular species.

Transuterine migration of the embryo also occurs in the mare. Ultrasound studies have shown that the equine conceptus is highly mobile within the uterine lumen between Days 11 and 15 with no movement detected after Day 17 (Ginther, 1983). Mobility is necessary to prevent luteolysis (McDowell et al., 1988). Coincidentally, from Days 8 to 20, the equine conceptus produces increasing

quantities of estrogens coinciding almost exactly with the period of increased conceptus mobility (Sharp et al., 1989). Ginther (1984) demonstrated that regardless of the side of ovulation, which occurred with equal frequency, final attachment occurred significantly more frequently in the right horn (66%) in barren and maiden mares but not in lactating mares. It is not known whether conceptus estrogens play a direct role in transuterine migration in mares. Both horses and pigs have a non-invasive epitheliochorial placenta and it is possible that similar mechanisms drive embryo motility, recognition and implantation.

Estrogen as a Mediator of Female Reproductive Function

Natural estrogens have 18 carbons (C18 steroids) with an aromatized A ring, hydroxylated at C3 and with a β -hydroxyl group at C17. Estrogens are produced by the granulosa cells of the ovary, the placenta, and by the Sertoli cells in the male. Estrogens exert a wide range of biological actions to regulate reproduction in the female reproductive system. Estrogen acts on both the tonic and surge centers of the hypothalamus via negative and positive feedback respectively, thus regulating the production and release of gonadotropins. Estrogen also acts directly on the anterior pituitary via negative feedback to regulate the release of gonadotropins. Estrogen induces sexual receptivity to the male and behavior associated with heat or estrus. Estrogen also supports secondary sex characteristics of the female.

In the uterus, estrogen is responsible for the initial preparation of pregnancy. Estrogen exerts a uterotrophic effect by enhancing endometrial glandular development which involves massive increases in RNA and protein synthesis, cellular division and growth. Stromal cells proliferate under the influence of estrogen. The endometrial epithelium increases in surface area and metabolic activity. In some species (primate and large farm animals), an increase in number and size of glandular invaginations of the stroma is seen. These estrogen-primed

cells secrete a watery fluid that contains a wide variety of proteins. The myometrium increases both its contractility and excitability and shows increased spontaneous activity under the influence of estrogen. In addition, estrogen potentiates the effects of oxytocin and $\text{PGF}_{2\alpha}$. Estrogen induces the expression of PR in the endometrium, thereby “priming” the uterus for the secretory phase when P_4 is the dominant hormone (Johnson and Everitt, 1995).

Estrogens have several actions within the ovary including maturation of follicles, increasing FSH and LH receptor expression in granulosa cells, and modulating steroid production in granulosa and thecal cells. Estrogen is luteolytic in ruminants but luteotrophic in swine.

Estrogens are necessary for mammary gland development. Before puberty, little mammary gland development has occurred. After puberty, under the influence of estrogen, lactiferous ducts spout and proliferate. At the tips of each branch, small, solid, masses of cells form which will later become milk producing alveoli.

Estrogens regulate cervical mucus production, with many species having a dramatic increase in mucus production at estrus. Additionally, estrogens enhance sperm penetration of mucus while P_4 inhibits penetration. In some mammals (humans, rodents), estrogens induce an increased mitotic activity in the columnar epithelium of the vagina, with a tendency to keratinize. Cyclic changes in the vagina, induced by estrogen and P_4 , result in bacterial generation of volatile aliphatic acids giving distinctive odors to vaginal secretions. In some species these odors may play a role in the male’s detection of females in estrus (Johnson and Everitt, 1995).

Biological effects of estrogens are mediated through specific receptors located within the cell nucleus that operate as ligand-induced transcription factors (Beato, 1989; O’Malley, 1990). Once bound by estrogens, the ER undergoes a conformational change, allowing the receptor to bind with high affinity to

chromatin and to modulate transcription of target genes (Murdoch and Gorski, 1991). Steroid hormone receptors consist of: a hypervariable N-terminal domain that contributes to the transactivation function, a highly conserved central domain responsible for specific DNA binding, dimerization, and nuclear localization, and a C-terminal domain involved in ligand binding and ligand-dependent transactivation function (Clark et al., 1992).

Until 1996, it was thought that there was only one form of the ER. In 1987, Koike et al. (1987) cloned the rat estrogen receptor which encoded a protein of 600 amino acids and had a calculated molecular weight of 67,029. Kuiper et al. (1996) cloned a novel rat ER cDNA from prostate which they named ER β subtype. ER β was characterized as encoding a protein of 485 amino acid residues with a calculated molecular weight of 54,200. Rat ER β protein is highly homologous to rat ER α , particularly in the DNA binding domain (> 90% amino acid identity) and in the C-terminal ligand-binding domain (55%). Kuiper et al. (1997) described the differences between ER α and ER β in relative ligand binding affinity and tissue distribution. Tissue distribution was found to be quite different between the two ER subtypes. ER α was found to have moderate to high expression in the uterus, testis, pituitary, ovary, kidney, epididymis, and adrenal. The prostate, ovary, lung, bladder, brain, uterus, and testis exhibited moderate to high expression of ER β . Ligand binding abilities were also different between the subtypes. Using competitive binding with 16 α -[¹²⁵I]ioda-17 β -estradiol, Kuiper et al (1997), demonstrated that most estrogenic substances or estrogenic antagonists tested competed for binding to both ER subtypes in a very similar preference and degree as follows; diethylstilbesterol > hexestrol > dienestrol > 4-OH-tamoxifen > 17 β -estradiol > coumesterol > estrone, 17 α -estradiol > nafoxidine, moxestrol > clomifene > estriol, 4-OH-estradiol > tamoxifen, 2-OH-estradiol, 5-androstene-3 β , 17 β -diol, genistein for ER α protein and dienestrol > 4-OH-tamoxifen > diethylstilbesterol > hexestrol > coumesterol > 17 β -estradiol > estrone, genestein

>estriol > nafoxidine, 5-androstene-3 β , 17 β -diol > 17 α -estradiol, clomifene, 2-OH-estradiol > 4-OH-estradiol, tamoxifen, moxestrol for ER β protein. These differences in relative ligand binding and tissue distribution could contribute to the selective action of ER agonists and antagonists in target tissues.

In species where estrogen is involved in both maternal recognition of pregnancy and embryo migration, embryonic estrogens interacting with the differentially expressed ER subtypes in the uterus and ovary may explain the multitude of physiologic effects exhibited by this hormone.

References

- Adam CL, Moir CE, Shiach P. Plasma progesterone concentrations in pregnant and non-pregnant llamas (*lama glama*). Vet Rec 1989; 125:618-620.
- Adams GP, Sumar J, Ginther OJ. Effects of lactational and reproductive status on ovarian follicular waves in llamas (*lama glama*). J Reprod Fert 1990; 90:535-545.
- Adams GP, Sumar J, Ginther OJ. Form and function of the corpus luteum in llamas. Animal Reprod Sci 1991; 24:127-138.
- Bazer FW. Establishment of pregnancy in sheep and pigs. Reprod Fertil Dev 1989; 1:237-242.
- Bazer FW. Mediators of maternal recognition of pregnancy in mammals. Proc Soc Exp Biol Med 1992; 199:373-384.
- Bazer FW, Vallet JL, Harney JP, Gross TS, Thatcher WW. Comparative aspects of maternal recognition of pregnancy between sheep and pigs. J Reprod Fertil 1989; 37(suppl):85-889.
- Beato M. Gene regulation by steroid hormones. Cell 1989; 56:335-344.
- Berglund LA, Sharp DC, Vernon MW, Thatcher WW. Effect of pregnancy status and collection technique on prostaglandin F in the uterine lumen of pony mares. J Reprod Fertil 1982; 32(suppl):335-341.
- Bourke DA, Adam CL, Kyle CE. Ultrasonography as an aid to controlled breeding in the llama (*lama glama*). Vet Rec 1992; 130:424-428.
- Bravo PW. Reproductive endocrinology of llamas and alpacas. Vet Clin North Am Food Anim Prac 1994; 10:265-279.
- Bravo W, Fowler ME. Basic physiology of reproduction in female llamas. Llamas 1990; 35-37.
- Bravo PW, Johnson LW. Reproductive physiology of the male camelid. Vet Clin North Am Food Anim Prac 1994; 10:259-264.
- Bravo PW, Sumar J. Laparoscopic examination of the ovarian activity in alpacas. Animal Reprod Sci 1989; 21:271-281.

Bravo PW, Varela MH. Prenatal development of the alpaca (*Lama pacos*). *Animal Reprod Sci* 1993; 32:245-252.

Bravo PW, Fowler ME, Stabenfeldt GH, Lasley BL. Endocrine responses in the llama to copulation. *Theriogenology* 1990a; 33(4):891-899.

Bravo PW, Fowler ME, Stabenfeldt GH, Lasley BL. Ovarian follicular dynamics in the llama. *Biol Reprod* 1990b; 43:579-585.

Bravo PW, Stabenfeldt GH, Lasley BL, Fowler ME. The effect of ovarian follicle size on pituitary and ovarian responses to copulation in domesticated South American Camelids. *Biol Reprod* 1991a; 45:553-559.

Bravo PW, Stabenfeldt GH, Fowler ME, Lasley BL. Urinary steroids in the periparturient and postpartum periods through early pregnancy in llamas. *Theriogenology* 1991b; 36:267-278.

Bravo PW, Stabenfeldt GH, Fowler ME, Lasley BL. Pituitary response to repeated copulation and/or gonadotropin-releasing hormone administration in llamas and alpacas. *Biol Reprod* 1992a; 47:884-888.

Bravo PW, Stabenfeldt GH, Fowler ME, Lasley BL, Frey RE. Testes development and testosterone concentrations in the llama (*Lama glama*). In: *Proceedings of 12th International Congress Animal Reproduction*, the Hague, the Netherlands, 1992b; 4:1698-1700.

Bravo PW, Fowler ME, Lasley BL. The postpartum llama: Fertility after parturition. *Biol Reprod* 1994; 51:1084-1087.

Bravo PW, Lasley BL, Fowler ME. Resumption of ovarian follicular activity and uterine involution in the postpartum llama. *Theriogenology* 1995; 44:783-791.

Clark JH, Schrader WT, O'Malley BW. Mechanisms of action of steroid hormones. In: Wilson J, Foster DW (eds.) *Textbook of Endocrinology*, WB Saunders Company, Philadelphia, PA, 1992; 35-90.

Del Campo MR, Del Campo CH, Ginther OJ. Vascular provisions for a local utero-ovarian cross-over pathway in New World Camelids. *Theriogenology* 1996; 46:983-991.

Dhindsa DS, Dzuick PJ, Norton HW. Time of transuterine migration and distribution of embryos in the pig. *Anat Rec* 1967; 159:325.

England BG, Foote WC, Cardoza AG, Matthews, DH, Riera S. Ovulation and corpus luteum function in the llama (*Lama glama*). J Endocr 1969; 45:505-513.

Fernandez-Baca S. Manipulation of reproductive functions in male and female New World camelids. Animal Reprod Sci 1993; 33:307-323.

Fernandez-Baca S, Hansel W, Novoa C. Embryonic mortality in the alpaca. Biol Reprod 1970a; 3:243-251.

Fernandez-Baca S, Hansel W, Novoa C. Corpus luteum function in the alpaca. Biol Reprod 1970b; 3:252-261.

Fernandez-Baca S, Madden DHL, Novoa C. Effect of different mating stimuli on induction of ovulation in the alpaca. J Reprod Fert 1970c; 22:261-267.

Fernandez-Baca S, Hansel W, Saatman R, Sumar J, Novoa C. Differential luteolytic effects of right and left uterine horns in the alpaca. Biol Reprod 1979; 20:586-595.

Fowler ME. Medicine and Surgery of South American Camelids. Ames, Iowa State University Press, 1989.

Fowler ME. Twinning in llamas. Llamas 1990; 4:35-38.

Fowler ME. Health care of the geriatric llama and alpaca. Vet Clin North Am Food Anim Prac 1994; 10(2): 391-399.

Fowler ME, Olander HJ. Fetal membranes and ancillary structures of llamas (*Lama glama*). Am J Vet Res 1990; 51:1495-1500.

Franklin WL: Biology, ecology and relationship to man of the South American camelids. In: Mammalian Biology in South America. Vol 6, Special Publication Series Pymatuning Laboratory of Ecology, University of Pittsburgh, Linesville, PA; 1981.

Geisert RD, Thatcher WW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig: III. Endometrial secretory response to estradiol valerate administered on day 11 of the estrous cycle. Biol Reprod 1982; 27:957-965.

Ginther OJ. Mobility of the early equine conceptus. Theriogenology 1983; 19:603-11.

Ginther OJ. Intrauterine movement of the early conceptus in barren and postpartum mares. *Theriogenology* 1984; 21:633-64.

Hafez ESE (Ed). *Reproduction in Farm Animals*. 6th edition, Lea and Febiger, Philadelphia, PA, 1993.

Helmer SD, Hansen PJ, Anthony RV, Thatcher WW, Bazer FW, Roberts RM. Identification of bovine trophoblast protein-1, a secretory protein immunologically related to ovine trophoblast protein-1. *J Reprod Fert* 1987; 79:83-91.

Hoffman E, Fowler ME. *The Alpaca Book: Management, Medicine, Biology and Fiber*. Herald, CA: Clay Press Inc; 1995.

Imakawa K, Anthony RV, Kazemi M, Marotti KR, Polites HG, Roberts RM. Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. *Nature* 1987; 330:377-379.

Johnson LW. Llama reproduction. *Vet Clin North Am Food Anim Prac* 1989; 5:159-182.

Johnson MH, Everitt BJ. *Essential Reproduction*. 4th edition, Blackwell Science, Oxford, England, 1995.

Koike S, Masaharu S, Muramatsu M. Molecular cloning and characterization of rat estrogen receptor cDNA. *Nucleic Acids Res* 1987; 15:2499-2513.

Kuiper GGJM, Enmark E, Peltö-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 1996; 93:5925-5930.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson, JA. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β . *Endocrinology* 1997; 138:863-870.

Leaman DW, Roberts RM. Genes for trophoblast interferons in sheep, goat, musk ox, and distribution of related genes among mammals. *J Interfer Res* 1992; 12:1-11.

Leipold HW, Hiraga T, Johnson LW. Congenital defects in the llama. *Vet Clin North Am Food Anim Prac* 1994; 10:401-419.

Leon, JB, Smith BB, Timm KI, LeCren G. Endocrine changes during pregnancy, parturition, and the early post-partum period in the llama (*lama glama*). J Reprod Fert 1990; 88:503-511.

Lichtenwalner AB, Woods GL, Weber JA. Ejaculatory pattern of llamas during copulation. Theriogenology 1996a; 46:285-291.

Lichtenwalner AB, Woods GL, Weber JA. Seminal collection, seminal characteristics and pattern of ejaculation in llamas. Theriogenology 1996b; 46:293-305.

McDowell KJ, Sharp DC, Grubaugh W, Thatcher WW, Wilcox CJ. Restricted conceptus mobility results in failure of pregnancy maintenance in mares. Biol Reprod 1988; 39:340-348.

Merkt H, Boer M, Schoon HA. The presence of an additional fetal membrane and its function in the newborn guanaco (*lama guanacoe*). Theriogenology 1988; 30:437-439.

Murdoch FE, Gorski J. The role of ligand in estrogen receptor regulation of gene expression. Mol Cell Endocrinol 1991; 78:C103-C108.

Novoa C. Reproduction in the camelidae. J Reprod Fert 1970; 22:3-20.

O'Malley B. The steroid receptor superfamily: more excitement predicted for future. Molecular Endocrinology 1990; 4:363-369.

Paul-Murphy J. Obstetrics, neonatal care, and congenital conditions. Vet Clin North Am Food Anim Prac 1989; 5:183-202.

Pollard JC, Littlejohn RP, Scott IC. The effects of mating on the sexual receptivity of female alpacas. Animal Reprod Sci 1994; 34:289-297.

Pontzer CH, Torres BA, Vallet JL, Bazer FW, Johnson HM. Antiviral activity of the pregnancy recognition hormone ovine trophoblast protein-1. Biochem Biophys Res Comm 1988; 152:801-807.

Pope WF, Maurer RR, Stormshak F. Intrauterine migration of the porcine embryo-Interaction of embryo, uterine flushings and indomethacin on myometrial function in vitro. J Anim Sci 1982a; 55:1169-1178.

Pope WF, Maurer RR, Stormshak F. Intrauterine migration of the porcine embryo-Influence of estradiol 17- β and histamine. *Biol Reprod* 1982b; 27:575-579.

Reed PJ. Studies on luteinizing hormone and gonadal steroids in male and female llamas (*Lama glama*). Corvallis, OR: Oregon State University; 1996. Thesis.

Roberts RM, Cross JC, Leaman DW. Interferons as hormones of pregnancy. *Endocrine Rev* 1992; 13:432-452.

San-Martin M, Copaira M, Zuniga J, Rodreguez R, Bustinza G, Acosta L. Aspects of reproduction in the alpaca. *J Reprod Fert* 1968; 16:395-399.

Sharp DC, McDowell KJ, Weithenauer J, Thatcher WW. The continuum of events leading to maternal recognition of pregnancy in mares. *J Reprod Fert* 1987; 37(suppl):101-107.

Smith CL, Peter AT, Pugh DG. Reproduction in llamas and alpacas: A review. *Theriogenology* 1994; 41: 573-592.

Spencer TE, George P, Becker WC, Ogle TF, Mirando MA, Bazer FW. Ovine interferon- τ regulates endometrial receptor for estrogen and oxytocin, but not progesterone. *Biol Reprod* 1995; 53:732-745.

Spencer TE, Bazer FW. Ovine interferon tau suppresses transcription of the estrogen receptor and oxytocin receptor genes in the ovine endometrium. *Endocrinology* 1996; 137:1144-1147.

Sumar J. Studies on reproductive pathology in alpacas. Uppsala, Sweden: Swedish University of Agricultural Science; 1983. Thesis.

Sumar J. Reproduction in alpacas and llamas. *Animal Reprod Sci* 1996; 42:405-415.

Sumar J, Bravo PW. In situ observation of the ovaries from llamas and alpacas. *J Vet Med Assoc* 1991; 199:1159.

Sumar J, Fredrikssen G, Alarcon V, Kindahl H, Edqvist L-E. Levels of 15-keto-13,14-dihydro-PGF $_{2\alpha}$, progesterone and oestradiol-17 β after induced ovulations in llamas and alpacas. *Acta Vet Scand* 1988; 29:339-346.

Sumar J, Bravo PW, Foote WC. Sexual receptivity and time of ovulation in alpacas. *Small Rum Res* 1993; 11:143-150.

Wathes DC, Lamming GE. The oxytocin receptor, luteolysis and the maintenance of pregnancy. *J Reprod Fertil* 1995; 49 (suppl):53-7.

Wiepz DW, Chapman RJ. Non-surgical embryo transfer and live birth in a llama. *Theriogenology* 1985; 24:251-257.

Wilson PR, Lapwood KR. Studies of reproductive development in Romney rams: II. LH and testosterone secretion following single or repeated doses of gonadotropin releasing hormone (GnRH). *Biol Reprod* 1979; 20:971-975.

CHAPTER 3

ESTRADIOL PRODUCTION BY THE PREIMPLANTATION LLAMA EMBRYO

Abstract

The embryonic signal that allows the continued function of the corpus luteum for pregnancy is not known in the llama. Llama embryos conceived on the right side migrate to the left uterine horn with 95-98% of pregnancies implanting in the left uterine horn. Other species that experience uterine migration, such as the horse and pig, have embryos that secrete large amounts of estradiol early in preimplantation development. The objective of this research was to determine if the llama embryo produces estradiol during the presumed period of maternal recognition of pregnancy. Llamas were superovulated with 1000 IU eCG and mated 7 d later (Day 0 = day of mating). Embryos were collected non-surgically on Days 7, 9, or 11 or at necropsy on Days 13 and 15. Embryos were cultured for 48 h in Ham's F-12 + 0.15% BSA. Medium was withdrawn every 24 h and replaced with fresh medium. Medium was assayed for estradiol-17 β by RIA. Measurable secretion of estradiol was detected in Day 7 embryos with levels increasing by day of gestation ($P < 0.05$). Estradiol production (pg/embryo) over the 48 h culture was greater ($P < 0.05$) by Day 11 embryos compared to Day 7 embryos. A dramatic rise in estradiol production was observed in Day 13 embryos. Estradiol production by Day 13 embryos increased approximately 50-fold ($P < 0.05$) compared to Day 11 embryos and was greatest ($P < 0.05$) by Day 15 embryos. Estradiol produced by the llama conceptus may play a role in maternal recognition of pregnancy and may also be involved in conceptus migration.

Introduction

The embryonic signal that initiates maternal recognition of pregnancy in the llama is not known. Llamas are induced ovulators with ovulation occurring 1.8 to 2 d

post-coitus (Adams et al., 1991; Bravo and Sumar, 1989; Bravo et al., 1990).

Serum progesterone concentrations ≥ 1 ng/ml are detectable by Day 4-5 (Day 0 = day of mating) and the corpus luteum (CL) starts to regress by approximately Day 11 or 12 (Adam et al., 1989) with females returning to receptive behavior as early as Day 13-14 (Fernandez-Baca et al, 1970). In pregnant females, a transient drop below 2 ng/ml is observed between Days 8-10 but plasma progesterone concentrations remain ≥ 2 ng/ml past Day 10 (Adams et al., 1991). Successive surges of 15-keto-13, 14-dihydro- $\text{PGF}_{2\alpha}$ are evident on Days 10-12 days following a sterile mating (Sumar et al., 1988). Maternal recognition of pregnancy would likely occur between Days 9-13 to prevent the destruction of the CL from prostaglandins.

The ruminant conceptus produces interferon-tau which acts on the uterine epithelium to suppress estrogen receptor and oxytocin receptor gene expression, thus preventing uterine release of luteolytic pulses of prostaglandins (Bazer et al., 1996). However, Leaman and Roberts (1992) did not find the interferon tau gene in the llama. Another possible candidate for the maternal recognition of pregnancy signal in llamas is estradiol. In the pig, maternal recognition of pregnancy occurs between Days 11-12 of gestation when the conceptus changes from a spherical to a filamentous form. Coinciding with this morphological change, is the initiation of estrogen production by the blastocyst which is considered the maternal recognition of pregnancy signal in pigs (Bazer and Thatcher, 1977). In addition, estradiol has been found to be important in the equidistant spacing of embryos in the pig uterus. Increased synthesis of estradiol by the pig embryo occurs concomitantly with embryo migration and increased myometrial activity in vitro (Pope et al., 1982a). In the mare, the conceptus migrates between uterine horns 12-14 times per day between Days 12-18 of pregnancy to inhibit endometrial $\text{PGF}_{2\alpha}$ production and this coincides with the production of increasing amounts of estradiol by the equine conceptus between Days 8-20 (Ginther, 1984).

In the llama, 95 to 98% of pregnancies are found in the left uterine horn with the CL located on the right ovary in approximately 50% of these pregnancies (Fernandez-Baca et al., 1970). Therefore, an embryo conceived on the right must migrate to the left horn prior to implantation (Fernandez-Baca et al., 1979; Bravo and Varela, 1993). Skidmore et al. (1994) observed aromatase activity and considerable quantities of estrogen synthesized by the camel conceptus between Days 10-33 after ovulation. The highest proportion of estrogen was in the form of estradiol in contrast to the higher estrone:estradiol ratio observed in pig (Perry et al., 1973) and horse embryos (Heap et al., 1982). The objective of this study was to determine if the llama conceptus produces estradiol during the presumed period of maternal recognition.

Material and Methods

Recovery of Embryos. A total of 19 reproductively normal females and 2 fertile male llamas from the Oregon State University-College of Veterinary Medicine herd were used for this project. All animals assigned to the project had no significant medical problems and were on current standard herd health procedures. Animals were maintained on pasture and supplemented with hay as needed. Males were housed in separate pens. Water was provided free choice.

Animals were superovulated to increase recovery of embryos. The superovulation protocol was a modification of the procedure used by Bourke et al. (1995). On Day 0, 5000 IU of human chorionic gonadotropin (hCG; Schein Pharmaceuticals, Florham Park, NJ) was administered i.m. Serum was collected by jugular puncture 4-6 d later and progesterone levels were determined by RIA to confirm ovulation. Serum progesterone concentrations were determined using a commercial solid phase radioimmunoassay kit (Diagnostic Products Corp, Coat-A-Count, Solid-Phase progesterone assay kit, Los Angeles, CA), previously validated for use in the llama (Leon et al., 1990). On Day 7, 1000 IU of equine chorionic gonadotropin (PMSG, Sioux Biochemical Corp., Sioux Center, IA) was

administered i.m. to females with confirmed ovulations. On Day 9, 500 μg $\text{PGF}_{2\alpha}$ (Estrumate; Miles Pharmaceuticals, Shawnee, KS) was injected i.m. to cause regression of any luteal tissue remaining from the previous cycle. Females were bred to one of the males 5 to 6 d after the Estrumate injection and again 48 h after the first breeding. hCG (5,000 IU) was administered i.m. at the time of the first breeding to insure ovulation of all follicles.

Embryos were collected non-surgically on Days 7, 9 and 11 after the first breeding (Day 0 = day of first mating). Animals were secured in a standard llama chute and injected with 20 to 40 mg xylazine (Miles Pharmaceuticals, Shawnee, KS) i.m. to achieve standing sedation. The tail head was shaved and surgically scrubbed. Lidocaine (0.22 mg/kg; Anthony Products Co., Arcadia, CA) was injected at the sacrococcygeal junction to achieve epidural anesthesia (Grubb et al., 1993). Days 13 and 15 embryos were collected at necropsy. Llamas were euthanized with Beuthanasia Solution® (1 ml/10 pounds; Schering Plough, Kenilworth, NJ) and the uterus was excised and flushed.

Embryos were recovered non-surgically on Days 7, 9 and 11 by flushing the uterus with Dulbecco's phosphate buffered saline containing 1% polyvinylpyrrolidone-10 (DPBS + PVP) and 10 ml/l antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, MO). Left and right uterine horns were flushed separately. Embryos were recovered from the flushing medium, and washed three times in microdrops of Ham's F-12 + 0.15% BSA (Hams F-12 + BSA; Sigma) under paraffin oil (Fisher Scientific Co., Tustin, CA). Each embryo was cultured in a 75 μl microdrop of Ham's F-12 + BSA under paraffin oil at 39° C in a humidified atmosphere of 5% CO_2 in air. At 24 and 48 h, each embryo was examined for morphology and 50 μl of medium was recovered and replaced with fresh medium. Collected medium was frozen at -20°C until analysis. Collections continued until at least 10 embryos at each time point were cultured for 48 hours. Days 13 (n=3) and 15 (n=1) embryos were collected at necropsy in a similar manner. After the

uterus was removed, the location of the CL was noted and each uterine horn was flushed with 60 ml of DPBS + PVP. All culture procedures were the same except Day 13 and 15 embryos were first sectioned (3-5 pieces), each section individually cultured in 100 and 200 μ l microdrops and 75 and 100 μ l of conditioned medium, respectively, were withdrawn daily and frozen for analysis.

Estradiol Determination. Estradiol concentrations in the conditioned medium were determined by radioimmunoassay at the Oregon Regional Primate Center (Beaverton, OR) using a protocol described by Goodman (1978). Samples were analyzed by RIA following an ether extraction. Recovery for all assays was at least 94%. The source of the antiserum is GDN No. 244 (or equivalent) from Dr. Gordon Niswender of Colorado State University. The lower limit of detection was 2 pg/ml. The inter-assay and intra-assay coefficients were 9% and 6%, respectively.

Statistical Analysis. Data were analyzed by repeated measures ANOVA comparing differences in estradiol production by day of gestation and time in culture. Differences in mean estradiol production between days of gestation and time in culture were analyzed by Fisher's least significant differences procedure. All analyses were performed using the NCSS statistical software program (Number Cruncher, Version 4.1, 1984, J.L. Hintze, Kaysville, UT).

Results

Recovery of Embryos. Nineteen females were superovulated with some females serving as repeated donors for a total of 29 embryo collections. A total of forty-five embryos were recovered and the mean number of embryos collected per successful flush was 2.6. The range of embryos recovered was 0-7. The number of embryos collected at each gestational age is reported in Table 3.1. Two Day 13 embryos were recovered from donors on a natural cycle. Twenty-nine embryos were cultured. The remaining embryos were used for other ongoing experiments.

Table 3.1. Recovery of embryos from superovulated donors.

	Day 7	Day 9	Day 11	Day 13	Day 15	Total
No. of flushes/ No. of animals	9/7	8/7	8/8	3/3	1/2	29/27 ^a
No. of embryos recovered:	11	18	12	3	1	45
No. of embryos cultured	9	10	6	3	1	29
Mean size	< 1 mm sphere	1-2 mm sphere	3-4 mm ovoid to 2-4 cm filament	6-8 cm filament	8-10 cm filament	

^aA total of 19 females were used. Four animals were flushed twice, 2 were flushed 3 times and the remaining 13 were flushed only once.

Estradiol Production by the Embryo. Embryos from all time points collected produced measurable quantities of estradiol-17 β . Some embryos were cultured for up to 264 h and remained viable as determined by trophoblastic vesicle formation. Estradiol production (pg/embryo) by Day 13 embryos during the 0-24 h culture interval and Day 15 embryos was greater ($P < 0.05$) compared to Day 7, 9 and 11 embryos and Day 13 embryos during the 24-48 h culture interval (Figure 3.1). Day 15 embryos produced more ($P < 0.05$) estradiol during 24-48 h compared to 0-24 h of culture but neither differed ($P > 0.10$) from estradiol production by Day 13 embryos during 0-24 h of culture. When averaged over the 48 h culture, estradiol production (pg/embryo) by Days 7 (1 ± 0.2), 9 (2.9 ± 0.5), and 11 (4.2 ± 1.5) embryos did not differ ($P > 0.10$) but was less ($P < 0.05$) compared to Days 13 (228.1 ± 99.5) and 15 (360.5 ± 69.7) embryos. Day 13 embryos produced approximately 50-fold more estradiol than Day 11 embryos. Estradiol production by Day 15 embryos was approximately 1.5 fold greater ($P < 0.05$) than Day 13 embryos. Because of the large variation associated with the analysis, Day 13 and 15 embryos were removed and the data re-analyzed. Estradiol production by Day 7 embryos during the 24-48 h culture interval was less ($P < 0.05$) compared to Day 9 embryos during the 24-48h and Day 11 embryos during the 0-24 and 24-48 h culture intervals. When averaged over the 48 h culture period, estradiol production by Day 11 embryos was greater ($P < 0.05$) than Day 7 embryos (Figure 3.2).

Discussion

Preimplantation llama embryos produce increasing amounts of estradiol-17 β during Days 7 through 15 of pregnancy. This period coincides with the period when maternal recognition of pregnancy must occur. Sumar et al. (1988) noted that successive surges of 15-keto-13, 14-dihydro-PGF_{2 α} are evident 10 to 12 days after a sterile mating in llamas and alpacas. The embryo must signal its presence to the maternal tract before the surges of prostaglandin initiate the luteolytic cascade. In

Figure 3.1. Estradiol production by preimplantation llama embryos collected on Days 7, 9, 11, 13, and 15. Data shown is from the first 48 h of culture. Estradiol production by Day 13 embryos during the 0-24 h culture interval and Day 15 embryos was greater ($P < 0.05$) compared to Day 7, 9 and 11 embryos and Day 13 embryos during the 24-48 h culture interval.

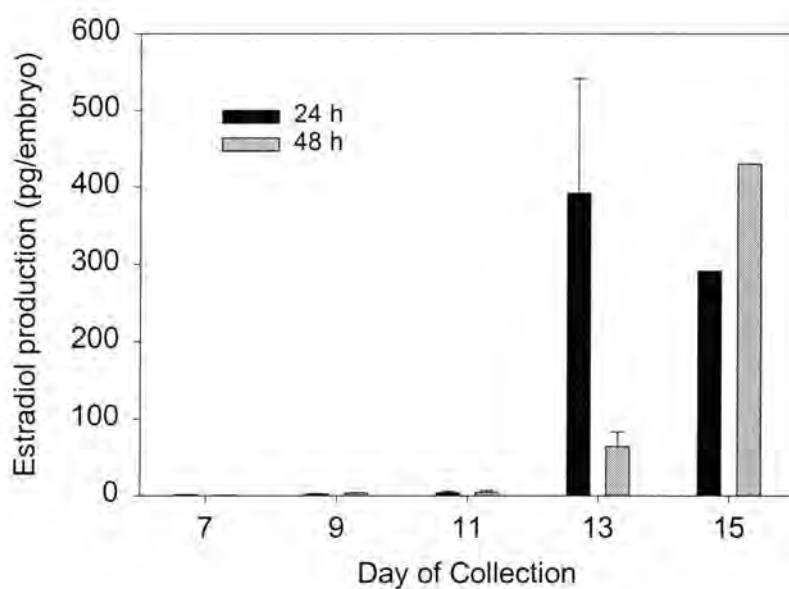
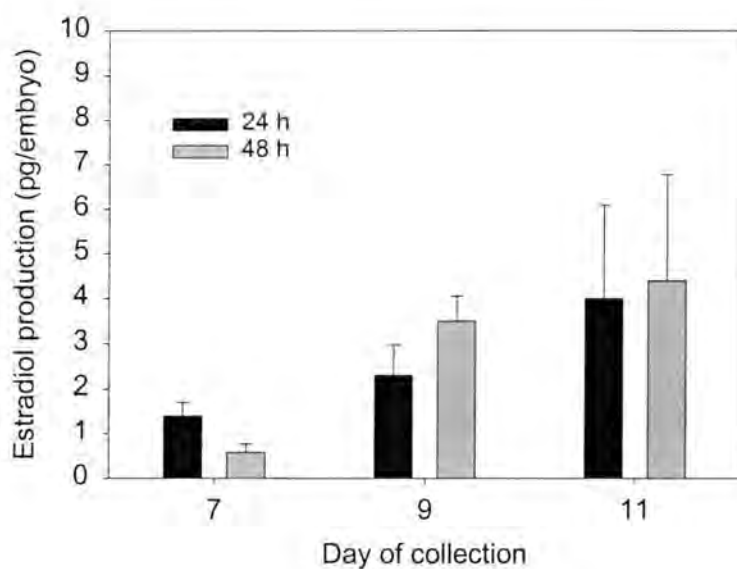


Figure 3.2. Estradiol production by preimplantation llama embryos collected on Days 7, 9 and 11. Data shown is from the first 48 hours in culture. When averaged over the 48 h culture period, estradiol production by Day 11 embryos was greater ($P < 0.05$) than Day 7 embryos.



the llama, a right-sided embryo must signal its presence to both the left and right uterine horns due to the systemic luteolytic effects of the left horn (Fernandez-Baca, et al., 1979). In this study, increasing levels of estradiol production by the llama preimplantation embryo were observed. Although there was not a statistically significant difference between Days 7, 9 and 11 embryos when Days 13 and 15 were included in the analysis, estradiol production increased by gestational age. The most dramatic increase in estradiol production occurred in Day 13 embryos. The estradiol surge observed on Day 13 would presumably occur too late to be involved in suppression of the prostaglandin surges. However, while estradiol levels were significantly lower on Day 11, they may still be adequate to interrupt the luteolytic cascade. In addition, the lower levels of estradiol secreted on Days 7, 9 and 11 may prepare the uterus and depress the surge of prostaglandins, while the Day 13 estradiol surge rescues the CL by having a direct luteotrophic effect on the CL. Further studies are needed to determine if estradiol has a direct effect on the CL.

Like the pig (Bazer and Thatcher, 1977), the increase in estradiol production is seen during a period of elongation. Llama embryos are approximately 1-2 mm spheres on Day 9, become ovoid (3-4 mm) or elongate up to 2-4 cm on Day 11 and reach 6-8 cm by Day 13. The peak in estradiol production seen on Day 13 is consistent with a rapid elongation phase.

Skidmore et al. (1994) observed aromatase activity and estrogen synthesis in dromedary camel embryos incubated with [^3H]androstendione. Estradiol was the major estrogen secreted. The onset of estrogen secretion in the camel embryo coincided with the observed time of luteolysis following a sterile mating and the authors suggested that this embryonic estrogen may be the signal required from the embryo to maintain luteal function.

Also similar to the sow and mare, llama embryos migrate, with right-sided embryos moving to the left uterine horn in almost all pregnancies. In the sow (Pope et al., 1982a) and the mare (Ginther, 1984), embryonic estradiol production

is increased during this migratory period. Intrauterine application of estradiol treated Silastic beads were able to extend the luteal phase in pigs by more than 3-6 days (LaForest and King, 1992). Pope et al. (1982b) demonstrated the migration of small spherical beads of Silastic glue containing physiological amounts of estradiol-17 β in the sow. It is possible that estradiol produced by the llama embryo is also involved in migration. One possible effect of estradiol could be a local increase in myometrial contractility resulting in contractions of the uterine horn thereby propelling the embryo to the contralateral side. Further studies are needed in the llama to determine if estradiol plays a direct role in embryo migration as it does in the pig.

In summary, llama embryos produce increasing quantities of estradiol during Days 7 through 15 of gestation. A dramatic rise in estradiol production occurs on Day 13 which correlates to a rapid elongation phase in the embryo. Whether estradiol is directly involved in maternal recognition of pregnancy and embryo migration still needs to be determined, however the present study has demonstrated estradiol production by the preimplantation llama embryo during the period these events occur.

References

- Adam CL, Moir CE, Shiach P. Plasma progesterone concentrations in pregnant and non-pregnant llamas (*lama glama*). Vet Rec 1989; 125:618-620.
- Adams GP, Sumar J, Ginther OJ. Form and function of the corpus luteum in llamas. Animal Reprod Sci 1991; 24:127-138.
- Bazer FW, Thatcher WW. Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin $F_2\alpha$ by the uterine endometrium. Prostaglandins 1977; 14:397-401.
- Bazer FW, Spencer TE, Ott TL. Placental Interferons. AJRI 1996; 35:297-308.
- Bourke D, Kyle CE, McEvoy TG, Young P, Adam CL. Superovulatory responses to eCG in Llamas (*lama glama*). Theriogenology 1995; 44:255-268.
- Bravo PW and Sumar J. Laparoscopic examination of the ovarian activity in alpacas. Animal Reprod Sci 1989; 21:271-281.
- Bravo PW, Fowler ME, Stabenfeldt GH, Lasley BL. Endocrine responses in the llama to copulation. Theriogenology 1990; 33(4):891-899.
- Bravo PW and Varela MH. Prenatal development of the alpaca (*lama pacos*). Animal Reprod Sci 1993; 32:245-252.
- Fernandez-Baca S, Hansel W, Novoa C. Embryonic mortality in the alpaca. Biol Reprod 1970; 3:243-251.
- Fernandez-Baca S, Hansel W, Saatman R, Sumar J, Novoa C. Differential luteolytic effects of right and left uterine horns in the alpaca. Biol Reprod 1979; 20:586-595.
- Ginther OJ. Intrauterine movement of the early conceptus in barren and postpartum mares. Theriogenology 1984; 21:633-64.
- Goodman RL. A quantitative analysis of the physiological role of estradiol and progesterone in the control of tonic and surge secretion of luteinizing hormone in the rat. Endocrinology 1978; 102:142-50.

Grubb TL, Riebold TW, Huber MJ. Evaluation of lidocaine, xylazine, and a combination of lidocaine and xylazine for epidural analgesia in llamas. JAVMA 1993; 203:1441-1444.

Heap RB, Hamon M, Allen WR. Studies on oestrogen synthesis by the equine conceptus. J Reprod Fert 1982; 32:343-352.

LaForest JP, King GJ. Effect of intrauterine application of oestradiol-17 β and prostaglandin E-2 on the porcine oestrous cycle and uterine endocrinology. J Reprod Fert 1992; 94:381-394.

Leaman DW, Roberts RM. Genes for trophoblast interferons in sheep, goat, musk ox, and distribution of related genes among mammals. J Interfer Res 1992; 12:1-11.

Leon, JB, Smith BB, Timm KI, LeCren G. Endocrine changes during pregnancy, parturition, and the early post-partum period in the llama (*Lama glama*). J Reprod Fert 1990; 88:503-511.

Perry JS, Heap RB, Amoroso EC. Steroid hormone production by pig blastocysts. Nature 1973; 245:45-47.

Pope WF, Maurer RR, Stormshak F. Intrauterine migration of the porcine embryo-Interaction of embryo, uterine flushings and indomethacin on myometrial function in vitro. J Anim Sci 1982a; 55:1169-1178.

Pope WF, Maurer RR, Stormshak F. Intrauterine migration of the porcine embryo-Influence of estradiol 17- β and histamine. Biol Reprod 1982b; 27:575-579.

Skidmore JA, Allen WR, Heap RB. Oestrogen synthesis by the peri-implantation conceptus of the one-humped camel (*Camelus dromedarius*). J Reprod Fertil 1994; 101:363-367.

Sumar J, Fredrikssen G, Alarcon V, Kindahl H, Edqvist L-E. Levels of 15-keto-13,14-dihydro-PGF2 α , progesterone and oestradiol-17 β after induced ovulations in llamas and alpacas. Acta Vet Scand 1988; 29:339-346.

CHAPTER 4

ADMINISTRATION OF ESTRADIOL DURING THE MID TO LATE LUTEAL PHASE EXTENDS THE CORPUS LUTEUM LIFESPAN AND ENHANCES PROGESTERONE PRODUCTION IN THE LLAMA

Abstract

Estradiol is a potential candidate for the embryonic substance responsible for maternal recognition of pregnancy in the llama. In a previous study, preimplantation llama embryos produced increasing amounts of estradiol during Days 7-15 of gestation with a dramatic increase on Day 13. Progressive surges of prostaglandin- $F_{2\alpha}$ initiate luteolysis between Days 10-12 if no signal is received from the llama embryo. The objective of this experiment was to determine if exogenous estradiol was luteotrophic when administered during the mid to late luteal phase. Thirty females were induced to ovulate with 5000 IU hCG (Day 0). Ovulation was confirmed on Day 5 by measuring serum progesterone. A progesterone level > 0.90 ng/ml was considered evidence of ovulation. Starting on Day 7 and continuing through Day 15, animals received daily injections i.m. of vehicle only ($n=11$), 5 ($n=7$) or 10 mg ($n=12$) estradiol benzoate (EB) dissolved in isopropylmyristate. Serum was collected immediately prior to each injection and also on days 16, 17, 18, 20, and 22. Sera were analyzed for progesterone with a commercial RIA kit. Mean progesterone levels were greater ($P < 0.05$) on Days 14, 15, 16, and 17 in llamas injected with 10 mg EB compared to llamas injected with vehicle only or 5 mg EB. These results demonstrate that exogenous estradiol enhances and partly extends luteal progesterone production. Estradiol produced by the preimplantation llama conceptus may play a role in maternal recognition of pregnancy and early luteal support.

Introduction

Maternal recognition of pregnancy requires a signal from the conceptus that disrupts the luteolytic cascade responsible for destruction of the corpus luteum (CL) and subsequent loss of progesterone support required for pregnancy. In ruminants, the conceptus produces interferon-tau which acts on the uterine epithelium to suppress estrogen receptor and oxytocin receptor gene expression, thus preventing uterine release of luteolytic pulses of prostaglandins (Bazer et al., 1996). Genes for trophoblast interferons have been found in sheep, goats, cattle, musk oxen, and giraffes, but not in llamas (Leaman and Roberts, 1992). The substance produced by the conceptus can also act directly on the CL leading to a direct luteotrophic effect. In the pig, estradiol produced by the conceptus functions in two ways. In pregnant pigs, the direction of uterine secretion of $\text{PGF}_{2\alpha}$ is exocrine, or into the uterine lumen, thereby preventing luteolysis, while in the non-pregnant pig, secretion is endocrine, or into the vasculature, inducing luteolysis (Bazer, 1992). Furthermore, administration of exogenous estrogens between Days 11 and 15 of the pig estrous cycle prolongs luteal lifespan significantly (Geisert et al., 1982). In the pig, initiation of estrogen synthesis by blastocysts and blastocyst elongation begin on Day 11 and coincide with the time of maternal recognition of pregnancy (Bazer and Thatcher, 1977).

Llamas are induced ovulators with ovulation occurring approximately 48 hours post coitus (Adams et al., 1991; Bravo and Sumar, 1989; Bravo et al., 1990). Serum progesterone concentrations increase to ≥ 1 ng/ml by Days 3-4 days post-ovulation. If pregnancy does not occur, progesterone levels drop rapidly by Days 8-10 post-ovulation and females can return to receptive behavior as early as 12 days after mating (Adam et al., 1989). Sumar et al. (1988) observed successive surges of 15-keto-13, 14-dihydro- $\text{PGF}_{2\alpha}$ 10 to 12 days after a sterile mating. Maternal recognition of pregnancy would likely occur between Days 9-13 post-coitus to prevent the destruction of the CL from uterine prostaglandins.

Estradiol is a possible candidate for maternal recognition of pregnancy in the llama. In a previous study, increasing concentrations of estradiol were produced by embryos collected on Days 7-15 post-coitus with a dramatic rise in estradiol production observed in embryos collected on Day 13, a time associated with blastocyst elongation. It is unknown whether estradiol is luteotrophic in the llama, thus the objective of this study was to determine if exogenous estradiol can extend the luteal phase in the llama.

Material and Methods

A total of 30 female llamas from the Oregon State University College of Veterinary Medicine herd were used for this project. All animals assigned to the project had no significant medical problems and were on current standard herd health procedures. Animals were maintained on pasture and supplemented with hay as needed. Water was provided free choice. A llama chute was used to secure the animals for all procedures.

To determine if estradiol extends the lifespan of the corpus luteum and has a role in maternal recognition of pregnancy, exogenous estradiol was administered and serum progesterone measured to evaluate extension of the luteal phase. On Day 0, 5000 IU of human chorionic gonadotropin (hCG; Schein Pharmaceuticals, Forham Park, NJ) intramuscularly (i.m.) to induce ovulation. Serum was collected by jugular puncture on Day 5 and progesterone analyzed by RIA to confirm ovulation. Ovulatory females were randomly assigned to one of three treatment groups. Estradiol benzoate (EB; Sigma Chemical Co., St. Louis, MO) dissolved in isopropylmyristate, 5 mg (n=7), 10 mg (n= 12), or vehicle only (n=11) was injected i.m. every 24 hours (rotating injection sites daily) on Days 7 through 15. Serum was obtained by jugular puncture daily on Days 7 through 15 just prior to injection of EB and on Days 16, 17, 18, 20, and 22. Serum was frozen at -20°C until analysis. Serum progesterone concentrations were determined using a commercial

solid phase radioimmunoassay kit(Diagnostic Products Corp, Coat-A Count, Solid-Phase progesterone assay kit, Los Angeles, CA), previously validated for use in the llama (Leon et al., 1990). The cross-reactivity of the antiserum was 100% to progesterone with the next highest cross-reactivities of: 9.0% to 5 α -pregnan-3,20-dione, 3.4% to 17 α -hydroxyprogesterone, 3.2% to 5 β -pregnan-3,20-dione, 2.2% to 11-deoxycorticosterone and less than 1% for all others. The inter- and intra-assay coefficients were 14% and 7.5%, respectively.

To confirm that the EB injections were elevating serum estradiol, a representative sampling (Days 5, 9, 15, and 20) from each of the treatment groups (n=3 each group) was assayed for estradiol. Serum estradiol concentrations were determined using a commercial competitive-binding radioimmunoassay kit(Diagnostic Systems Lab, Webster, Tx) which specifically measures estradiol 17 β . The cross-reactivity of the antiserum was 100% to estradiol-17 β with the next highest cross-reactivities of 6.1% to equilenin, 3.4% to estrone, and all others were less than 2%. To determine the assay's effectiveness in detecting estradiol benzoate, estradiol benzoate (0-5,000 pg/ml) was added to pooled llama serum (from non-experimental animals) and the samples were included in the assay. Estradiol concentrations from the serum of the experimental animals were determined both from a standard curve using standards provided in the kit and from the EB spiked serum samples.

Progesterone data were analysed by repeated measures ANOVA and differences in means between days were analyzed by Fisher's least significant differences procedure. All analyses were performed using the NCSS statistical software program (Number Cruncher, Version 4.1, 1984, J.L. Hintze, Kaysville, UT).

Results

A preliminary study with 11 female llamas was performed using EB dissolved in soybean oil. All experimental procedures and analysis were described as above.

The results are illustrated in Figure 4.1. No differences ($P > 0.10$) in progesterone production were observed among the treatments. However, when compared to previous reports (Leon et al., 1990; Adam et al., 1989) progesterone levels obtained from animals receiving vehicle only were much higher than expected. Soybean oil vehicle may have had an estrogenic effect in addition to the EB (Setchell et al., 1984). Another possibility was that the hCG used to induce ovulation may have had a luteotrophic effect. A subsequent experiment was performed to test these possibilities. A 2X2 factorial experiment was designed with 8 animals receiving either hCG or breeding to a vasectomized male for ovulation induction. In addition, soybean oil or saline was administered in an identical treatment design ($n=2$ each block) as described in the Materials and Methods. No differences ($P > 0.10$) were detected in either the method of ovulation induction or in soybean oil versus saline (Figure 4.2). Despite no apparent treatment effect by soybean oil, the experiment was repeated using isopropylmyristate as the vehicle. Initially, a total of 23 females were tested. Because all treated groups shared the same pasture and to test the possibility of a potential pheromone effect among the treated groups, an additional experiment with 7 animals was performed with treatment groups at separate sites. One group ($n=4$) received 10 mg EB in isopropylmyristate and a separate group ($n=3$) received vehicle only at a site approximately 0.5 miles away. The treatment protocol was identical as described above. Similar results were obtained in this separate study and the results were pooled. The results are presented in Figure 4.3. From the pooled data of 30 animals, mean progesterone levels were greater ($P < 0.05$) on Days 14, 15, 16, and 17 in llamas injected with 10 mg EB compared to llamas injected with vehicle only or 5 mg EB. The progesterone levels of the vehicle only treated animals are higher than expected based on previous published results (Adam et al., 1989 and Leon et al., 1990). However, an elevation in progesterone levels is observed in the 10 mg treatment group as compared to the 5 mg and vehicle only groups on days when luteolysis should have commenced.

Figure 4.1. Serum progesterone levels in non-pregnant, ovulatory llamas receiving vehicle only, 5 mg or 10 mg EB in soybean oil injections from Days 7-15 post-hCG. Data points represent mean progesterone levels by day and treatment group. No differences were detected among treatment groups ($P > 0.10$). Pooled SEM are 1.7, 2.0, and 1.7 for vehicle only, 5 mg EB, and 10 mg EB, respectively.

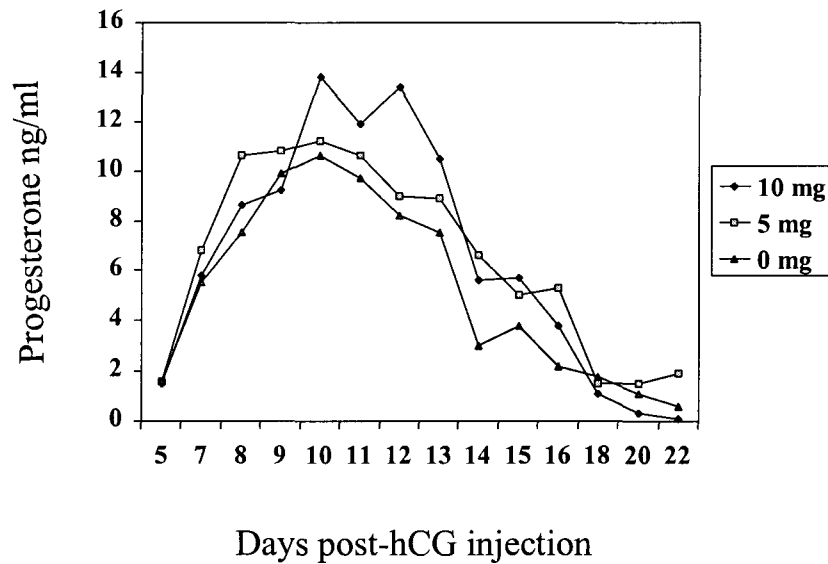


Figure 4.2. Serum progesterone levels in non-pregnant, ovulatory llamas receiving soybean oil or saline injections from Days 7-15 post-mating/hCG. Data points represent mean progesterone levels by day and treatment group. No significant differences were detected among treatment groups ($P > 0.10$). Pooled SEM was 7.3.

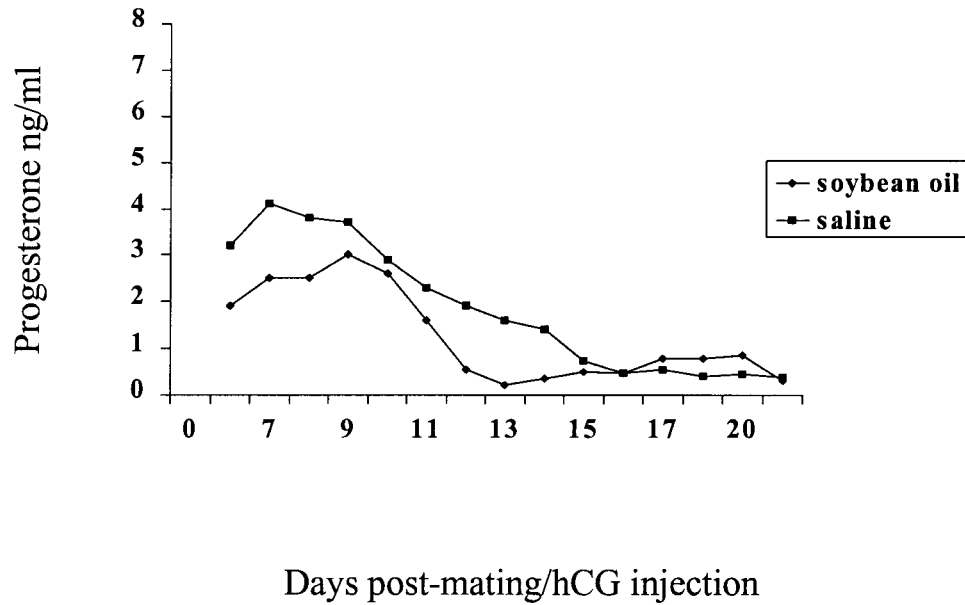
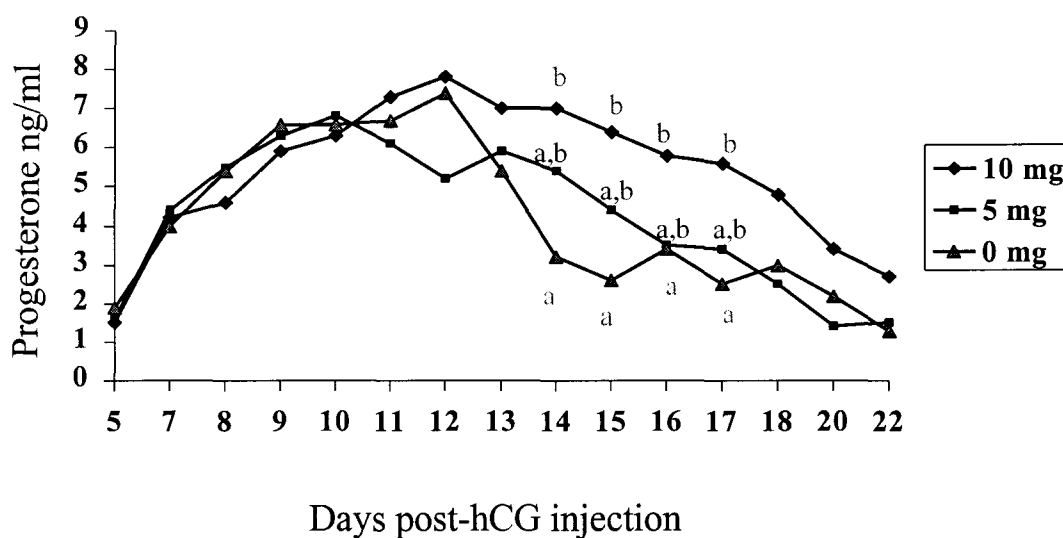


Figure 4.3. Serum progesterone levels in non-pregnant, ovulatory llamas receiving vehicle only, 5 mg or 10 mg EB in isopropylmyristate injections from Days 7-15 post-hCG. Data points represent mean progesterone levels by day and treatment group. Differences were detected on Days 14, 15, 16, and 17 in animals receiving 10 mg EB ($P < 0.05$) compared to 5 mg EB and vehicle only. Pooled SEM were 0.9, 1.1, and 0.8 for vehicle only, 5 mg EB, and 10 mg EB, respectively.

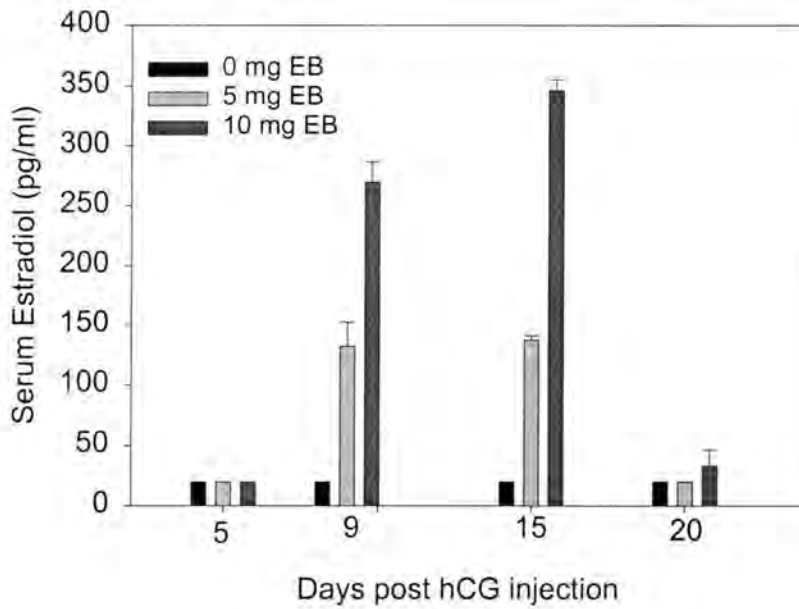


To confirm that EB was elevating serum estradiol, serum estradiol levels were assayed. The results are presented in Figure 4.4. The assay was able to detect approximately one-tenth the estradiol benzoate actually present in the serum. However, the results clearly demonstrate that serum levels in the 10 EB mg treatment group were approximately twofold to that of estradiol in the 5 mg EB treatment group, while the vehicle only group had estradiol levels below the limits of detection of the assay.

Discussion

This study was designed to determine if exogenous estradiol is luteotrophic in the llama. A previous study demonstrated the production of estradiol by llama blastocysts collected on Days 7-15 post-coitus. Estradiol produced by the conceptus during this time may be responsible for the inhibition of luteolytic events initiated by $\text{PGF}_{2\alpha}$. Administration of exogenous estrogen results in maintenance of the CL of the pig whose conceptus also produces estrogens (Geisert et al., 1982). Data from this experiment suggest that exogenous estrogen is luteotrophic in the llama. Progesterone levels were significantly elevated on Days 14-17 in females receiving 10 mg EB daily. The normal luteal phase would be expected to last only 14 days with progesterone levels approaching zero by this time (Adams et al., 1991). Early in the luteal phase (Days 8-13), progesterone levels were not significantly different among treatment groups. There was a high degree of variability between animals in terms of progesterone secretion. In addition, the progesterone levels of the control animals were higher than expected and also some control animals had an extended luteal phase. A possibility for an elevated progesterone level would be the presence of a double ovulation. Approximate 10% to 15% of ovulatory cycles result in multiple ovulations in llamas (Fernandez-Baca, 1993; Bravo et al., 1990; Adam et al., 1989). Using ultrasound to track CL size and number would have been helpful in an experiment of this type to correlate to

Figure 4.4. Serum estradiol levels on Days 5, 9, 15, and 20 post-hCG in non-pregnant, ovulatory llamas receiving 0, 5, or 10 mg EB in isopropylmyristate from Days 7-15 post-hCG. Data points represent mean estradiol levels by day and treatment group. Differences were detected among all treatment groups on Days 9 and 15 ($P < 0.01$).



progesterone levels. In addition, administration of exogenous estradiol past Day 15 may have exhibited more dramatic differences between treatment groups in terms of progesterone secretion.

Administration of exogenous estrogens between Days 11 and 15 of the estrous cycle prolongs luteal function in pigs to an average of about 120 days (Geisert et al., 1982). Ford and Christenson (1991) demonstrated a protective effect of estradiol 17- β when co-implanted with PGF_{2 α} into pig corpora lutea. It is unknown if estradiol has a direct luteotrophic effect on the CL in the llama or if the effect is indirect via inhibition of uterine prostaglandin surges. The llama has a short luteal phase compared to other farm species. Ovulation occurs approximately 2 days post-coitus and luteolysis begins approximately 10-12 days after mating (Sumar et al., 1988). The llama embryo has a very short time period to initiate events to block the luteolytic cascade. Our laboratory has previously shown that llama embryos produce estradiol during the time when maternal recognition of pregnancy would need to occur. Similar to the pig, llama embryos produce increasing amounts of estradiol at the time of blastocyst elongation and this corresponds to the time of maternal recognition of pregnancy in the pig (Bazer and Thatcher, 1977). A mechanism similar to the estradiol influence in the pig may be involved in maternal recognition of pregnancy in the llama.

In conclusion, 10 mg/day EB was able to significantly increase progesterone levels compared to 5 mg/day and vehicle only groups on Days 14-17, 2 days past the last EB injection. This period is also 3-4 days past the time when luteolysis would be expected to be complete. Estradiol may have a direct effect on the CL or it may have an indirect effect by suppressing uterine prostaglandin release.

References

- Adam CL, Moir CE, Shiach P. Plasma progesterone concentrations in pregnant and non-pregnant llamas (*Lama glama*). Vet Rec 1989; 125:618-620.
- Adams GP, Sumar J, Ginther OJ. Form and function of the corpus luteum in llamas. Animal Reprod Sci 1991; 24:127-138.
- Bazer FW. Mediators of maternal recognition of pregnancy in mammals. Proc Soc Exp Biol Med 1992; 199:373-384.
- Bazer FW, Thatcher WW. Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin $F_2\alpha$ by the uterine endometrium. Prostaglandins 1977; 14:397-401.
- Bazer FW, Spencer TE, Ott TL. Placental Interferons. AJRI 1996; 35:297-308.
- Bravo PW and Sumar J. Laparoscopic examination of the ovarian activity in alpacas. Animal Reprod Sci 1989; 21:271-281.
- Bravo PW, Fowler ME, Stabenfeldt GH, Lasley BL. Endocrine responses in the llama to copulation. Theriogenology 1990; 33(4):891-899.
- Fernandez-Baca S. Manipulation of reproductive functions in male and female New World camelids. Animal Reprod Sci 1993; 33:307-323.
- Fernandez-Baca S, Hansel W, Novoa C. Embryonic mortality in the alpaca. Biol Reprod 1970; 3:243-251.
- Fernandez-Baca S, Hansel W, Saatman R, Sumar J, Novoa C. Differential luteolytic effects of right and left uterine horns in the alpaca. Biol Reprod 1979; 20:586-595.
- Ford SP, Christenson LK. Direct effects of oestradiol-17 β and prostaglandin E-2 in protecting pig corpora lutea from a luteolytic dose of prostaglandin F-2 α . J Reprod Fert 1991; 93:203-209.
- Geisert RD, Thatcher WW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig: III. Endometrial secretory response to estradiol valerate administered on day 11 of the estrous cycle. Biol Reprod 1982; 27:957-965.

Heap RB, Hamon M, Allen WR. Studies on oestrogen synthesis by the equine conceptus. J Reprod Fert 1982; 32:343-352

LaForest JP, King GJ. Effect of intrauterine application of oestradiol-17 β and prostaglandin E-2 on the porcine oestrous cycle and uterine endocrinology. J Reprod Fert 1992; 94:381-394.

Leaman DW, Roberts RM. Genes for trophoblast interferons in sheep, goat, musk ox, and distribution of related genes among mammals. J Interfer Res 1992; 12:1-11.

Leon, JB, Smith BB, Timm KI, LeCren G. Endocrine changes during pregnancy, parturition, and the early post-partum period in the llama (*lama glama*). J Reprod Fert 1990; 88:503-511.

Perry JS, Heap RB, Amoroso EC. Steroid hormone production by pig blastocysts. Nature 1973; 245:45-47.

Setchell KD, Borriello SP, Hulme P, Kirk DN, Axelson M. Nonsteroidal estrogens of dietary origin; possible roles in hormone-dependent disease. Am J Clin Nutr 1984; 403:569-578.

Skidmore JA, Allen WR, Heap RB. Oestrogen synthesis by the peri-implantation conceptus of the one-humped camel (*Camelus dromedarius*). J Reprod Fertil 1994; 101:363-367.

Sumar J, Fredrikssen G, Alarcon V, Kindahl H, Edqvist L-E. Levels of 15-keto-13,14-dihydro-PGF2 α , progesterone and oestradiol-17 β after induced ovulations in llamas and alpacas. Acta Vet Scand 1988; 29:339-346.

CHAPTER 5

EXPRESSION OF ESTROGEN RECEPTORS α AND β IN THE PREGNANT AND NON-PREGNANT LLAMA UTERUS AND CORPUS LUTEUM

Abstract

Similar to the horse and pig, llama embryos produce increasing quantities of estradiol during the presumed period of maternal recognition of pregnancy. Llama embryos also exhibit transuterine migration with 95% of pregnancies implanting in the left uterine horn. Approximately 50% of these pregnancies are supported by a corpus luteum (CL) on the right ovary. It is possible that similar mechanisms are involved in pregnancy recognition and embryo migration among these three species. Two types of estrogen receptor (ER) have been characterized, ER α and ER β . The two subtypes exhibit differential expression by tissue and reproductive stage and also have different ligand-binding affinities. To determine estradiol's potential role in pregnancy recognition and embryo migration, differences in ER α and ER β expression were evaluated in the llama CL and uterus during Days 7-13 post-mating (Day 0 = day of mating) in pregnant and non-pregnant llamas. ER subtype expression was evaluated using Reverse Transcription-Polymerase Chain Reactions and comparisons were made by days post-mating and reproductive status (pregnant versus non-pregnant) for CL, uterus and endometrium, and side (right versus left uterine horn) for endometrium and uterus. ER subtype expression was also evaluated in non-ovulated and juvenile females. No differences ($P > 0.10$) in expression of ER α or ER β were observed in CL due to reproductive status or within Days 7-11 post-mating. However, there was a significant linear regression ($P < 0.05$) for both ER α , which increased from Days 7-11, and ER β , which decreased from Days 7-11. Expression of ER α in ovary was higher ($P < 0.05$) compared to Day 7 and Day 9 CL. When tissue from Day 13 CL was included in the analysis, ER α expression was lower ($P < 0.05$) on Day 9 compared to Day 11

pregnant females. In uterus, ER α expression decreased ($P < 0.01$) from Days 7-11 and was lower in pregnant compared to non-pregnant uterus ($P < 0.10$). Expression of ER β was higher in pregnant versus non-pregnant uterus ($P < 0.10$). There were no differences ($P > 0.10$) in expression of ER α or ER β in endometrium by reproductive status or days post-mating. Analysis of pregnant uterus revealed a decrease ($P < 0.01$) in ER α expression from Days 7-13, but no difference ($P > 0.10$) in right versus left side. No differences ($P > 0.10$) were observed in expression of ER α or ER β in pregnant endometrium by days post-mating or right versus left side, however a trend of greater ER α expression in the right versus left uterine horn was observed ($P=0.20$). A decreased expression of ER α was suggested ($P = 0.12$) in juvenile uterus compared to pubertal females. Expression of ER β in the non-pregnant uterus was different ($P < 0.05$) in non-ovulated and juvenile females compared to Days 7 and 11 post-mating. In summary, expression of ER α and ER β in llama CL and ER α in uterus and endometrium are affected by reproductive status and days post-mating. The combination of differential expression of ER subtypes and estradiol production by the conceptus suggests a role for estradiol in maternal recognition of pregnancy and embryo migration in the llama.

Introduction

Luteolysis in the ruminant, initiated by the endometrium, is dependent upon the production of uterine prostaglandins which are released in pulses during late diestrus if a signal from a conceptus is not received by the endometrium (McCracken et al., 1984). Prostaglandin-F $_{2\alpha}$ (PGF $_{2\alpha}$) released by the endometrium is responsible for events leading to regression of the corpus luteum (CL). Endometrial PGF $_{2\alpha}$ moves into the uterine vasculature where it transfers via countercurrent exchange to the ipsilateral ovarian artery. PGF $_{2\alpha}$ then travels to the ovary and initiates a cascade of luteolytic events resulting in regression of the CL. Llamas are induced ovulators and exhibit a similar luteolytic mechanism. Sumar et

al. (1988) observed progressive surges of $\text{PGF}_{2\alpha}$ 10-12 d after a sterile mating in llamas. Similar to the ruminant, $\text{PGF}_{2\alpha}$ travels via the vasculature and can transfer to the ovarian artery by countercurrent exchange. However, the llama is unique in that the left uterine horn exerts both systemic and local luteolytic effects while the right horn exerts only a local effect (Fernandez-Baca et al., 1979). Because of this differential effect, luteolysis of a CL on the right side is initiated by $\text{PGF}_{2\alpha}$ from both the left and right uterine horns. If an embryo is present in the right uterine horn, it must signal its presence to both uterine horns to interrupt the luteolytic cascade. In fact, it has been demonstrated that the llama embryo migrates from the right uterine horn and implants in the left uterine horn. Fernandez-Baca et al. (1970) observed an implantation rate of 95% in the left uterine horn with 50% of left-sided pregnancies supported by a CL on the right in the alpaca. This evidence supports the theory that an embryo conceived on the right must migrate to the left horn prior to implantation. Del Campo et al. (1996) examined the unique vascular anatomy of the llama and alpaca uterus and observed that 95% (30/32) had a large branch of the right uterine artery that entered the left horn and a large venous branch of the left uterine vein that crossed-over to the right uterine vein. Thus, prostaglandins from the left uterine horn could transfer to vasculature on the right and initiate luteolysis of a contralateral CL. There is no corresponding right uterine vein that crosses over, thus the right uterine horn can only initiate luteolysis of the ipsilateral CL. In the remaining 5% (2/32) the vasculature was reversed. It is not known whether these animals with a reversed vasculature exhibit right-sided implantations.

The conceptus signal that initiates maternal recognition of pregnancy is not known in the llama. Coincidental timing of luteolytic events and embryo migration suggest that the substance produced by the conceptus may be involved in both events. Previous studies in this lab demonstrated that preimplantation llama embryos produce increasing quantities of estradiol from Days 7-15 of gestation and

that exogenous estradiol had a luteotrophic effect. Estradiol is produced by embryos of other species that exhibit migration. The mare conceptus migrates between uterine horns 12-14 times per day between Days 12-18 which coincides with the production of increasing amounts of estradiol by the conceptus (Ginther, 1984). Pope et al. (1982) observed increased synthesis of estradiol by the pig embryo at the corresponding time of embryo migration and increased myometrial activity.

The myometrium increases contractility, excitability and shows increased spontaneous activity under the influence of estrogen. Estrogen potentiates the effects of oxytocin and $\text{PGF}_{2\alpha}$ by up-regulating oxytocin (OTR) and $\text{PGF}_{2\alpha}$ receptor expression (Hixon and Flint, 1987; Beard and Lamming, 1994). It is possible that embryonic estrogen induces a local contractility of the uterus resulting in propulsion of the embryo in species that exhibit embryo migration.

Two different types of estrogen receptor (ER) have been characterized. Rat $\text{ER}\alpha$ encodes a protein of 600 amino acids and has a calculated molecular weight of 67,029 (Koike et al., 1987). Kuiper et al. (1996) cloned a novel rat ER cDNA from prostate, designated $\text{ER}\beta$ subtype, that encodes a protein of 485 amino acid residues with a calculated molecular weight of 54,200. Rat $\text{ER}\beta$ protein is highly homologous to rat $\text{ER}\alpha$, particularly in the DNA binding domain (> 90% amino acid identity) and in the C-terminal ligand-binding domain (55%). $\text{ER}\alpha$ and $\text{ER}\beta$ were found to be differentially expressed based on tissue type (Kuiper et al., 1997). Differential expression of ER may play a role in embryo migration in species whose conceptuses produce estradiol.

Llamas, horses and pigs have a non-invasive epitheliochorial placenta and it is possible that similar mechanisms drive embryo motility, maternal recognition and implantation. Therefore, the objective of these experiments was to examine the relative expression of $\text{ER}\alpha$ and $\text{ER}\beta$ in the llama CL and uterus during the period of embryo migration and maternal recognition of pregnancy.

Material and Methods

Tissue Collection. A total of 23 reproductively normal adult female llamas, 2 juvenile females (ages 3 and 6 months) and 3 males (2 fertile and 1 sterile/vasectomized) from the Oregon State University College of Veterinary Medicine (OSU-CVM) herd were used for this project. All animals assigned to the project had no significant medical problems and were on current standard herd health procedures. Animals were maintained on pasture and supplemented with hay as needed. Males were housed in separate pens. Water was provided free choice.

In order to determine expression of ER α and ER β , CL and uterine tissues were collected. Tissues from females mated to fertile males (pregnant females) were collected on Days 7, 9, 11 (n=3 each day), and 13 (n=2), (Day 0 = day of mating). Females were euthanized with Beuthanasia Solution® (1 ml/10 pounds; Schering Plough, Kenilworth, NJ) at OSU-CVM prior to tissue collection. Immediately after euthanasia, the uterus was removed. Location of the CL was noted and each horn of the uterus was flushed with 60 ml of Dulbecco's phosphate buffered saline containing 1% polyvinylpyrrolidone-10 (DPBS + PVP) and 10 ml/l antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, MO). Left and right uterine horns were flushed separately. Uterine flushes were examined to confirm the presence of an embryo. Immediately after flushing the uterus the following tissues (approximately 1g each) were collected: endometrium, full-thickness uterine horn and CL. Tissues were placed in individual microcentrifuge tubes and snap frozen immediately in ethanol/dry ice. Samples were kept at -80 °C until analyzed. Tissues from sterile-mated animals (non-pregnant females) were also collected on Days 7, 9 and 11 (n=3 each day) following mating to a vasectomized male. Euthanasia was conducted as described. The uterus was not flushed in sterile-mated animals, however endometrium, full-thickness uterine horn, and CL were collected. The above tissues were also collected from non-exposed, non-ovulated females (n=2) except a section of ovary was collected instead of CL.

Full-thickness uterine horn samples were also collected from 2 juvenile females (ages 3 and 6 months). All tissues were analyzed in the same manner.

Reverse Transcription-Polymerase Chain Reactions (RT-PCR). To evaluate gene expression, RNA was recovered from tissues using acid guanidinium thiocyanate/phenol/chloroform extraction as described by Chomczynski and Sacchi (1987). RNA was reconstituted in autoclaved distilled water and stored at -80° C. RNA was quantified and purity assessed by UV-spectrophotometry. RT-PCR was conducted following procedures described by Arcellano-Panlilio and Schultz (1993). Equivalent amounts of total RNA (1 µg) from each tissue sample were incubated with 0.5 µg oligo (dT)₁₂₋₁₈ primers (Gibco BRL, Grand Island, NY) for 10 min at 70° C in a total volume of 12 µl and quick chilled on ice. A mixture of 4 µl 5X first-strand buffer (250mM Tris HCL, pH 8.3, 375 mM KCl, and 15 mM MgCl₂), 2 µl 0.1 M dithiothreitol (DTT), 1 µl 10 mM dNTP mix (10 mM each dATP, dGTP, dTTP, and dCTP), and 1 µl (200 U) Superscript II reverse transcriptase (Gibco BRL) was added and the mixture incubated at 42° C for 90 minutes followed by a 10 minute soak at 95° C. The reaction mixture was diluted with sterile distilled water for a total volume of 50 µl cDNA. A total volume of 5 µl of each cDNA was used for amplification by PCR using 1U Taq DNA Polymerase (Promega, Madison, WI) in a 50 µl reaction volume containing 2.0 mM Tris-HCl (pH 8.0), 10.0 mM KCl, 0.01 mM EDTA, 0.1 mM DTT, 5% glycerol, 0.05% Tween®20, 0.05% Nonidet®-40, 2.5mM MgCl₂, 0.2 mM dNTP mix, and 2.0 µM oligonucleotide primers specific for ERα, ERβ, and β-actin. The reaction mix was overlaid with mineral oil and amplified in a thermal cycler. PCR conditions were: 1) an initial 4 min soak at 94°C 2) 30 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 60°C and extension for 2 min at 72°C and 3) incubation at 72°C for 7 min. Day 13.5 non-pregnant bovine endometrium served as the positive control for ERα, bovine ovary for ERβ, and water in place of RT product was the negative control. Primer pairs used in amplification were designed

from published bovine (ER α and ER β) and mouse (β -actin) sequences. Primer pairs were synthesized at the Oregon State University Center for Gene Research and Biotechnology Central Services Laboratory (Table 5.1). PCR products were resolved on a 2% agarose gel containing 0.5 μ g/ml ethidium bromide. Gels were photographed using a MP-40 Polaroid Land camera and positive/negative film (Polaroid 665). Film was exposed under UV light for 45 seconds. The negatives were processed as described in the product insert. Briefly, the negative was soaked for 30-60 seconds in a sodium sulfite cleaning solution followed by a 5-minute wash under running water. The negative was then dipped in 2% PhotoFlo and allowed to dry. Each sample was assayed in duplicate with β -actin serving as a loading control. The intensity and area of the amplified signals were analyzed by scanning and computing densitometry (Hoefer GS-300 Scanning Densitometer; and GS-350h Data System). Relative amounts of ER subtypes were compared to β -actin by comparing relative densities of the bands on the photographic negative. Relative concentrations of products are expressed as the ratio of ER subtype to β -actin. PCR products were sequenced for each ER subtype and β -actin. Representative RT-PCR products from each primer pair were purified using a PCR QIAquick Spin Purification Kit (Qiagen, Valencica, CA) and sequenced at the Oregon State University Center for Gene Research and Biotechnology Central Services Laboratory.

Statistical Analysis. Receptor expression in the CL was evaluated by 2-way ANOVA where days after mating and reproductive status were the major effects. Endometrial and uterine tissues were analyzed in a 3 X 2 X 2 ANOVA where days after mating (7, 9, 11), reproductive status (pregnant, non-pregnant) and uterine side (right, left) were the major effects. Data were partitioned by reproductive status and tissue type (uterus versus endometrium) to include pregnant Day 13, non-ovulated and juvenile tissues and were analyzed by 2-way ANOVA where

Table 5.1. PCR primer sequences for ER α , ER β , and β -actin.

Primer name	Primer sequence	PCR fragment size (bp)	Reference:
ER α	5' primer = GCGATCCTGATGATTGGTCT (21 nt) 3' primer = CATCTCCAGCAGCAGGTCAT (21 nt)	477	Malayer and Woods, 1998
ER β	5' primer = CACCTCTCTCCTTTAGCCATCC (22 nt) 3' primer = CAGCAGCAGCTCTTTCACCTCG (21 nt)	528	Rosenfeld, et al., 1999
β -actin	5' primer = CGTGGGCCCTAGGCACCA (21 nt) 3' primer = TTGGCCTTAGGGTTCAGGGGGG (22 nt)	243	Tokunaga et al., 1986

days after mating and uterine side were the major effects. Orthogonal contrasts and Duncan's NMRT were used to compare days after mating within subsets of data. All analyses were performed using the NCSS statistical software program (Number Cruncher, Version 4.1, 1984, J.L. Hintze, Kaysville, UT).

Results

Llama ER subtype sequences. RT-PCR amplified a 477 nucleotide fragment for ER α and a 528 nucleotide fragment for ER β (Figures 5.1 and 5.2). Comparison of the llama ER α fragment to published sequences revealed sequence homologies to ER α of 93.5%, 92.4%, and 92.2% in the pig, human and cow, respectively. Comparison of the llama ER β fragment to published sequences revealed sequence homologies to ER β of 92.1%, and 88.4% in the cow and human, respectively (FASTA search; Pearson and Lipman, 1988). Representative ethidium bromide stained agarose gels depicting expression of ER α , ER β , and β -actin in pregnant and non-pregnant, uterus, endometrium and CL are provided in Figures 5.3, 5.4 and 5.5.

Analysis of ER subtype expression in CL. No differences were detected in either ER α or ER β expression in CL within Days 7-11 or by pregnancy status ($P > 0.10$). However, a significant linear regression was observed for both ER α and ER β , with ER α increasing and ER β decreasing from Days 7-11 (Figure 5.6). Differences ($P < 0.05$) in ER α expression were detected in ovarian tissue compared to pooled pregnant and non-pregnant Days 7 and 9 CL, with ER α expression in ovary being greater than CL as a general trend (Figure 5.7a). No differences ($P > 0.10$) were detected in expression of ER β by day among ovary and pooled pregnant and non-pregnant Days 7, 9, 11, and 13 CL (Figure 5.7b). Using pooled CL data, orthogonal contrasts revealed a significant decrease ($P < 0.10$) in ER α expression in Day 7 compared to Days 9, 11 and 13 CL (Figure 5.8). However, because Day 13 pregnant CL did not have a corresponding non-pregnant

Figure 5.1. Llama ER α nucleotide sequence.

1	TGCGGATCCT	GATGATTGGT	CTTGTCTGGC	GCTCCATGGA	GCACCCAGGG
51	AAGCTCCTGT	TTGCTCCTAA	CTTGCTCCTA	GACAGGAACC	AGGGAAAATG
101	TGTCGAGGGC	ATGGTGGAGA	TCTTTGACAT	GTTGCTGGCC	ACGTCGTCTC
151	GGTTCCGCAT	GATGAATCTC	CAGGGAGAGG	AGTTTGTGTG	CCTCAAATCC
201	ATCATTTTGC	TTAATTCTGG	AGTGTACACA	TTTCTGTCCA	GCACCCTGAA
251	GTCTCTAGAA	GAGAAGGACC	ACATCCACCG	TGTCCTGGAC	AAGATCATAG
301	ACACCTTGAT	CCATCTGATG	GCCAAGGCGG	GCCTCAGTCT	GCAGCAGCAG
351	CACCGGCGCC	TGGCCCAGCT	CCTCCTCATC	CTCTCCCACT	TCAGACACAT
401	GAGTAACAAA	GGCATGGAGC	ATCTATATAA	CATGAAGTGC	AAGAACGTGG
451	TGCCCAACTG	TTACAAA			

Figure 5.2. Llama ER β nucleotide sequence.

1	ATTGCCAGTC	GTCACCTTCTG	TATACAGAAC	CTCAAAAGAG	TCCTTGGTGT
51	GAAGCAAGAT	CACTAGAACA	CACCCTACCT	GTGAACAGAG	AGACACTGAA
101	AAGGAAGGCC	AGTGGGAGCA	GCTGTGCCAG	CCCTGTTACT	AGTCCAAGTT
151	CAAAGAGGGA	TGCTCACTTC	TGCGCTGTCT	GCAGCGATTA	TGCGTCGGGA
201	TACCACTATG	GAGTCTGGTC	GTGTGAAGGA	TGTAAGGCCT	TTTTTAAAAG
251	AAGCATTCAA	GGACATAATG	ATTATATTTG	TCCAGCTACA	AACCAGTGTA
301	CAATAGATAA	GAACCGGCGT	AAAAGCTGCC	AGGCCTGCCG	ACTTCGGAAG
351	TGCTATGAAG	TCGGAATGGT	GAAGTGTGGC	TCCCGGAGAG	AAAGGTGTGG
401	GTACCGCCTA	GTGCGAAGGC	AGAGAAATTC	TGAAGAGCAG	CTGCATTGCC
451	TGAGCAAAGC	CAAGAAAAAT	GGTGGACATG	TGACACCAGT	GAAAGAACTG
501	TGCTGGT				

Figure 5.3. Gene expression of ER α , ER β and β -actin in the pregnant and non-pregnant llama uterus. **Lane 1:** water, **lanes 2 and 3:** Day 7 pregnant uterus, right and left sides, **lanes 4 and 5:** Day 9 pregnant uterus, right and left sides, **lanes 6 and 7:** Day 11 pregnant uterus, right and left sides, **lane 8:** DNA ladder, **lane 9:** water, **lanes 10 and 11:** Day 7 non-pregnant uterus, right and left sides, **lanes 12 and 13:** Day 9 non-pregnant uterus, right and left sides, **lanes 14 and 15:** Day 11 non-pregnant uterus, right and left sides. **A.** ER α . **B.** ER β . **C.** β -actin.

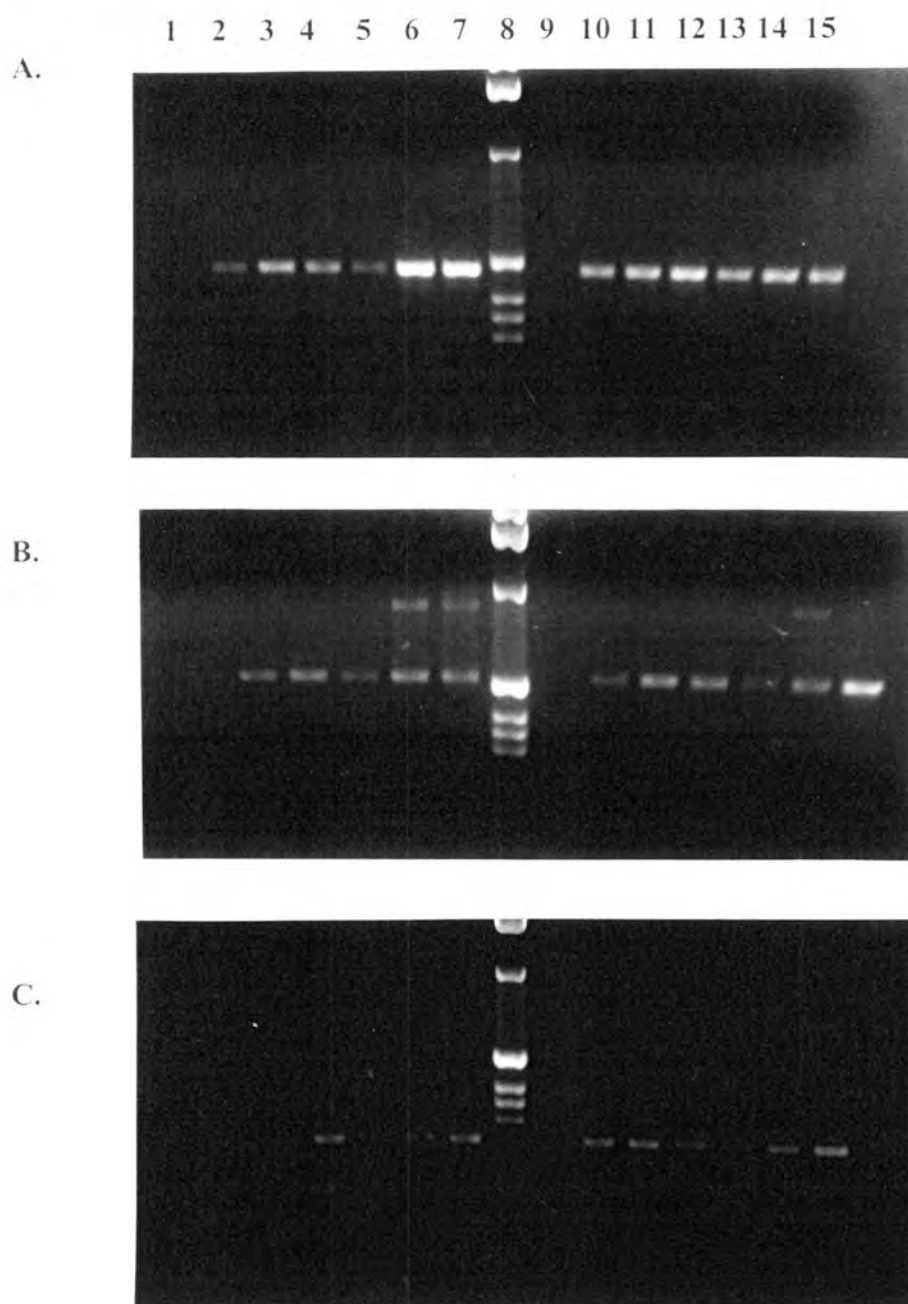


Figure 5.4. Gene expression of ER α , ER β and β -actin in the pregnant and non-pregnant llama endometrium. **Lane 1:** water, **lanes 2 and 3:** Day 7 pregnant endometrium, right and left sides, **lanes 4 and 5:** Day 9 pregnant endometrium, right and left sides, **lanes 6 and 7:** Day 11 pregnant endometrium, right and left sides, **lane 8:** DNA ladder, **lane 9:** water, **lanes 10 and 11:** Day 7 non-pregnant endometrium, right and left sides, **lanes 12 and 13:** Day 9 non-pregnant endometrium, right and left sides, **lanes 14 and 15:** Day 11 non-pregnant endometrium, right and left sides. **A.** ER α . **B.** ER β . **C.** β -actin.

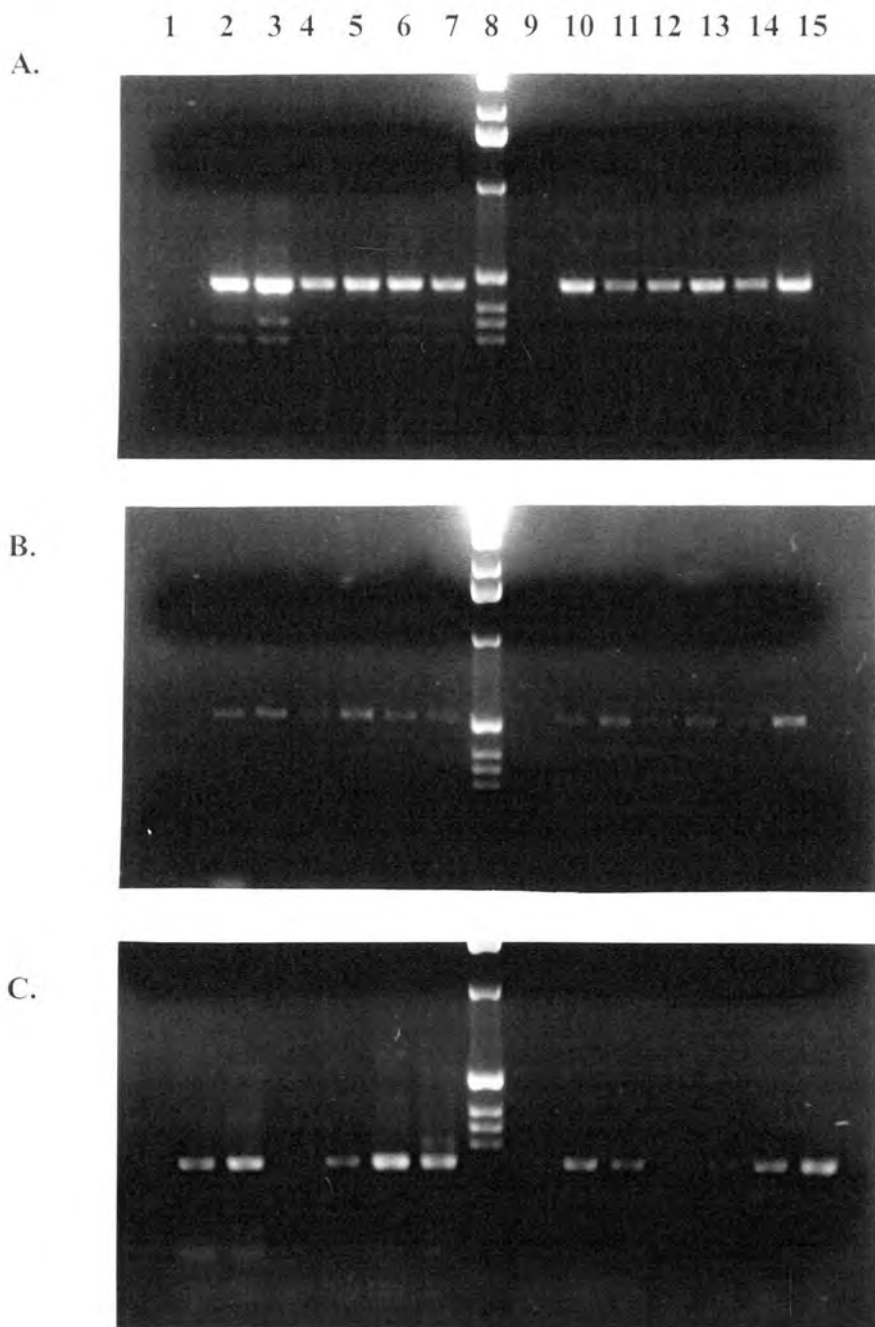


Figure 5.5. Gene expression of ER α , ER β and β -actin in the pregnant and non-pregnant llama CL and ovary. **Lane 1:** water, **lane 2:** Day 7 pregnant CL, **lane 3:** Day 9 pregnant CL, **lane 4:** Day 11 pregnant CL, **lane 5:** DNA ladder, **lane 6:** Day 7 non-pregnant CL, **lane 7:** Day 9 non-pregnant CL, **lane 8:** Day 11 non-pregnant CL, **lane 9:** ovary from follicular phase female.

A. ER α . B. ER β . C. β -actin.

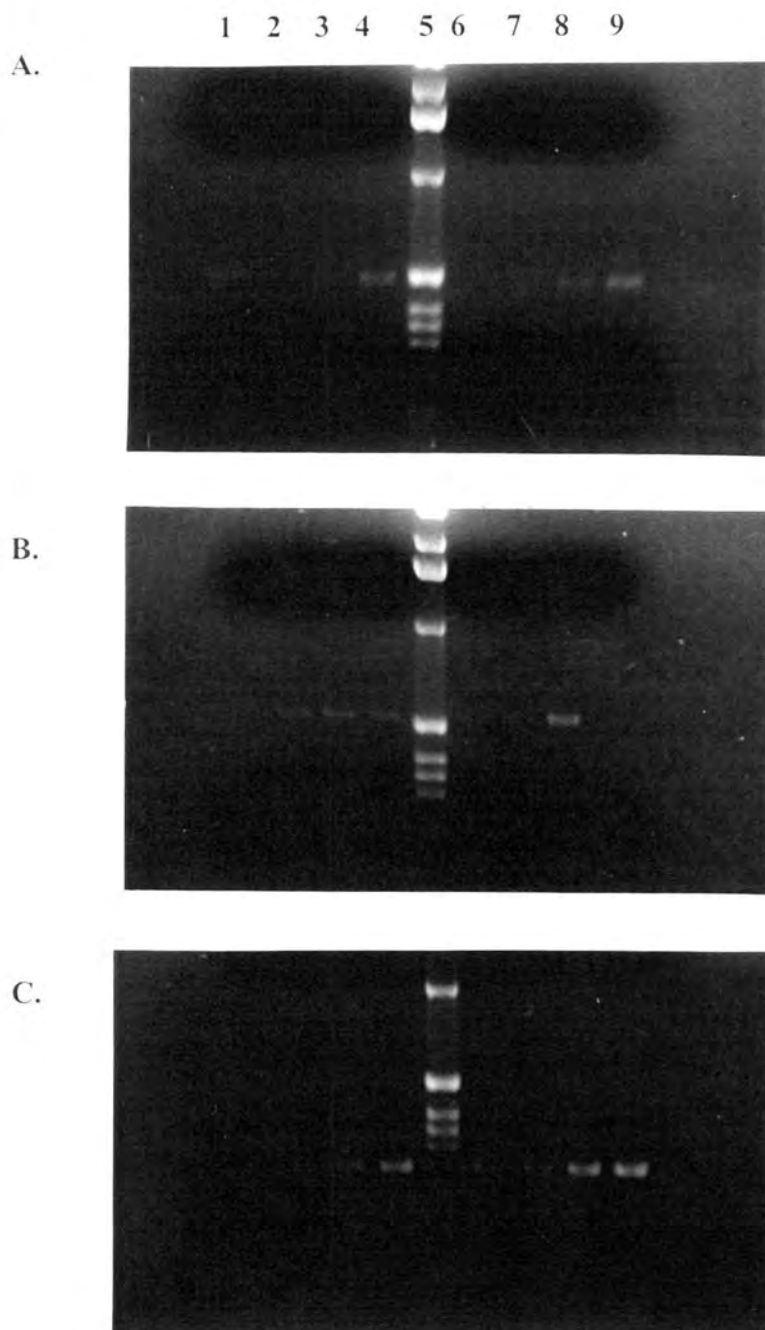


Figure 5.6. Expression of ER α and ER β in llama CL on Days 7, 9 and 11 post-mating (n=6, 4 and 6, respectively). Graphs depict the means of each receptor subtype calculated from scanning densitometry data and are expressed as a percentage of β -actin expression.

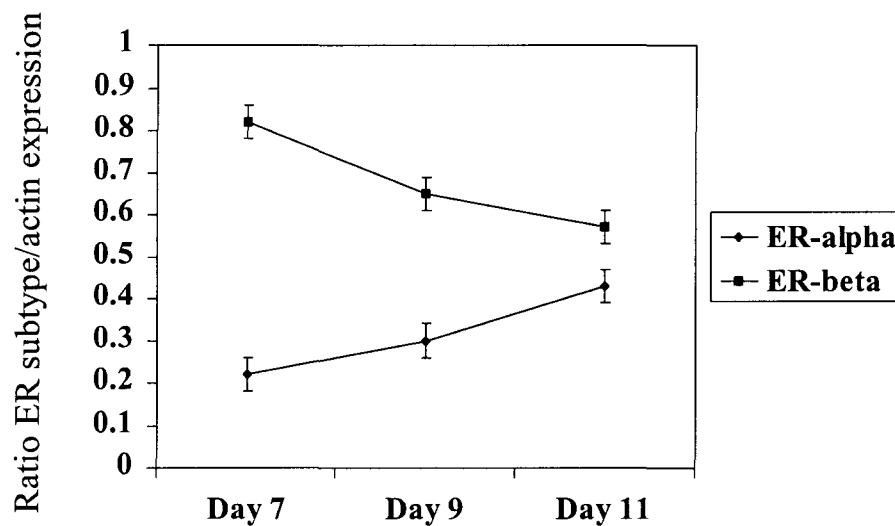
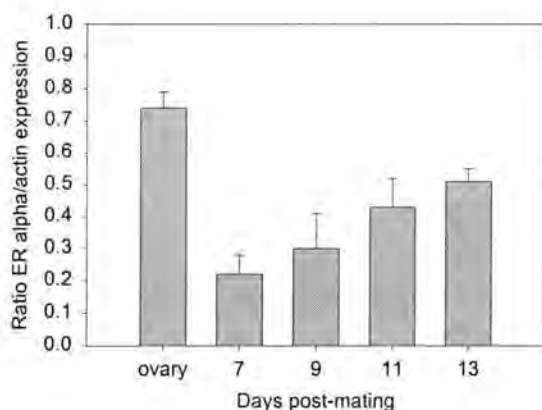


Figure 5.7. Expression of ER α and ER β in pregnant and non-pregnant llama CL on Days 7, 9, 11 and 13 post-mating (n=6, 4, 6, and 2 respectively) and ovary recovered from follicular phase females (n=3). Graphs depict the means of each receptor subtype calculated from scanning densitometry data and are expressed as a percentage of β -actin expression.

A. ER α



B. ER β

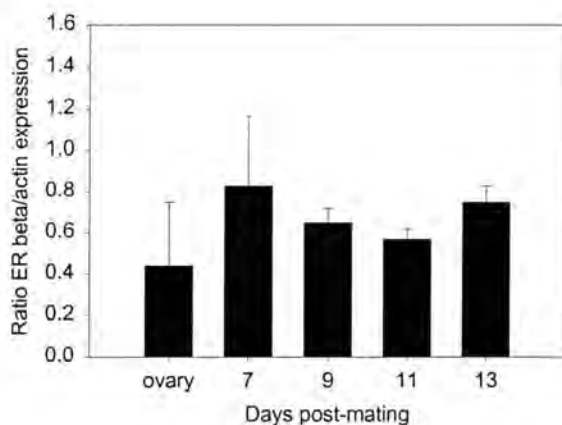
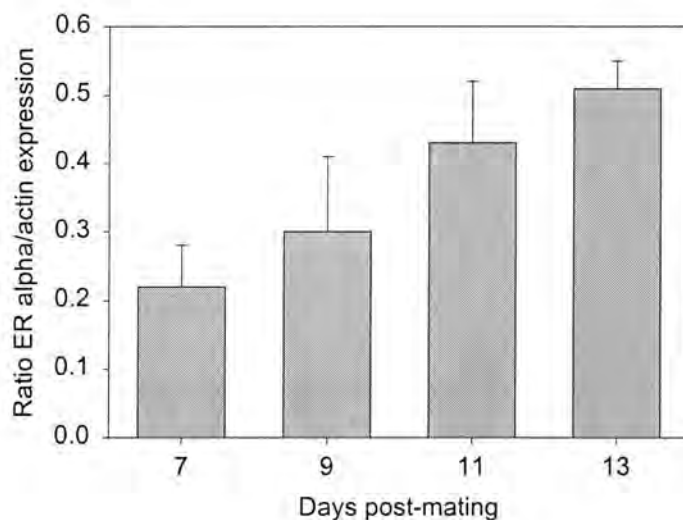


Figure 5.8. Expression of ER α in pregnant and non-pregnant llama CL on Days 7, 9, 11 and 13 post-mating (n=6, 4, 6 and 2, respectively). Graphs depict the means of each receptor subtype calculated from scanning densitometry data and are expressed as a percentage of β -actin expression. Day 7 expression was lower than Days 9, 11 and 13 ($P = 0.08$, orthogonal contrast).



control, the data were re-analyzed with the non-pregnant CL data omitted. A reduction ($P < 0.05$) in ER α expression was observed in pregnant Day 9 CL compared to Day 11 (Figure 5.9). No differences ($P > 0.10$) by day post-mating or pregnancy status were observed for ER β expression in the pregnant CL.

Analysis of ER subtype expression in the endometrium and uterus.

Separate analyses were performed on endometrium and uterus in all pubertal animals. Differences between ER expression in uterus versus endometrium were used as an indication of ER expression in myometrium. ER α expression in uterus decreased ($P < 0.01$) from Days 7-11 (3.0 ± 0.2 , 2.1 ± 0.3 and 1.6 ± 0.2 , respectively) and was higher ($P = 0.08$) in non-pregnant versus pregnant animals (2.5 ± 0.2 versus 2.0 ± 0.2 , respectively) (Figure 5.10a). No differences ($P > 0.10$) in ER α expression were detected in the right versus left side. ER β expression was greater ($P = 0.09$) in pregnant versus non-pregnant uterus (0.6 ± 0.1 versus 0.4 ± 0.1 , respectively) (Figure 5.10b). No differences ($P > 0.10$) were detected in endometrium by days post-mating, reproductive status, or right versus left side in either ER α or ER β expression (Figures 5.11a and 5.11b).

ER α expression declined ($P < 0.10$) in pregnant uterus from Days 7 to 13 post-mating. All days were significantly lower than Day 7 (Figure 5.12a). No differences ($P > 0.10$) were detected in ER β expression by days post-mating or right versus left side in pregnant uterus (Figure 5.12b). No differences ($P > 0.10$) were detected in expression of ER α or ER β in pregnant days 7-13 endometrium by days post-mating or right versus left sides (Figures 5.13a and 5.13b). However, there was a consistent tendency for greater ER α expression in the right versus left side (4.4 ± 0.8 versus 3.0 ± 0.8 , respectively; $P = 0.20$).

ER α expression in juvenile uterus tended to be lower ($P = 0.12$) than in uteri recovered from Days 7, 9 and 11 non-pregnant and non-ovulated females (Figure 5.14a). Expression of ER β was lower ($P < 0.05$) in Day 9 non-pregnant uterus

Figure 5.9. Expression of ER α in llama CL on Days 7, 9, 11 and 13 of pregnancy (n=3, 2, 3 and 2 respectively). Graphs depict the means of each receptor subtype calculated from scanning densitometry data and are expressed as a percentage of β -actin expression. Day 9 expression was lower than Day 11 ($P < 0.05$, Duncan's NMRT).

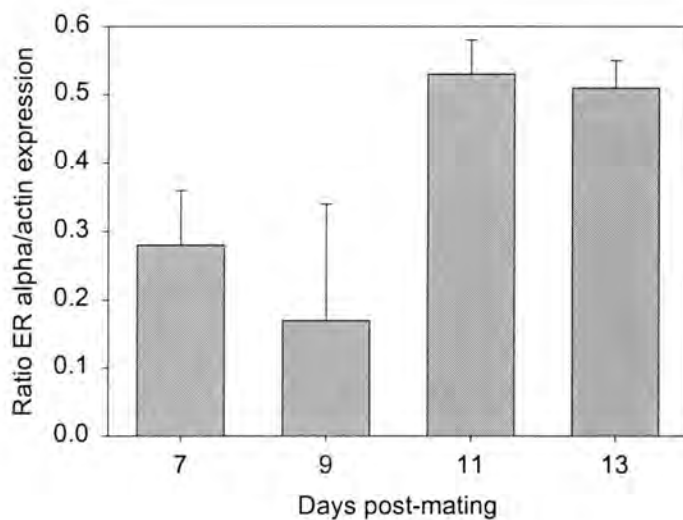
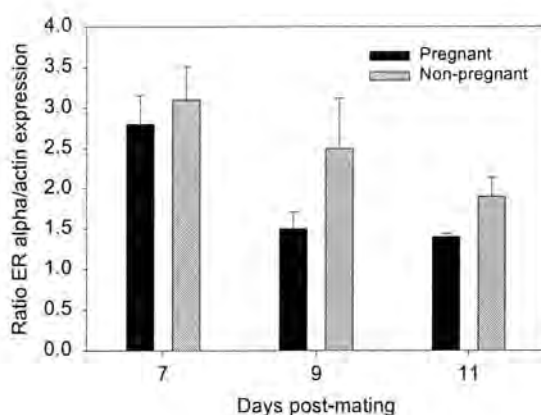


Figure 5.10. Expression of ER α and ER β in llama uterus on Days 7, 9 and 11 post-mating ($n = 6/6$, $2/4$ and $6/6$, respectively). Graphs depict the means of each receptor subtype calculated from scanning densitometry data and are expressed as a percentage of β -actin expression. Differences by day (decreasing expression over time; $P = 0.01$) and reproductive status (non-pregnant > pregnant; $P = 0.075$) were detected in ER α expression and a difference by reproductive status (pregnant > non-pregnant; $P = 0.086$) was detected in ER β expression.

A. ER α



B. ER β

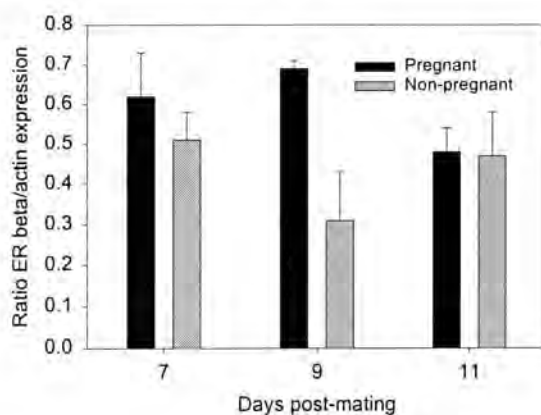
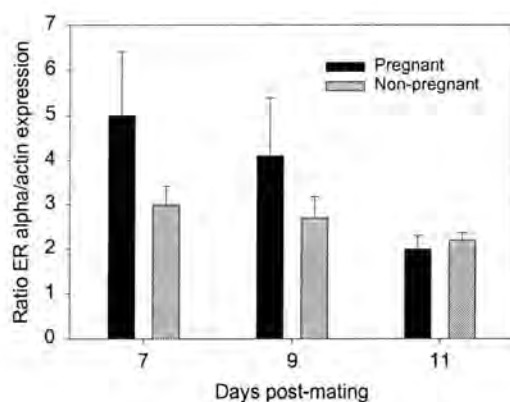


Figure 5.11. Expression of ER α and ER β in llama endometrium on Days 7, 9 and 11 post-mating (n=6/6, 2/6 and 6/6, respectively). Graphs depict the means of each receptor subtype calculated from scanning densitometry data and are expressed as a percentage of β -actin expression. No differences were detected by day post-mating or reproductive status in either ER α or ER β expression ($P > 0.10$).

A. ER α



B. ER β

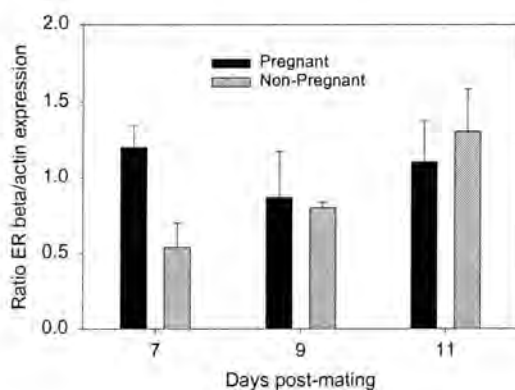
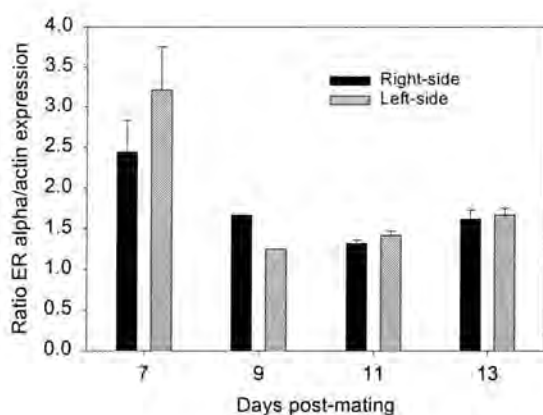


Figure 5.12. Expression of ER α and ER β in pregnant llama uterus, Days 7, 9, 11 and 13 post-mating (n=3, 1, 3 and 2 respectively). Graphs depict the means of each receptor subtype calculated from scanning densitometry data and are expressed as a percentage of β -actin expression. A difference by Day (Day 7 > 9, 11, and 13) was detected in ER α expression ($P < 0.01$). No differences were detected in expression of ER β) by day of gestation or in expression of ER α or β in the right versus left side ($P > 0.10$).

A. ER α



B. ER β

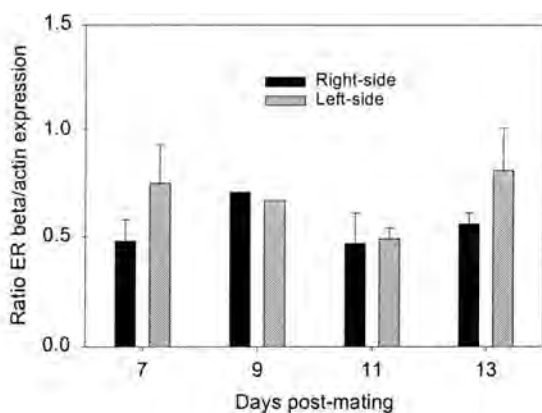
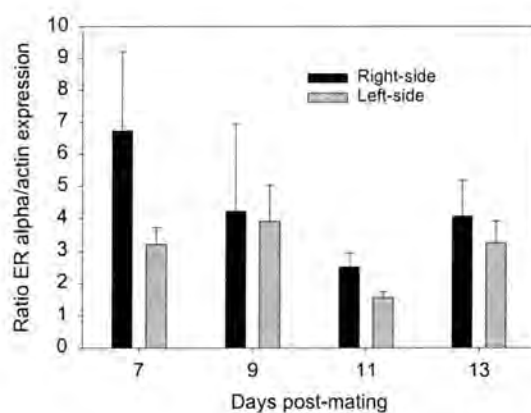
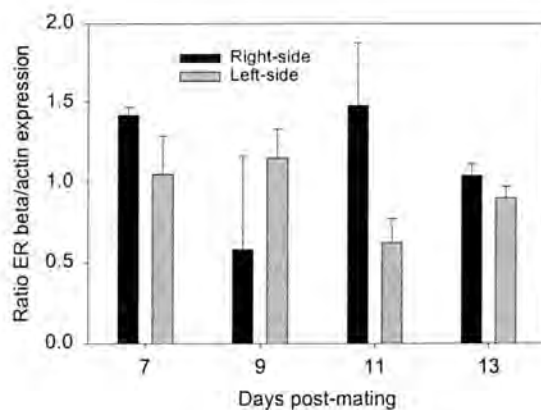


Figure 5.13. Expression of ER α and ER β in pregnant llama endometrium on Days 7, 9, 11 and 13 post-mating (n=3, 3, 3 and 2 respectively). Graphs depict the means of each receptor subtype calculated from scanning densitometry data and are expressed as a percentage of β -actin expression. No differences were detected in expression of ER α or ER β by day of gestation or right versus left side ($P > 0.10$).

A. ER α



B. ER β



compared to Days 7 and 11. ER β expression in uterus was lower ($P < 0.05$) in non-ovulated and juvenile females compared to Days 7 or 11 post-mating non-pregnant females (Figure 5.14b). Expression of ER β was not detectable in either of the juvenile females and was observed in one non-ovulated female. No differences in ER α expression ($P > 0.10$) were detected in non-pregnant endometrium in right versus left side or among Days 7, 9, 11 post-mating, non-ovulated and juvenile females (Figure 5.15a). ER β expression was not detected in non-ovulated females (Figure 5.15b). ER β expression in non-pregnant Days 7 and 9 endometrium was lower ($P < 0.05$) compared to Day 11 (Figure 5.16). A summary of the relative expression patterns of ER subtypes is provided in Table 5.2.

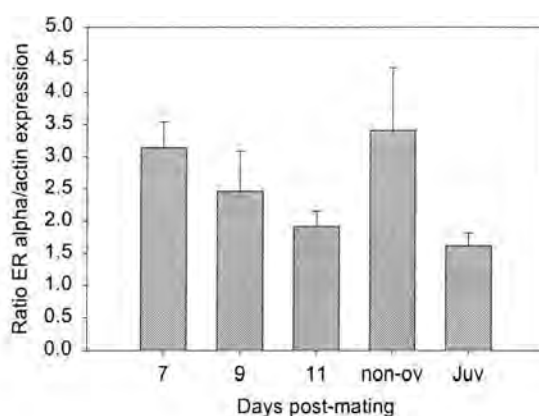
Discussion

The llama ER α and ER β sequences cloned in this experiment revealed close homology with published ER α and ER β sequences in human, pig and cow. This was to be expected as the region chosen for the primer pair to flank included the DNA binding domain in both ER subtypes. Rosenfeld et al. (1999) described amino acid sequence homologies of 100% in the DNA binding domain of ER β in bovine, human, mouse and rat. Even though ER α and ER β have high homologies in their DNA binding domains (Kuiper et al., 1996), both llama ER α and ER β sequences matched specifically to previously published ER α and ER β sequences.

Expression of both ER subtypes was noteworthy in the CL. While not significantly different between days, ER α increased by days post-mating while ER β decreased. The role of estrogens mediating events during the luteal phase and the different roles of the estrogen receptors in these events is not well understood. Estrogens increase granulosa cell numbers and IGF-1 production by the granulosa cells, maintain the FSH receptor, induce expression of the LH receptor, augment aromatase activity and subsequent estradiol production, and attenuate granulosa cell apoptosis (Krege et al., 1998). In the rat, immunohistochemical localization of the

5.14. Expression of ER α and ER β in uteri recovered from non-pregnant llamas on Days 7, 9 and 11 post-mating, non-ovulated and juvenile females (n=6, 4, 6, 4, and 4 respectively). Graphs depict the means of each receptor subtype calculated from scanning densitometry data and are expressed as a percentage of β -actin expression. A suggestive difference was detected in expression of ER α in the juvenile uterus as compared to Days 7, 9, 11 post-mating and non-ovulated females ($P = 0.12$, orthogonal contrast). Expression of ER β was different in non-ovulated and juvenile females compared to Days 7 and 11 post-mating ($P < 0.05$).

A. ER α



B. ER β

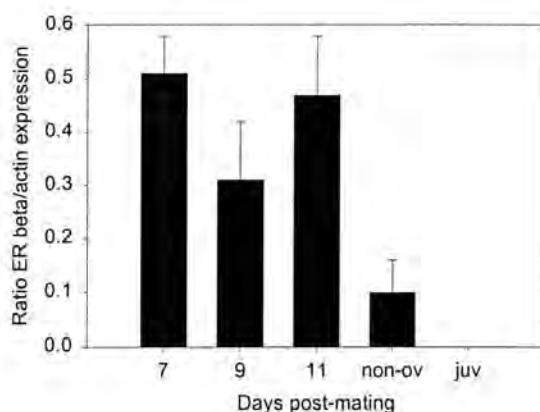
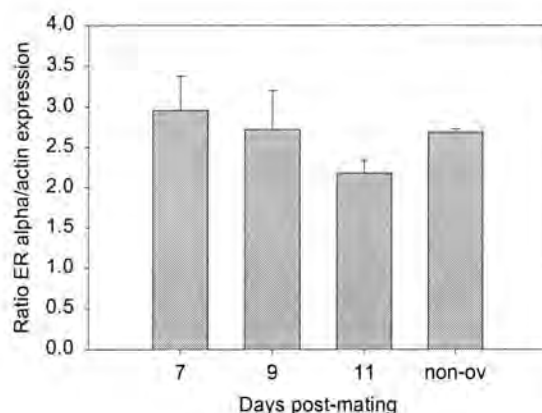


Figure 5.15. Expression of ER α and ER β in endometrium recovered from non-pregnant llamas on Days 7, 9 and 11 post-mating, and non-ovulated females (n=6, 2, 6 and 4 respectively). Graphs depict the means of each receptor subtype calculated from scanning densitometry data and are expressed as a percentage of β -actin expression. No differences were detected in expression of ER α by days post-mating. Expression of ER β was different in non-ovulated females compared to females on Days 7, 9 and 11 post-mating ($P < 0.05$).

A. ER α



B. ER β

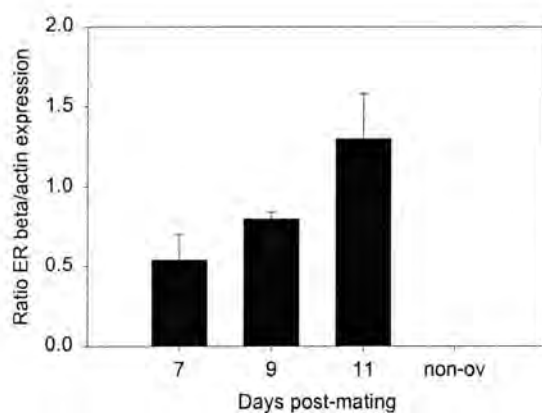


Figure 5.16. Expression of ER β in non-pregnant llama endometrium on Days 7, 9 and 11 post-mating (n=6, 2 and 6 respectively). Pooled SEM was 0.13. Graphs depict the means of each receptor subtype calculated from scanning densitometry data and are expressed as a percentage of β -actin expression. Expression of ER β was different in Day 11 versus Days 7 and 9 (P = 0.10).

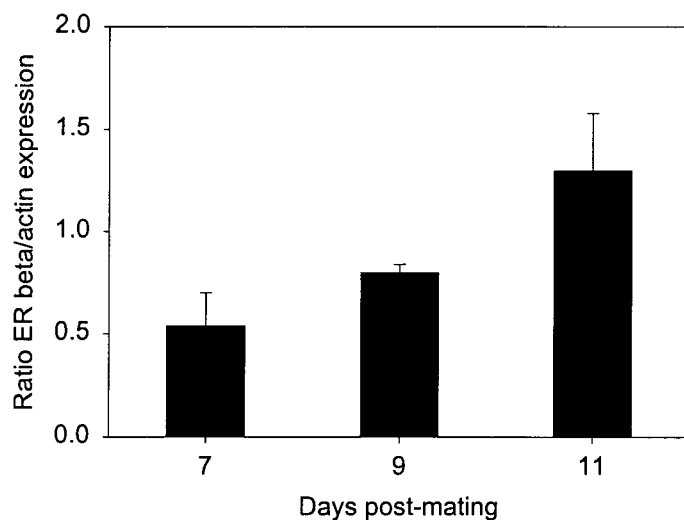


Table 5.2. Summary of ER α and ER β expression in the llama CL, uterus, and endometrium (day refers to days post-mating and reproductive status refers to pregnant versus non-pregnant).

	ER α expression	ER β expression
CL	Increased from Days 7-11	Decreased from Days 7-11
Ovary	Ovary > Days 7, 9, 11, 13 CL	Not different from CL
Uterus	Decreased from Days 7-11 Non-pregnant > Pregnant	Not different by day Pregnant > non-pregnant
Endometrium	No differences by day or reproductive status	No differences by day or reproductive status
Pregnant uterus	Day 7 > 9, 11, 13 Right side = left side	No differences by day Right side = left side
Pregnant endometrium	No differences by day Right side = left side	No differences by day Right side = left side
Non-pregnant uterus	No differences by day	No differences by day
Non-pregnant endometrium	No differences by day	Day 11 > Days 7 and 9
Juvenile uterus	Juvenile < pubertal animals	Not detectable
Non-ovulated controls	No difference from ovulated animals	Non-ovulated < Days 7 and 11 non-pregnant females

two estrogen receptor sub-types revealed immunoreactive ER β in the granulosa cells of primary, secondary, and mature follicles. but not in thecal, luteal or interstitial cells, oocytes, or germinal epithelium. Immunoreactive ER α was detected in the germinal epithelial, thecal, and interstitial but not granulosa cells (Sar and Welsch, 1999). Rosenfeld et al. (1999) detected bovine ER β mRNA and protein in granulosa cells of antral follicles in various stages of follicular growth using in situ hybridization and immunohistochemistry.

Kuiper et al., (1997) described the differences between ER α and ER β in relative ligand binding affinity and tissue distribution. It was found that the tissue distribution was quite different between the two ER subtypes. ER α was found to have moderate to high expression in the uterus, testis, pituitary, ovary, kidney, epididymis, and adrenal. The prostate, ovary, lung, bladder, brain, uterus, and testis exhibited moderate to high expression of ER β . The results of this experiment detected ER α expression in both the CL and ovary, with expression of ER β slightly higher than ER α in the CL although relative expression was low for both. Expression of ER α in the ovary appeared to be slightly higher than ER β .

Previous studies demonstrated that the llama embryo produces estradiol and that estradiol may be luteotrophic in the llama. If estradiol is mediating a direct luteotrophic effect on the CL, then differential expression of ER may play a role in controlling these events. Further studies are needed to determine if differential expression of ER subtypes plays a role in CL development and maintenance, especially in species such as the pig in which estradiol is known to be luteotrophic.

This study demonstrated a down regulation of ER α in the uterus from Days 7-11 post-mating. This decrease was not evident in the endometrium. Therefore, this decrease is probably a result of decreasing ER α message in the myometrium. This agrees with previous studies in which estrogen receptors are down regulated during the luteal phase (Wathes and Hamon, 1993). Interestingly in this experiment, only ER α was decreased during this time period, while ER β did not exhibit a difference

by days post-mating in the uterus or endometrium. In addition, ER α expression was higher in non-pregnant animals, while ER β expression was higher in pregnant animals. In species that experience embryo migration, estrogens are postulated to play a role in these events (Pope et al., 1982). If embryonic estradiol is mediating embryo migration, it may be acting through either ER subtype perhaps by up-regulating OTR or PGF_{2 α} receptor expression. Because llama embryos exhibit one-way migration from the right to the left uterine horn, it was expected that there might be a differential expression of ER by side. It was hypothesized that a higher number of estrogen receptors in the right side could induce a greater local contractility of the right uterine horn, thus propelling the embryo to the left side. While a significant difference by side was not observed, a trend was evident with greater expression of ER subtypes in right-sided endometrium. In general, ER β expression was low in all tissues analyzed and more sensitive techniques may be better able to detect differences.

Pubertal non-pregnant animals exhibited higher expression of ER α in uterus than juvenile females. ER β expression was not detected in either juvenile uterus. Further analysis is needed to determine if ER β message is absent before puberty in the uterus. ER β was not detected in the endometrium of non-ovulated females. Again, further analysis is needed to determine if ER β message is expressed only in the luteal endometrium.

In conclusion, differential expression of ER α and ER β was detected in CL, uterus and endometrium of the pregnant and non-pregnant llama. It is possible that estradiol produced by the embryo is involved in both embryo migration and maternal recognition of pregnancy, and that these events are mediated by differential expression of ER subtypes.

References

- Arcellana-Panlilio MY, Schultz GA. Analysis of messenger RNA. In: Guide to Techniques in Mouse Development. Methods of Enzymology (Eds. Wassarman PM, DePamphilis ML), New York:Academic Press 1993; 225:303-328.
- Beard AP, Lamming GE. Oestradiol concentration and the development of the uterine oxytocin receptor and oxytocin-induced PGF_{2α} release in ewes. J Reprod Fert 1994; 100:143-150.
- Chomczynski P, Sacchi N. Single step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156-159.
- Fernandez-Baca S, Hansel W, Novoa C. Embryonic mortality in the alpaca. Biol Reprod 1970; 3:243-251.
- Ginther OJ. Intrauterine movement of the early conceptus in barren and postpartum mares. Theriogenology 1984; 21:633-64.
- Hixon JE, Flint APF. Effects of a luteolytic dose of oestradiol benzoate on uterine oxytocin receptor concentrations, phosphoinositide turnover and prostaglandin F-2α secretion in sheep. J Reprod Fert 1987; 78:457-467.
- Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson, JA, Smithies O. Generation and reproductive phenotypes of mice lacking estrogen receptor β. Proc Natl Acad Sci USA 1998; 95:15677-15682.
- Kuiper GGJM, Enmark E, Peltö-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 1996; 93:5925-5930.
- Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson, JA. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 1997; 138:863-870.
- McCracken JA, Schramm W, Okulicz WC. Hormone receptor control of pulsatile secretion of PGF_{2α} from ovine uterus during luteolysis and its abrogation in early pregnancy. Anim Reprod Sci 1984; 7:31-56.
- Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. PNAS 1988; 85:2444-2448.

Pope WF, Maurer RR, Stormshak F. Intrauterine migration of the porcine embryo-Influence of estradiol 17- β and histamine. *Biol Reprod* 1982b; 27:575-579.

Rosenfeld CS, Yuan X, Manikkam M, Calder MD, Garverick HA, Lubahn DB. Cloning, sequencing and localization of bovine estrogen receptor- β within the ovarian follicle. *Biol Reprod* 1999; 60:691-697.

Sar M, Welsch F. Differential expression of estrogen receptor- β and estrogen receptor- α in the rat ovary. *Endocrinology* 1999; 140:963-971.

Tokunaga K, Taniguchi H, Yoda K, Shimizu M, Sakiyama S. Nucleotide sequence of a full-length cDNA for mouse cytoskeletal β -actin mRNA. *Nucl Acids Res* 1986; 14:2829.

Wathes DC, Hamon M. Localization of oestradiol, progesterone and oxytocin receptors in the uterus during the oestrus cycle and early pregnancy of the ewe. *J Endocrin* 1993; 138:479-491.

CHAPTER 6

SUMMARY

Embryonic mortality is a significant factor contributing to reduced reproductive efficiency in many mammals. As with other farm species, llamas and alpacas experience a high incidence of early embryonic mortality. Because the llama embryo is known to migrate from the right to the left uterine horn, with approximately 95% of pregnancies occurring in the left uterine horn, improper embryo migration may be a factor in early pregnancy loss in the llama. The results reported from these studies will hopefully clarify events occurring during early embryonic development and maternal recognition of pregnancy.

The first study demonstrated that llama embryos produce increasing quantities of estradiol during Days 7 through 15 of gestation. A dramatic rise in estradiol production occurs on Day 13 which correlates to a rapid elongation phase in the embryo. The coincidental timing of maternal recognition of pregnancy and embryo migration suggest that a substance secreted by the preimplantation llama embryo may mediate both events. Whether estradiol is directly involved in maternal recognition of pregnancy and embryo migration still needs to be determined, however the present study demonstrated estradiol production by the preimplantation llama embryo during the period that these events occur.

The second study demonstrated a potentially luteotrophic role for estradiol in the llama. Progesterone levels were significantly increased on Days 14-17 in females receiving 10 mg/day EB on Days 7-15 of an induced luteal phase as compared to females who received 5 mg/day EB and vehicle only. This prolonged luteal phase extends past the day of the last injection and is also 3-4 days past the time when luteolysis would be expected to be complete. Estradiol may have a direct effect on the CL or it may have an indirect effect by suppressing uterine prostaglandin release.

The final study demonstrated differential expression of ER α and ER β in the CL, uterus and endometrium of the pregnant and non-pregnant llama. ER α increased by days post-mating while ER β decreased in the llama CL. Down regulation of ER α in the uterus was observed from Days 7-11 post-mating. However, only ER α was decreased during this time period. ER β did not exhibit a difference by days post-mating in the uterus or endometrium. ER α expression was higher in non-pregnant animals, while ER β expression was higher in pregnant animals. Pubertal non-pregnant animals exhibited higher expression of ER α than juvenile females in uterus. ER β was not detected in the endometrium of non-ovulated females.

It is possible that estradiol produced by the embryo is involved in both embryo migration and maternal recognition of pregnancy, and that these events are mediated by differential expression of ER subtypes.

BIBLIOGRAPHY

Adam CL, Moir CE, Shiach P. Plasma progesterone concentrations in pregnant and non-pregnant llamas (*lama glama*). Vet Rec 1989; 125:618-620.

Adams GP, Sumar J, Ginther OJ. Effects of lactational and reproductive status on ovarian follicular waves in llamas (*lama glama*). J Reprod Fert 1990; 90:535-545.

Adams GP, Sumar J, Ginther OJ. Form and function of the corpus luteum in llamas. Animal Reprod Sci 1991; 24:127-138.

Arcellana-Panlilio MY, Schultz GA. Analysis of messenger RNA. In: Wassarman PM, DePamphilis ML (eds.), *Guide to Techniques in Mouse Development. Methods of Enzymology*. New York: Academic Press; 1993: 225:303-328.

Ayalon N. A review of embryonic mortality in cattle. J Reprod Fert 1978; 54:483-493.

Bazer FW. Establishment of pregnancy in sheep and pigs. Reprod Fertil Dev 1989; 1:237-242.

Bazer FW. Mediators of maternal recognition of pregnancy in mammals. Proc Soc Exp Biol Med 1992; 199:373-384.

Bazer FW, Vallet JL, Harney JP, Gross TS, Thatcher WW. Comparative aspects of maternal recognition of pregnancy between sheep and pigs. J Reprod Fertil 1989; 37(suppl):85-889.

Beard AP, Lamming GE. Oestradiol concentration and the development of the uterine oxytocin receptor and oxytocin-induced PGF_{2α} release in ewes. J Reprod Fert 1994; 100:143-150.

Beato M. Gene regulation by steroid hormones. Cell 1989; 56:335-344.

Berglund LA, Sharp DC, Vernon MW, Thatcher WW. Effect of pregnancy status and collection technique on prostaglandin F in the uterine lumen of pony mares. J Reprod Fertil 1982; 32 (suppl):335-341.

Brownan, DL. Pastoral nomadism in the Andes. Current Anthropology 1974; 15:188-196.

Bourke DA, Adam CL, Kyle CE. Ultrasonography as an aid to controlled breeding in the llama (*lama glama*). Vet Rec 1992; 130:424-428.

Bourke D, Kyle CE, McEvoy TG, Young P, Adam CL. Superovulatory responses to eCG in Llamas (*lama glama*). Theriogenology 1995; 44:255-268.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-254.

Bravo PW. Reproductive endocrinology of llamas and alpacas. Vet Clin North Am Food Anim Prac 1994; 10:265-279.

Bravo W, Fowler ME. Basic physiology of reproduction in female llamas. Llamas 1990; 35-37.

Bravo PW, Johnson LW. Reproductive physiology of the male camelid. Vet Clin North Am Food Anim Prac 1994; 10:259-264.

Bravo PW, Sumar J. Laparoscopic examination of the ovarian activity in alpacas. Animal Reprod Sci 1989; 21:271-281.

Bravo PW, Varela MH. Prenatal development of the alpaca (*lama pacos*). Animal Reprod Sci 1993; 32:245-252.

Bravo PW, Sumar J, Rivera SG, Foote WC. Reproductive wastage in the female alpaca. In: *Improving Reproductive Performance of Small Ruminants*. Utah State University, Logan 1987; 155-159.

Bravo PW, Fowler ME, Stabenfeldt GH, Lasley BL. Endocrine responses in the llama to copulation. Theriogenology 1990; 33(4):891-899.

Bravo PW, Fowler ME, Stabenfeldt GH, Lasley BL. Ovarian follicular dynamics in the llama. Biol Reprod 1990; 43:579-585.

Bravo PW, Stabenfeldt GH, Lasley BL, Fowler ME. The effect of ovarian follicle size on pituitary and ovarian responses to copulation in domesticated South American Camelids. Biol Reprod 1991; 45:553-559.

Bravo PW, Stabenfeldt GH, Fowler ME, Lasley BL. Urinary steroids in the periparturient and postpartum periods through early pregnancy in llamas. Theriogenology 1991; 36:267-278.

Bravo PW, Stabenfeldt GH, Fowler ME, Lasley BL. Pituitary response to repeated copulation and/or gonadotropin-releasing hormone administration in llamas and alpacas. *Biol Reprod* 1992a; 47:884-888.

Bravo PW, Stabenfeldt GH, Fowler ME, Lasley BL, Frey RE. Testes development and testosterone concentrations in the llama (*Lama glama*). In: *Proceedings of 12th International Congress Animal Reproduction, the Hague, the Netherlands*, 1992b; 4:1698-1700.

Bravo PW, Fowler ME, Lasley BL. The postpartum llama: Fertility after parturition. *Biol Reprod* 1994; 51:1084-1087.

Bravo PW, Lasley BL, Fowler ME. Resumption of ovarian follicular activity and uterine involution in the postpartum llama. *Theriogenology* 1995; 44:783-791.

Chomczynski P, Sacchi N. Single step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162:156-159.

Clark JH, Schrader WT, O'Malley BW. Mechanisms of action of steroid hormones. In: Wilson J, Foster DW (eds.) *Textbook of Endocrinology*, WB Saunders Company, Philadelphia, PA, 1992; 35-90.

Del Campo MR, Del Campo CH, Ginther OJ. Vascular provisions for a local utero-ovarian cross-over pathway in New World Camelids. *Theriogenology* 1996; 46:983-991.

Dhindsa DS, Dzuick PJ, Norton HW. Time of transuterine migration and distribution of embryos in the pig. *Anat Rec* 1967; 159:325.

Edey, TN. Embryo Mortality. In: Tomes GJ, Robertson DE, Lightfoot RJ, Haresign W (eds), *Sheep Breeding*. London: Butterworths; 1979: 315-325.

England BG, Foote WC, Cardoza AG, Matthews, DH, Riera S. Ovulation and corpus luteum function in the llama (*lama glama*). *J Endocr* 1969; 45:505-513.

England BG, Foote WC, Cardoza AG, Matthews, DH, Riera S. Oestrus and mating behaviour in the llama (*lama glama*). *Anim Behav* 1971; 19:722-726.

Fernandez-Baca S. Manipulation of reproductive functions in male and female New World camelids. *Animal Reprod Sci* 1993; 33:307-323.

Fernandez-Baca S, Hansel W, Novoa C. Embryonic mortality in the alpaca. *Biol Reprod* 1970a; 3:243-251.

Fernandez-Baca S, Hansel W, Novoa C. Corpus luteum function in the alpaca. *Biol Reprod* 1970b; 3:252-261.

Fernandez-Baca S, Madden DHL, Novoa C. Effect of different mating stimuli on induction of ovulation in the alpaca. *J Reprod Fert* 1970c; 22:261-267.

Fernandez-Baca S, Hansel W, Saatman R, Sumar J, Novoa C. Differential luteolytic effects of right and left uterine horns in the alpaca. *Biol Reprod* 1979; 20:586-595.

Fowler ME. *Medicine and Surgery of South American Camelids*. Ames, Iowa State University Press, 1989.

Fowler ME. Twinning in llamas. *Llamas* 1990; 4:35-38.

Fowler ME. Health care of the geriatric llama and alpaca. *Vet Clin North Am Food Anim Prac* 1994; 10(2): 391-399.

Fowler ME, Olander HJ. Fetal membranes and ancillary structures of llamas (*Lama glama*). *Am J Vet Res* 1990; 51:1495-1500.

Franklin WL: Biology, ecology and relationship to man of the South American camelids. In: *Mammalian Biology in South America*. Vol 6, Special Publication Series Pymatuning Laboratory of Ecology, University of Pittsburgh, Linesville, PA; 1982

Geisert RD, Thatcher WW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig: III. Endometrial secretory response to estradiol valerate administered on day 11 of the estrous cycle. *Biol Reprod* 1981; 27:957-965.

Ginther OJ. Mobility of the early equine conceptus. *Theriogenology* 1983; 19:603-11.

Ginther OJ. Intrauterine movement of the early conceptus in barren and postpartum mares. *Theriogenology* 1984; 21:633-64.

Goodman RL. A quantitative analysis of the physiological role of estradiol and progesterone in the control of tonic and surge secretion of luteinizing hormone in the rat. *Endocrinology* 1978; 102:142-50.

Gordon I. *Controlled Reproduction in Horses, Deer, and Camelids*. University Press, Cambridge, UK, 1997:189-208.

Grubb TL, Riebold TW, Huber MJ. Evaluation of lidocaine, xylazine, and a combination of lidocaine and xylazine for epidural analgesia in llamas. *JAVMA* 1993; 203:1441-1444.

Hafez ESE (Ed). *Reproduction in Farm Animals*. 6th edition, Lea and Febiger, Philadelphia, PA, 1993.

Heap RB, Hamon M, Allen WR. Studies on oestrogen synthesis by the equine conceptus. *J Reprod Fert* 1982; 32:343-352

Helmer SD, Hansen PJ, Anthony RV, Thatcher WW, Bazer FW, Roberts RM. Identification of bovine trophoblast protein-1, a secretory protein immunologically related to ovine trophoblast protein-1. *J Reprod Fert* 1987; 79:83-91

Hixon JE, Flint APF. Effects of a luteolytic dose of oestradiol benzoate on uterine oxytocin receptor concentrations, phosphoinositide turnover and prostaglandin F-2 α secretion in sheep. *J Reprod Fert* 1987; 78:457-467.

Hoffman E, Fowler ME. *The Alpaca Book: Management, Medicine, Biology and Fiber*. Herald, CA : Clay Press Inc; 1995.

Imakawa K, Anthony RV, Kazemi M, Marotti KR, Polites HG, Roberts RM. Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophoblast. *Nature* 1987; 330:377-379.

Johnson LW. Llama reproduction. *Vet Clin North Am Food Anim Prac* 1989; 5:159-182.

Johnson MH, Everitt BJ. *Essential Reproduction*. 4th edition, Blackwell Science, Oxford, England, 1995.

Koike S, Masaharu S, Muramatsu M. Molecular cloning and characterization of rat estrogen receptor cDNA. *Nucleic Acids Res* 1987; 15:2499-2513.

Kuiper GGJM, Enmark E, Peltö-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 1996; 93:5925-5930.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson, JA. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β . *Endocrinology* 1997; 138:863-870.

LaForest JP, King GJ. Effect of intrauterine application of oestradiol-17 β and prostaglandin E-2 on the porcine oestrous cycle and uterine endocrinology. *J Reprod Fert* 1992; 94:381-394.

Leaman DW, Roberts RM. Genes for trophoblast interferons in sheep, goat, musk ox, and distribution of related genes among mammals. *J Interfer Res* 1992; 12:1-11.

Leipold HW, Hiraga T, Johnson LW. Congenital defects in the llama. *Vet Clin North Am Food Anim Prac* 1994; 10:401-419.

Leon, JB, Smith BB, Timm KI, LeCren G. Endocrine changes during pregnancy, parturition, and the early post-partum period in the llama (*lama glama*). *J Reprod Fert* 1990; 88:503-511.

Lichtenwalner AB, Woods GL, Weber JA. Ejaculatory pattern of llamas during copulation. *Theriogenology* 1996a; 46:285-291.

Lichtenwalner AB, Woods GL, Weber JA. Seminal collection, seminal characteristics and pattern of ejaculation in llamas. *Theriogenology* 1996b; 46:293-305.

Malayer JR, Woods VM. Development of oestrogen receptor expression and hormone response in the uterus of the bovine fetus. *J Reprod Fertil* 1998a; 112:289-300.

Malayer JR, Woods VM, Biby AG. Expression of estrogen receptor beta mRNA in the bovine uterus (abstract). *Biol Reprod* 1998b; 58 (suppl 1):143.

McCracken JA, Schramm W, Okulicz WC. Hormone receptor control of pulsatile secretion of PGF_{2 α} from ovine uterus during luteolysis and its abrogation in early pregnancy. *Anim Reprod Sci* 1984; 7:31-56.

McDowell KJ, Sharp DC, Grubaugh W, Thatcher WW, Wilcox CJ. Restricted conceptus mobility results in failure of pregnancy maintenance in mares. *Biol Reprod* 1988; 39:340-348.

Merkt H, Boer M, Schoon HA. The presence of an additional fetal membrane and its function in the newborn guanaco (*lama guanacoe*). *Theriogenology* 1988; 30:437-439.

Murra JV. Herds and herders in the Incan state. In: Leeds A, Vayda AP (eds.), *Man, Culture, and Animals*, Washington D.C.: American Association for the Advancement of Science, 1965: Pub no. 78.

Murdoch FE, Gorski J. The role of ligand in estrogen receptor regulation of gene expression. *Mol Cell Endocrinol* 1991; 78:C103-C108

Novoa C. Reproduction in the camelidae. *J Reprod Fert* 1970; 22:3-20.

Novoa C and Wheeler JC. Llamas and alpacas. In Mason IL (ed.), *Evolution of Domestic Animals*, London: Longman Press; 1988: 116-144.

O'Malley B. The steroid receptor superfamily: more excitement predicted for future. *Molecular Endocrinology* 1990; 4:363-369.

Paul-Murphy J. Obstetrics, neonatal care, and congenital conditions. *Vet Clin North Am Food Anim Prac* 1989; 5:183-202.

Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. *PNAS* 1988; 85:2444-2448.

Perry JS, Heap RB, Amoroso EC. Steroid hormone production by pig blastocysts. *Nature* 1973; 245:45-47.

Pollard JC, Littlejohn RP, Scott IC. The effects of mating on the sexual receptivity of female alpacas. *Animal Reprod Sci* 1994; 34:289-297.

Pontzer CH, Torres BA, Vallet JL, Bazer FW, Johnson HM. Antiviral activity of the pregnancy recognition hormone ovine trophoblast protein-1. *Biochem Biophys Res Comm* 1988; 152:801-807.

Pope WF, Maurer RR, Stormshak F. Intrauterine migration of the porcine embryo-Interaction of embryo, uterine flushings and indomethacin on myometrial function in vitro. *J Anim Sci* 1982a; 55:1169-1178.

Pope WF, Maurer RR, Stormshak F. Intrauterine migration of the porcine embryo-Influence of estradiol 17- β and histamine. *Biol Reprod* 1982b; 27:575-579.

- Reed PJ. Studies on luteinizing hormone and gonadal steroids in male and female llamas (*Lama glama*). Corvallis, OR: Oregon State University; 1996. Thesis.
- Roberts RM, Cross JC, Leaman DW. Interferons as hormones of pregnancy. *Endocrine Rev* 1992; 13:432-452.
- Rosenfeld CS, Yuan X, Manikkam M, Calder MD, Garverick HA, Lubahn DB. Cloning, sequencing and localization of bovine estrogen receptor- β within the ovarian follicle. *Biol Reprod* 1999; 60:691-697.
- San-Martin M, Copaira M, Zuniga J, Rodreguez R, Bustinza G, Acosta L. Aspects of reproduction in the alpaca. *J Reprod Fert* 1968; 16:395-399.
- Sar M, Welsch F. Differential expression of estrogen receptor- β and estrogen receptor- α in the rat ovary. *Endocrinology* 1999; 140:963-971.
- Setchell KD, Borriello SP, Hulme P, Kirk DN, Axelson M. Nonsteroidal estrogens of dietary origin; possible roles in hormone-dependent disease. *Am J Clin Nutr* 1984, 40:569-578.
- Sharp DC, McDowell KJ, Weithenauer J, Thatcher WW. The continuum of events leading to maternal recognition of pregnancy in mares. *J Reprod Fertil* 1987; 37(suppl):101-107.
- Skidmore JA, Allen WR, Heap RB. Oestrogen synthesis by the peri-implantation conceptus of the one-humped camel (*Camelus dromedarius*). *J Reprod Fertil* 1994; 101:363-367.
- Smith CL, Peter AT, Pugh DG. Reproduction in llamas and alpacas: A review. *Theriogenology* 1994; 41: 573-592.
- Spencer TE, George P, Becker WC, Ogle TF, Mirando MA, Bazer FW. Ovine interferon- τ regulates endometrial receptor for estrogen and oxytocin, but not progesterone. *Biol Reprod* 1995; 53:732-745.
- Spencer TE, Bazer FW. Ovine interferon tau suppresses transcription of the estrogen receptor and oxytocin receptor genes in the ovine endometrium. *Endocrinology* 1996; 137:1144-1147.
- Sumar J. Studies on reproductive pathology in alpacas. Uppsala, Sweden: Swedish University of Agricultural Science; 1983. Thesis.

Sumar J. Reproduction in alpacas and llamas. *Animal Reprod Sci* 1996; 42:405-415.

Sumar J, Bravo PW. In situ observation of the ovaries from llamas and alpacas. *J Vet Med Assoc* 1991; 199:1159.

Sumar J, Fredrikssen G, Alarcon V, Kindahl H, Edqvist L-E. Levels of 15-keto-13,14-dihydro-PGF 2α , progesterone and oestradiol-17 β after induced ovulations in llamas and alpacas. *Acta Vet Scand* 1988; 29:339-346.

Sumar J, Bravo PW, Foote WC. Sexual receptivity and time of ovulation in alpacas. *Small Rum Res* 1993; 11:143-150.

Tokunaga K, Taniguchi H, Yoda K, Shimizu M, Sakiyama S. Nucleotide sequence of a full-length cDNA for mouse cytoskeletal β -actin mRNA. *Nucl Acids Res* 1986; 14:2829.

Vidal-Rioja LA, Zambelli A, Semorle L. An assesment of the relationships among species of Camelidae by satellite DNA comparison. *Hereditas* 1994; 121:283-290.

Wathes DC, Lamming GE. The oxytocin receptor, luteolysis and the maintenance of pregnancy. *J Reprod Fertil* 1995; 49 (suppl):53-7.

Wathes DC, Hamon M. Localization of oestradiol, progesterone and oxytocin receptors in the uterus during the oestrus cycle and early pregnancy of the ewe. *J Endocrin* 1993; 138:479-491.

Wheeler J. Ancient alpacas and modern myths. *Alpacas*, 1994; Winter:19-21.

Wiepz DW, Chapman RJ. Non-surgical embryo transfer and live birth in a llama. *Theriogenology* 1985; 24:251-257.

Wilson PR, Lapwood KR. Studies of reproductive development in Romney rams: II. LH and testosterone secretion following single or repeated doses of gonadotropin releasing hormone (GnRH). *Biol Reprod* 1979; 20:971-975.